THE PROKARYOTES

Third Edition

A Handbook on the Biology of Bacteria:

Ecophysiology and Biochemistry

Edited by
MARTIN DWORKIN (EDITOR-IN-CHIEF)
STANLEY FALKOW
EUGENE ROSENBERG
KARL-HEINZ SCHLEIFER
ERKO STACKEBRANDT

Volume 2

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Martin Dworkin (Editor-in-Chief), Stanley Falkow, Eugene Rosenberg,
Karl-Heinz Schleifer, Erko Stackebrandt (Editors)

Springer

Editor-in-Chief
Professor Dr. Martin Dworkin
Department of Microbiology
University of Minnesota
Box 196
University of Minnesota
Minneapolis, MN 55455-0312
USA

Editors
Professor Dr. Stanley Falkow
Department of Microbiology
and Immunology
Stanford University Medical School
299 Campus Drive, Fairchild D039
Stanford, CA 94305-5124
USA

Professor Dr. Eugene Rosenberg
Department of Molecular Microbiology
and Biotechnology
Tel Aviv University
Ramat-Aviv 69978
Israel

Professor Dr. Karl-Heinz Schleifer
Department of Microbiology
Technical University Munich
80290 Munich
Germany

Professor Dr. Erko Stackebrandt
DSMZ- German Collection of Microorganisms
and Cell Cultures GmbH
Mascheroder Weg 1b
38124 Braunschweig
Germany

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Library of Congress Control Number: 91017256

Volume 2
ISBN-10: 0-387-25492-7
ISBN-13: 978-0387-25492-0
e-ISBN: 0-387-30742-7
Print + e-ISBN: 0-387-33491-2
DOI: 10.1007/0-387-30742-7

Volumes 1–7 (Set)
ISBN-10: 0-387-25499-4
ISBN-13: 978-0387-25499-9
e-ISBN: 0-387-30740-0
Print + e-ISBN: 0-387-33488-2

Printed on acid-free paper.

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Printed in Singapore. (BS/KYO)

9 8 7 6 5 4 3 2 1

springer.com

Preface

Each of the first two editions of *The Prokaryotes* took a bold step. The first edition, published in 1981, set out to be an encyclopedic, synoptic account of the world of the prokaryotes—a collection of monographic descriptions of the genera of bacteria. The Archaea had not yet been formalized as a group. For the second edition in 1992, the editors made the decision to organize the chapters on the basis of the molecular phylogeny championed by Carl Woese, which increasingly provided a rational, evolutionary basis for the taxonomy of the prokaryotes. In addition, the archaea had by then been recognized as a phylogenetically separate and distinguishable group of the prokaryotes. The two volumes of the first edition had by then expanded to four. The third edition was arguably the boldest step of all. We decided that the material would only be presented electronically. The advantages were obvious and persuasive. There would be essentially unlimited space. There would be no restrictions on the use of color illustrations. Film and animated descriptions could be made available. The text would be hyperlinked to external sources. Publication of chapters would be seriati—the edition would no longer have to delay publication until the last tardy author had submitted his or her chapter. Updates and modifications could be made continuously. And, most attractively, a library could place its subscribed copy on its server and make it available easily and cheaply to all in its community. One hundred and seventy chapters have thus far been presented in 16 releases over a six-year period. The virtues and advantages of the online edition have been borne out. But we failed to predict the affection that many have for holding a bound, print version of a book in their hands. Thus, this print version of the third edition shall accompany the online version.

We are now four years into the 21st century. Indulge us then while we comment on the challenges, problems and opportunities for microbiology that confront us.

Moselio Schaechter has referred to the present era of microbiology as its third golden age—the era of "integrative microbiology." Essentially all microbiologists now speak a common language. So that the boundaries that previously separated subdisciplines from each other have faded: physiology has become indistinguishable from pathogenesis; ecologists and molecular geneticists speak to each other; biochemistry is spoken by all; and—mirabile dictu!—molecular biologists are collaborating with taxonomists.

But before these molecular dissections of complex processes can be effective there must be a clear view of the organism being studied. And it is our goal that these chapters in *The Prokaryotes* provide that opportunity.

There is also yet a larger issue. Microbiology is now confronted with the need to understand increasingly complex processes. And the modus operandi that has served us so successfully for 150 years—that of the pure culture studied under standard laboratory conditions—is inadequate. We are now challenged to solve problems of multimembered populations interacting with each other and with their environment under constantly variable conditions. Carl Woese has pointed out a useful and important distinction between empirical, methodological reductionism and fundamentalist reductionism. The former has served us well; the latter stands in the way of our further understanding of complex, interacting systems. But no matter what kind of synoptic systems analysis emerges as our way of understanding host–parasite relations, ecology, or multicellular behavior, the understanding of the organism as such is sine qua non. And in that context, we are pleased to present to you the third edition of *The Prokaryotes*.

Martin Dworkin
Editor-in-Chief

Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These four volumes on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

This edition of *The Prokaryotes* recognizes the almost unbelievable impact that the work of Carl Woese has had in defining a phylogenetic basis for the microbial world. The concept that the ribosome is a highly conserved structure in all cells and that its nucleic acid components may serve as a convenient reference point for relating all living things is now generally accepted. At last, the phylogeny of prokaryotes has a scientific basis, and this is the first serious attempt to present a comprehensive treatise on prokaryotes along recently defined phylogenetic lines. Although evidence is incomplete for many microbial groups, these volumes make a statement that clearly illuminates the path to follow.

There are basically two ways of doing research with microbes. A classical approach is first to define the phenomenon to be studied and then to select the organism accordingly. Another way is to choose a specific organism and go where it leads. The pursuit of an unusual microbe brings out the latent hunter in all of us. The intellectual challenges of the chase frequently test our ingenuity to the limit. Sometimes the quarry repeatedly escapes, but the final capture is indeed a wonderful experience. For many of us, these simple rewards are sufficiently gratifying so that we have chosen to spend our scientific lives studying these unusual creatures. In these endeavors many of the strategies and tools as well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

In this school, the principles of the selective, enrichment culture technique have been developed and diversified; they have been a major force in designing and applying new principles for the capture and isolation of microbes from nature. For me, the "organism approach" has provided rewarding adventures. The organism continually challenges and literally drags the investigator into new areas where unfamiliar tools may be needed. I believe that organism-oriented research is an important alternative to problem-oriented research, for new concepts of the future very likely lie in a study of the breadth of microbial life. The physiology, biochemistry, and ecology of the microbe remain the most powerful attractions. Studies based on classical methods as well as modern genetic techniques will result in new insights and concepts.

To some readers, this edition of the *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and

prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the “nitrosome,” a unique nitrogen-fixing organelle. Study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become “dog-eared” and worn as students seek basic information for the hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, i.e., from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator these volumes should generate excitement.

Happy hunting!

Ralph S. Wolfe
Department of Microbiology
University of Illinois at Urbana-Champaign

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Contributors

Sharon L. Abbott
Microbial Diseases Laboratory
Berkeley, CA 94704
USA

Aharon Abeliovich
Department of Biotechnology Engineering
Institute for Applied Biological Research
Environmental Biotechnology Institute
Ben Gurion University
84105 Beer-Sheva
Israel

Soman N. Abraham
Director of Graduate Studies in Pathology
Departments of Pathology, Molecular Genetics and Microbiology, and Immunology
Duke University Medical Center
Durham, NC 27710
USA

David G. Adams
School of Biochemistry and Microbiology
University of Leeds
Leeds LS2 9JT
UK

Ben Adler
Monash University
Faculty of Medicine, Nursing and Health Sciences
Department of Microbiology
Clayton Campus
Victoria, 3800
Australia

Raymond Akhurst
CSIRO Entomology
Black Mountain
ACT 2601 Canberra
Australia

Rudolf Amann
Max Planck Institute for Marine Microbiology
D-28359 Bremen
Germany

Burt E. Anderson
Department of Medical Microbiology and Immunology
College of Medicine
University of South Florida
Tampa, FL 33612
USA

Robert E. Andrews
Department of Microbiology
University of Iowa
Iowa City, IA 52242
USA

Garabed Antranikian
Technical University Hamburg-Harburg
Institute of Technical Microbiology
D-21073 Hamburg
Germany

David R. Arahal
Colección Española de Cultivos Tipo (CECT)
Universidad de Valencia
Edificio de Investigación
46100 Burjassot (Valencia)
Spain

M. J. Arduino
Center for Infectious Diseases
Centers for Disease Control
Atlanta, GA 30333
USA

Judith Armitage
Department of Biochemistry
Microbiology Unit
University of Oxford
OX1 3QU Oxford
UK

Ingo Autenrieth
Institut für Medizinische Mikrobiologie
Universitatsklinikum Tuebingen
D-72076 Tuebingen
Germany

Hans-Dietrich Babenzien
Leibniz-Institut für Gewässerökologie und Binnenfischereiim Forschungsverbund Berlin
12587 Berlin
Germany

Werner Back
Lehrstuhl für Technologie der Brauerei I
Technische Universität München
D-85354 Freising-Weihenstephan
Germany

Margo Baele
Department of Pathology
Bacteriology and Poultry Diseases
Faculty of Veterinary Medicine
Ghent University
B-9820 Merelbeke
Belgium

Jose Ivo Baldani
EMBRAPA-Agrobiology
Centro Nacional de Pesquisa de Agrobiologia
Seropedica, 23851-970
CP 74505 Rio de Janeiro
Brazil

David L. Balkwill
Department of Biomedical Sciences
College of Medicine
Florida State University
Tallahassee, FL 32306-4300
USA

Horia Banciu
Department of Biotechnology
Delft University of Technology
2628 BC Delft

Tammy Bannerman
School of Allied Medical Professions
Division of Medical Technology
The Ohio State University
Columbus, OH 43210
USA

Bonnie L. Bassler
Department of Molecular Biology
Princeton University
Princeton, NJ 08544-1014
USA

Linda Baumann
School of Nursing
Clinical Science Center
University of Wisconsin
Madison, WI 53792-2455
USA

Paul Baumann
Department of Microbiology
University of California, Davis
Davis, CA 95616-5224
USA

Edward A. Bayer
Department of Biological Chemistry
Weizmann Institute of Science
Rehovot 76100
Israel

Dennis A. Bazylinski
Department of Microbiology, Immunology and Preventive Medicine
Iowa State University
Ames, IA 55001
USA

Jan Hendrick Becking
Stichting ITAL
Research Institute of the Ministry of Agriculture and Fisheries
6700 AA Wageningen
The Netherlands

Robert Belas
The University of Maryland Biotechnology Institute
Center of Marine Biotechnology
Baltimore, MD 21202
USA

Birgitta Bergman
Department of Botany
Stockholm University
SE-106 91 Stockholm
Sweden

Kathryn Bernard
Special Bacteriology Section
National Microbiology Laboratory
Health Canada
Winnipeg R3E 3R2
Canada

Jean-François Bernardet
Unité de Virologie et Immunologie Moléculaires
Institut National de la Recherche Agronomique (INRA)
Domaine de Vilvert
78352 Jouy-en-Josas cedex
France

Costanzo Bertoldo
Technical University Hamburg-Harburg
Institute of Technical Microbiology
D-21073 Hamburg
Germany

Bruno Biavati
Istituto di Microbiologia Agraria
40126 Bologna
Italy

Magne Bisgaard
Department of Veterinary Microbiology
Royal Veterinary and Agricultural University
1870 Frederiksberg C
Denmark

William Bishai
Departments of Molecular Microbiology and Immunology, International Health, and Medicine
Center for Tuberculosis Research
Johns Hopkins School of Hygiene and Public Health
Baltimore, MD 21205-2105
USA

Johanna Björkroth
Department of Food and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki
FIN-00014 Helsinki
Finland

Eberhard Bock
Institute of General Botany
Department of Microbiology
University of Hamburg
D-22609 Hamburg
Germany

Noel Boemare
Ecologie Microbienne des Insectes et
Interactions Hôte-Pathogène
UMR EMIP INRA-UMII
IFR56 Biologie cellulaire et Porcessus infectieux
Université Montpellier II
34095 Montpellier
France

Antje Boetius
Max-Planck-Institut für Marine Mikrobiologie
D-28359 Bremen
Germany

Adam S. Bonin
Portland State University
Portland OR 97207
USA

David R. Boone
Department of Biology
Environmental Science and Engineering
Oregon Graduate Institute of Science and Technology
Portland State University
Portland, OR 97207-0751
USA

Diana Borenshtein
Massachusetts Institute of Technology
Cambridge, MA 02139-4307
USA

Edward J. Bottone
Division of Infectious Diseases
The Mount Sinai Hospital
One Gustave L. Levy Place
New York, NY 10029
USA

Timothy L. Bowen
Department of Microbiology
University of Georgia
Athens, GA 30602
USA

John P. Bowman
Australian Food Safety Centre for Excellence
School of Agricultural Science
Hobart, Tasmania, 7001
Australia

John A. Breznak
Department of Microbiology and Molecular Genetics
Michigan State University
East Lansing, MI 48824-1101
USA

Stephanie Bringer-Meyer
Institut Biotechnologie
Forschungszentrum Jülich
D-52425 Jülich
Germany

Sylvain Brisse
Unité Biodiversité des Bactéries Pathogènes Emergentes
U 389 INSERM
Institut Pasteur
75724 Paris
France

Thomas D. Brock
Department of Bacteriology
University of Wisconsin-Madison
Madison, WI 53706
USA

Robert Brubaker
Department of Microbiology
Michigan State University
East Lansing, MI 48824
USA

Andreas Brune
Max Planck Institute for Terrestrial Microbiology
Marburg
Germany

Brita Bruun
Department of Clinical Microbiology
Hillerød Hospital
DK 3400 Hillerød
Denmark

Carmen Buchrieser
Laboratoire de Génomique des Microorganismes Pathogènes
Institut Pasteur
75724 Paris
France

Hans-Jürgen Busse
Institut für Bakteriology, Mykologie, und Hygiene
Veterinärmedizinische Universität Wien
A-1210 Vienna
Austria

Patrick Butaye
CODA-CERVA-VAR
1180 Brussels
Belgium

Gerald I. Byrne
Department of Medical Microbiology and Immunology
University of Wisconsin—Madison
Madison, WI 53706
USA

Didier Cabanes
Department of Immunology and Biology of Infection
Molecular Microbiology Group
Institute for Molecular and Cellular Biology
4150-180 Porto
Portugal

Melissa Caimano
Center for Microbial Pathogenesis
and
Department of Pathology
and
Department of Genetics and Development
University of Connecticut Health Center
Farmington, CT 06030-3205
USA

Ercole Canale-Parola
Department of Microbiology
University of Massachusetts
Amherst, MA 01003
USA

Elisabeth Carniel
Laboratoire des *Yersinia*
Institut Pasteur
75724 Paris
France

Colleen M. Cavanaugh
Bio Labs
Harvard University
Cambridge, MA 02138
USA

Jiann-Shin Chen
Department of Biochemistry
Virginia Polytechnic Institute and State University—Virginia Tech
Blacksburg, VA 24061-0308
USA

Zhongying Chen
Department of Biology
University of North Carolina
Chapel Hill, NC 27514
USA

Qi Cheng
University of Western Sydney
Penrith South
NSW 1797
Australia

Henrik Christensen
Department of Veterinary Microbiology
Royal Veterinary and Agricultural University
Denmark

Nicholas P. Cianciotto
Department of Microbiology and Immunology
Northwestern University School of Medicine
Chicago, IL
USA

Dieter Claus
Deutsche Sammlung von Mikroorganismen
D-3300 Braunschweig-Stockheim
Germany

P. Patrick Cleary
Department of Microbiology
University of Minnesota Medical School
Minneapolis, MN 55455
USA

Yehuda Cohen
Department of Molecular and Microbial Ecology
Institute of Life Science
Hebrew University of Jerusalem
91904 Jerusalem
Israel

Matthew D. Collins
Institute of Food Research
Reading Lab, Early Gate
UK

Guy Cornelis
Microbial Pathogenesis Unit
Université Catholique de Louvain and
Christian de Duve Institute of Cellular Pathology
B1200 Brussels
Belgium

Pascale Cossart
Unité des Interactions Bactéries-Cellules
INSERM U604
Institut Pasteur
75724 Paris
France

Michael Cotta
USDA-ARS North Regional Research Center
Peoria, IL 61604-3902
USA

Ronald L. Crawford
Food Research Center
University of Idaho
Moscow, ID 83844-1052
USA

Cecil S. Cummins
Department of Anaerobic Microbiology
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061
USA

Heribert Cypionka
Institut für Chemie und Biologie des Meeres
Fakultät 5, Mathematik und Naturwissenschaften
Universität Oldenburg
D-26111 Oldenburg
Germany

Milton S. da Costa
M. Fernanda Nobre
Centro de Neurociências e Biologia Celular
Departamento de Zoologia
Universidade de Coimbra
3004-517 Coimbra
Portugal

Rolf Daniel
Department of General Microbiology
Institute of Microbiology and Genetics
37077 Göttingen
Germany

Seana Davidson
University of Washington
Civil and Environmental Engineering
Seattle, WA 98195-2700
USA

Scott C. Dawson
Department of Molecular and Cellular Biology
University of California-Berkeley
Berkeley, CA 94720
USA

Dirk de Beer
Max-Planck-Institute for Marine Microbiology
D-28359 Bremen
Germany

Jan A.M. de Bont
Department of Food Science
Agricultural University
6700 EV Wageningen
The Netherlands

Maria H. Deinema
Laboratory of Microbiology
Agricultural University
6703 CT Wageningen
The Netherlands

Jozef de Ley
Laboratorium voor Microbiologie en Microbiële Genetica
Rijksuniversiteit Ghent
B-9000 Ghent
Belgium

Edward F. DeLong
Science Chair
Monterey Bay Aquarium Research Institute
Moss Landing, CA 95039
USA

Arnold L. Demain
Department of Biology
Massachusetts Institute of Technology
Cambridge, MA 02139
USA

Uwe Deppenmeier
Department of Biological Sciences
University of Wisconsin
Milwaukee, WI 53202
USA

Paul de Vos
Department of Biochemistry, Physiology and Microbiology
Universiteit Gent
B-9000 Gent
Belgium

Luc Devriese
Faculty of Veterinary Medicine
B982 Merelbeke
Belgium

Floyd E. Dewhirst
Forsyth Dental Center
140 Fenway
Boston, MA 02115
USA

Leon M. T. Dicks
Department of Microbiology
University of Stellenbosch
ZA-7600 Stellenbosch
South Africa

Michael P. Doyle
College of Agricultural and Environmental Sciences
Center for Food Safety and Quality Enhancement
University of Georgia
Griffin, GA 30223-1797
USA

Harold L. Drake
Department of Ecological Microbiology
BITOEK, University of Bayreuth
D-95440 Bayreuth
Germany

Patrick R. Dugan
Idaho National Engineering Laboratory
EG & G Idaho
Idaho Falls, ID 83415
USA

Paul V. Dunlap
Department of Molecular
Cellular and Developmental Biology
University of Michigan
Ann Arbor, MI 48109-1048
USA

Olivier Dussurget
Unité des Interactions Bactéries-Cellules
INSERM U604
Institut Pasteur
75724 Paris
France

Martin Dworkin
University of Minnesota Medical School
Department of Microbiology
Minneapolis, MN 55455
USA

Jürgen Eberspächer
Institut fur Mikrobiologie
Universitat Hohenheim
D-7000 Stuttgart 70
Germany

Paul H. Edelstein
Department of Pathology and Laboratory Medicine
University of Pennsylvania Medical Center
Philadelphia, PA 19104-4283
USA

Wolfgang Eder
Lehrstuhl für Mikrobiologie
Universität Regensburg
93053 Regensburg
Germany

Karen Elkins
CBER/FDA
Rockville, MD 20852
USA

Craig D. Ellermeier
Department of Microbiology
University of Illinois
Urbana, IL 61801
and
Department of Molecular and Cellular Biology
Harvard University
Cambridge, MA 02138
USA

Lyudmila I. Evtushenko
All-Russian Collection of Microorganisms
Institute of Biochemistry and Physiology of the Russian, Academy of Sciences
Puschino
Moscow Region, 142290
Russia

Takayuki Ezaki
Bacterial Department
Gifu University Medical School
40 Tsukasa
Machi Gifu City
Japan

Solly Faine
Monash University
Faculty of Medicine, Nursing and Health Sciences
Department of Microbiology
Clayton Campus
Victoria, 3800
Australia

J. J. Farmer, III
Center for Infectious Diseases
Centers for Disease Control
Atlanta, GA 30333
USA

W. Edmund Farrar
Department of Medicine
Medical University of South Carolina
Charleston, SC 29425
USA

Mariano E. Fernandez Miyakawa
California Animal Health and Food Safety Laboratory
University of California, Davis
San Bernardino, CA 92408
USA

Doran L. Fink
Edward Mallinckrodt Department of Pediatrics and Department of Molecular Microbiology
Washington University School of Medicine
St. Louis, Missouri 63110
USA

Jacqueline Fletcher
Department of Entomology and Plant Pathology
Oklahoma State University
Stillwater, OK
USA

Robert Forster
Bio-Products and Bio-Processes Program
Agriculture and Agri-Food Canada
Lethbridge Research Centre
Lethbridge T1J 4B1
Canada

M. Pilar Francino
Evolutionary Genomics Department
DOE Joint Genome Institute
Walnut Creek, CA 94598
USA

Charles M. A. P. Franz
Institute of Hygiene and Toxicology
BFEL
D-76131 Karlsruhe
Germany

David N. Fredricks
VA Palo Alto Healthcare System
Palo Alto, CA 94304
USA

J. K. Fredrickson
Pacific Northwest National Laboratory
Richland, Washington 99352
USA

Bärbel Friedrich
Institut für Biologie/Mikrobiologie
Homboldt-Universität zu Berlin
Chaussesstr. 117
D-10115 Berlin
Germany

Dagmar Fritze
Deutsche Sammlung von Mikroorganismen
D-3300 Braunschweig-Stockheim
Germany

John A. Fuerst
Department of Microbiology and Parasitology
University of Queensland
Brisbane
Queensland 4072
Australia

Hiroshi Fukushima
Public Health Institute of Shimane Prefecture
582-1 Nishihamasada, Matsue
Shimane 690-0122
Japan

Jean-Louis Garcia
Laboratoire ORSTOM de Microbiologie des Anaérobies
Université de Provence
CESB-ESIL
13288 Marseille
France

Ferran Garcia-Pichel
Associate Professor
Arizona State University
Tempe, AZ 85281
USA

Arnold Geis
Institut für Mikrobiologie
Bundesanstalt für Milchforschung
D-24121 Kiel
Germany

Caroline Attardo Genco
Department of Medicine
Section of Infectious Diseases and Department of Microbiology
Boston University School of Medicine
Boston, MA 02118
USA

Yves Germani
Institut Pasteur
Unité Pathogénie Microbienne Moléculaire
and
Réseau International des Instituts Pasteur
Paris 15
France

Frank C. Gibson
Department of Medicine
Section of Infectious Diseases
and ‘
Department of Microbiology
Boston University School of Medicine
Boston, MA 02118
USA

Monique Gillis
Laboratorium voor Mikrobiologie
Universiteit Gent
B-9000 Gent
Belgium

Stephen Giovannoni
Department of Microbiology
Oregon State University
Corvallis, OR 97331
USA

Frank Oliver Glöckner
Max-Planck-Institut für Marine Mikrobiologie
D-28359 Bremen
Germany

Ulf B. Göbel
Institut für Mikrobiologie und Hygiene
Universitaetsklinikum Chariteacute
Humboldt-Universitaet zu Berlin
D-10117 Berlin
Germany

José M. González
Department de Microbiologia y Biologia Celular
Facultad de Farmacia
Universidad de La Laguna
38071 La Laguna, Tenerife
SPAIN

Michael Goodfellow
School of Biology
Universtiy of Newcastle
Newcastle upon Tyre NE1 7RU
UK

Friedrich Götz
Facultät für Biologie
Institut für Microbielle Genetik
Universität Tübingen
D-72076 Tübingen
Germany

Hans-Dieter Görtz
Department of Zoology
Biologisches Institut
Universität Stuttgart
D-70569 Stuttgart
Germany

Gerhard Gottschalk
Institut für Mikrobiologie und Genetik
Georg-August-Universität Göttingen
D-37077 Göttingen
Germany

P. H. Graham
Department of Soil, Water, and Climate
St. Paul, MN 55108
USA

Paul A. Granato
Department of Microbiology and Immunology
State University of New York Upstate Medical University
Syracus, NY 13210
USA

Peter N. Green
NCIMB Ltd
AB24 3RY Aberdeen
UK

James R. Greenwood
Bio-Diagnostics Laboratories
Torrance, CA 90503
USA

Francine Grimont
Unite 199 INSERM
Institut Pasteur
75724 Paris
France

Patrick A. D. Grimont
Institut Pasteur
75724 Paris
France

Roland Gross
Institut für Mikrobiologie
Johann Wolfgang Goethe-Universität
Frankfurt am Main
Germany

Ji-Dong Gu
Laboratory of Environmental Toxicology
Department of Ecology & Biodiversity
and
The Swire Institute of Marine Science
University of Hong Kong
Hong Kong SAR
P.R. China
and
Environmental and Molecular Microbiology
South China Sea Institute of Oceanography
Chinese Academy of Sciences
Guangzhou 510301
P.R. China

Françoise Guinet
Laboratoire des *Yersinia*
Institut Pasteur
75724 Paris
France

Michael Gurevitz
Department of Botany
Life Sciences Institute
Tel Aviv University
Ramat Aviv 69978
Israel

H. M. Hafez
Institute of Poultry Diseases
Free University Berlin
Berlin
German

Auli Haikara
VTT Biotechnology
Tietotie 2, Espoo
Finland

Walter P. Hammes
Institute of Food Technology
Universität Hohenheim
D-70599 Stuttgart
Germany

Satoshi Hanada
Research Institute of Biological Resources
National Institute of Advanced Industrial
Science and Technology (AIST)
Tsukuba 305-8566
Japan

Hans H. Hanert
Institut für Mikrobiologie
Technische Univeristät Braunschweig
D-3300 Braunschweig
Germany

Michael Hannig
Lehrstuhl für Mikrobiologie
Universität Regensburg
D-93053 Regensburg
Germany

Theo A. Hansen
Microbial Physiology (MICFYS)
Groningen University
Rijksuniversiteit Groningen
NL-9700 AB Groningen
The Netherlands

Jeremy M. Hardie
Department of Oral Microbiology
School of Medicine & Dentistry
London E1 2AD
UK

Timothy Harrah
Bioengineering Center
Tufts University
Medford, MA 02155
USA

Anton Hartmann
GSF-National Research Center for
Environment and Health
Institute of Soil Ecology
Rhizosphere Biology Division
D-85764 Neuherberg/Muenchen
Germany

Sybe Hartmans
Department of Food Science
Agricultural University Wageningen
6700 EV Wageningen
The Netherlands

Patricia Hartzell
Department of Microbiology, Molecular Biology, and Biochemistry
University of Idaho
Moscow, ID 83844-3052
USA

Sydney M. Harvey
Nichols Institute Reference Laboratories
32961 Calle Perfecto
San Juan Capistrano, CA 92675
USA

John P. Hays
Department of Medical Microbiology and Infectious Diseases
Erasmus MC
3015 GD Rotterdam
The Netherlands

Reiner Hedderich
Max Planck Institute für Terrestriche Mikrobiologie
D-35043 Marburg
Germany

Brian P. Hedlund
Department of Biological Sciences
University of Nevada, Las Vegas
Las Vegas, NV 89154-4004
USA

Robert A. Heinzen
Department of Molecular Biology
University of Wyoming
Laramie, WY 82071-3944
USA

Ilkka Helander
VTT Biotechnology
Tietotie 2, Espoo
Finland

H. Ernest Hemphill
Department of Biology
Syracuse University
Syracuse, NY 13244
USA

Christian Hertel
Institute of Food Technology
Universität Hohenheim
D-70599 Stuttgart
Germany

Robert B. Hespell
Northern Regional Research Center, ARS
US Department of Agriculture
Peoria, IL 61604
USA

F. W. Hickman-Brenner
Center for Infectious Diseases
Centers for Disease Control
Atlanta, GA 30333
USA

Donald C. Hildebrand
Department of Plant Pathology
University of California-Berkeley
Berkeley, CA 94720
USA

Gregory Hinkle
Department of Botany
University of Massachusetts
Amherst, MA 01003
USA

K. H. Hinz
Clinic for Poultry
School of Veterinary Medicine
D-30559 Hannover
Germany

Peter Hirsch
Institut für Allgemeine Mikrobiologie
Universität Kiel
D-2300 Kiel
Germany

Tor Hofstad
Department of Microbiology and Immunology
University of Bergen
N-5021 Bergen
Norway

Michael J. Hohn
Lehrstuhl für Mikrobiologie
Universität Regensburg
D-93053 Regensburg
Germany

Barry Holmes
Central Public Health Laboratory
National Collection of Type Cultures
London NW9 5HT
UK

Wilhelm H. Holzapfel
Federal Research Centre of Nutrition
Institute of Hygiene and Toxicology
D-76131 Karlsruhe
Germany

Harald Huber
Lehrstuhl für Mikrobiologie
Universität Regensburg
D-93053 Regensburg
Germany

Robert Huber
Lehrstuhl für Mikrobiologie
Universität Regensburg
D-93053 Regensburg
Germany

Celia J. Hugo
Department of Microbial, Biochemical and Food Biotechnology
University of the Free State
Bloemfontein
South Africa

Meredith Hullar
University of Washington
Seattle, WA
USA

Thomas Hurek
Laboratory of General Microbiology
University Bremen
28334 Bremen
Germany

Johannes F. Imhoff
Marine Mikrobiologie
Institut für Meereskunde an der Universität Kiel
D-24105 Kiel
Germany

Ralph Isberg
Department of Molecular Biology and Microbiology
Tufts University School of Medicine
Boston, MA 02111
USA

Elena P. Ivanova
Senior Researcher in Biology
Laboratory of Microbiology
Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences
690022 Vladivostok
Russia

Rainer Jaenicke
6885824 Schwalbach a. Ts.
Germany
and
Institut für Biophysik und Physikalische Biochemie
Universität Regensburg
Regensburg
Germany
and
School of Crystallography
Birbeck College
University of London
London, UK

J. Michael Janda
Microbial Diseases Laboratory
Division of Communicable Disease Control
California Department of Health Services
Berkeley, CA 94704-1011
USA

Holger W. Jannasch
Woods Hole Oceanographic Institution
Woods Hole, MA 02543
USA

Christian Jeanthon
UMR CNRS 6539–LEMAR
Institut Universitaire Europeen de la Mer
Technopole Brest Iroise
29280 Plouzane
France

Cheryl Jenkins
Department of Microbiology
University of Washington
Seattle, WA 98195
USA

John L. Johnson
Department of Anaerobic Microbiology
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061
USA

Dorothy Jones
Department of Microbiology
University of Leicester, School of Medicine
Lancaster LE1 9HN
UK

Piet J. Jooste
Department of Biotechnology and Food Technology
Tshwane University of Technology
Pretoria 0001
South Africa

Edouard Jurkevitch
Department of Plant Pathology and Microbiology
Faculty of Agriculture
Food & Environmental Quality Services
The Hebrew University
76100 Rehovot
Israel

Clarence I. Kado
Department of Plant Pathology
University of California, Davis
Davis, CA 95616-5224
USA

Dale Kaiser
Department of Biochemistry
Stanford University School of Medicine
Stanford, CA 94305-5329
USA

Murat V. Kalayoglu
Department of Medical Microbiology and Immunology
University of Wisconsin—Madison
Madison, WI 53706
USA

Peter Kämpfer
Institut für Angewandte Mikrobiologie
Justus Liebig-Universität
D-35392 Gießen
Germany

David Kaplan
Department of Chemcial and Biological Engineering
Tufts University
Medford, MA 02115
USA

Yoshiaki Kawamura
Department of Microbiology
Regeneration and Advanced Medical Science
Gifu University Graduate School of Medicine
Gifu 501-1194
Japan

Ronald M. Keddie
Craigdhu
Fortrose
Ross-shire IV 10 8SS
UK

Donovan P. Kelly
University of Warwick
Department of Biological Sciences
CV4 7AL Coventry
UK

Melissa M. Kendall
Department of Biology
Portland State University
Portland, OR 97207-0751
USA

Karel Kersters
Laboratorium voor Mikrobiologie
Department of Biochemistry
Physiology and Microbiology
Universiteit Gent
B-9000 Gent
Belgium

Nadia Khelef
Unité des Interactions Bactéries-Cellules
INSERM U604
Institut Pasteur
75724 Paris
France

Kumiko Kita-Tsukamoto
Ocean Research Institute
University of Tokyo
Tokyo 164
Japan

Oliver Klimmek
Johann Wolfgang Goethe-Universität Frankfurt
Institut für Mikrobiologie
D-60439 Frankfurt
Germany

Wesley E. Kloos
Department of Genetics
North Carolina State University
Raleigh, NC 27695-7614
USA

Miloslav Kocur
Czechoslovak Collection of Microorganisms
J.E. Purkyně University
662 43 Brno
Czechoslovakia

Paul Kolenbrander
National Institute of Dental Research
National Institute of Health
Bethesda, MD 20892-4350
USA

Kazuo Komagata
Laboratory of General and Applied Microbiology
Department of Applied Biology and Chemistry
Faculty of Applied Bioscience
Tokyo University of Agriculture
Tokyo, Japan

Hans-Peter Koops
Institut für Allgemeine Botanik
Abteilung Mikrobiologie
Universität Hamburg
D-22069 Hamburg
Germany

Noel R. Krieg
Department of Biology
Virginia Polytechnic Institute
Blacksburg, VA 24061-0406
USA

Achim Kröger
Institut für Mikrobiologie
Biozentrum Niederursel
D-60439 Frankfurt/Main
Germany

Reiner Michael Kroppenstedt
Deutsche Sammlung von Mikroorganismen und Zellkulturen
D-3300 Braunschweig
Germany

Terry Ann Krulwich
Department of Biochemistry
Mount Sinai School of Medicine
New York, NY 10029
USA

J. Gijs Kuenen
Department of Biotechnology
Delft University of Technology
2628BC Delft
The Netherlands

Daisy A. Kuhn
Department of Biology
California State University
Northridge, CA 91330
USA

Hidehiko Kumagai
Division of Applied Sciences
Graduate School of Agriculture
Kyoto University
Kitashirakawa
606 8502 Kyoto
Japan

Barbara N. Kunkel
Department of Biology
Washington University
St. Louis, MO 63130
USA

Kirsten Küsel
Department of Ecological Microbiology
BITOEK, University of Bayreuth
D-95440 Bayreuth
Germany

David P. Labeda
Microbial Genomics and Bioprocessing Research Unit
National Center for Agricultural Utilization Research
Agricultural Research Service
U.S. Department of Agriculture
Peoria, IL 61604
USA

Edward T. Lally
Leon Levy Research Center for Oral Biology
University of Pennsylvania
Philadelphia, Pennsylvania, 19104-6002
USA

Bart Lambert
Plant Genetic Systems N.V.
J. Plateaustraat 22
B-9000 Ghent
Belgium

Raphael Lamed
Department of Molecular Microbiology and Biotechnology
George S. Wise Faculty of Life Sciences
Tel Aviv University
Ramat Aviv 69978
Israel

Giancarlo Lancini
Consultant, Vicuron Pharmaceutical
21040 Gerenzano (Varese)
Italy

Jan W. M. la Rivière
Institut für Mikrobiologie
Universität Göttingen
D-3400 Göttingen
Germany

Jared R. Leadbetter
Environmental Science and Engineering
California Institute of Technology
Pasadena, CA 91125-7800
USA

Donald J. LeBlanc
ID Genomics
Pharmacia Corporation
Kalamazoo, MI 49001
USA

Marc Lecuit
Unité des Interactions Bactéries-Cellules
INSERM U604
Institut Pasteur
75724 Paris
France

Adrian Lee
School of Microbiology & Immunology
University of New South Wales
Sydney, New South Wales
2052 Australia

Natuschka Lee
Lehrstuhl für Mikrobiologie
Technische Universität München
D-85350 Freising
Germany

Susan Leschine
Department of Microbiology
University of Massachusetts
Amherst, MA 01003-5720
USA

Na (Michael) Li
Division of Biostatistics
School of Public Health
University of Minnesota
Minneapolis, MN 55455
USA

Mary E. Lidstrom
Department of Chemical Engineering
University of Washington
Seattle, WA 98195
USA

Wolfgang Liebl
Institut für Mikrobiologie und Genetik
Georg-August-Universität
D-37077 Göttingen
Germany

Franz Lingens
Institut fur Mikrobiologie
Universitat Hohenheim
D-7000 Stuttgart 70
Germany

Puspita Lisdiyanti
Laboratory of General and Applied Microbiology
Department of Applied Biology and Chemistry
Faculty of Applied Bioscience
Tokyo University of Agriculture
Tokyo, Japan

Derek Lovley
Department of Microbiology
University of Massachusetts
Amherst, MA 01003
USA

Wolfgang Ludwig
Lehrstuhl für Mikrobiologie
Technische Universität München
D-85350 Freising
Germany

David Lyerly
TechLab, Inc.
Corporate Research Center
Blacksburg VA 24060-6364
USA

Janet I. Macinnes
University of Guelph
Guelph N1G 2W1
Canada

Michael T. Madigan
Department of Microbiology
Mailcode 6508
Southern Illinois University
Carbondale, IL 62901-4399
USA

Luis Angel Maldonado
School of Biology
Universidad Nacional Autonoma de Mexico (UNAM)
Instituto de Ciencias del Mar y Limnologia
Ciudad Universitaria CP
04510 Mexico DF
Mexico

Jim Manos
The University of Maryland Biotechnology Institute
Center of Marine Biotechnology
Baltimore, MD 21202

Lynn Margulis
Department of Botany
University of Massachusetts
Amherst, MA 01003
USA

Kevin C. Marshall
School of Microbiology
University of New South Wales
Kensington
New South Wales 2033
Australia

Esperanza Martinez-Romero
Centro de Investigacion sobre Fijacion de Nitrogeno
Cuernavaca Mor
Mexico

Vitor A. P. Martins dos Santos
Gesellschaft für Biotechnologische Forschung
Division of Microbiology
Braunschweig D-38124
Germany

Vega Masignani
IRIS, Chiron SpA
53100 Siena
Italy

Paola Mattarelli
Istituto di Microbiologia Agraria
40126 Bologna
Italy

Carola Matthies
Department of Ecological Microbiology
BITOEK, University of Bayreuth
D-95440 Bayreuth
Germany

Ann G. Matthysse
Department of Biology
University of North Carolina
Chapel Hill, NC 27599
USA

Megan E. McBee
Biological Engineering Division
Massachusetts Institute of Technology
Cambridge, MA
USA

Bruce A. McClane
Department of Molecular Genetics and Biochemistry
University of Pittsburgh School of Medicine
Pittsburgh, PA 15261
USA

Zoe P. McKiness
Department of Organic and Evolutionary Biology
Harvard University
Cambridge, MA 02138
USA

Ulrich Melcher
Department of Biochemistry and Molecular Biology
Oklahoma State University
Stillwater, OK
USA

Jianghong Meng
Nutrition and Food Science
University of Maryland
College Park, MD 20742-7521
USA

Valery V. Mikhailov
Pacific Institute of Bioorganic Chemistry
Far-Eastern Branch of the Russian Academy of Sciences
690022 Vladivostok
Russia

Melissa B. Miller, Ph.D.
Department of Pathology and Laboratory Medicine
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599
USA

Michael F. Minnick
Division of Biological Sciences
University of Montana
Missoula, MT 59812-4824
USA

Ralph Mitchell
Laboratory of Microbial Ecology
Division of Engineering and Applied Sciences
Harvard University
Cambridge, MA 02138
USA

Cesare Montecucco
Professor of General Pathology
Venetian Institute for Molecular Medicine
35129 Padova
Italy

Edward R. B. Moore
The Macaulay Institute
Environmental Sciences Group
Aberdeen AB158QH
UK
and
Culture Collection University of Göteborg (CCUG)
Department of Clinical Bacteriology
University of Göteborg
Göteborg SE-416 43
Sweden

Nancy A. Moran
University of Arizona
Department of Ecology and Evolutionary Biology
Tucson, AZ 85721
USA

Edgardo Moreno
Tropical Disease Research Program (PIET)
Veterinary School, Universidad Nacional
Costa Rica

Ignacio Moriyón
Department of Microbiology
University of Navarra
32080 Pamplona
Spain

Annette Moter
Institut für Mikrobiologie und Hygiene
Universitaetsklinikum Chariteacute
Humboldt-Universität zu Berlin
D-10117 Berlin
Germany

Eppe Gerke Mulder
Laboratory of Microbiology
Agricultural University
6703 CT Wageningen
The Netherlands

Yasuyoshi Nakagawa
Biological Resource Center (NBRC)
Department of Biotechnology
National Institute of Technology and Evaluation
Chiba 292-0818
Japan

Francis Nano
Department of Biochemistry & Microbiology
University of Victoria
Victoria V8W 3PG
Canada

Kenneth H. Nealson
Department of Earth Sciences
University of Southern California
Los Angeles, CA 90033
USA

Douglas C. Nelson
Department of Microbiology
University of California, Davis
Davis, CA 95616
USA

Klaus Neuhaus
Department of Pediatrics, Infection, Immunity, and Infectious Diseases Unit
Washington University School of Medicine
St. Louis, MO 63110
USA

Diane G. Newell
Veterinary Laboratory Agency (Weybridge)
Addlestone
New Haw
Surrey KT1 53NB
UK

Irene L. G. Newton
Department of Organismic and Evolutionary Biology
Harvard University
Cambridge, MA 02138
USA

S.A. Nierzwicki-Bauer
Department of Biology
Rensselaer Polytechnic Institute
Troy, NY
USA

M. Fernanda Nobre
Departamento de Zoologia
Universidade de Coimbra
3004-517 Coimbra
Portugal

Philippe Normand
Laboratoire d'Ecologie Microbienne
UMR CNRS 5557
Université Claude-Bernard Lyon 1
69622 Villeurbanne
France

Steven J. Norris
Department of Pathology and Laboratory Medicine and Microbiology and Molecular Genetics
University of Texas Medical Scvhool at Houston
Houston, TX 77225
USA

Howard Ochman
Department of Biochemistry and Molecular Biophysics
University of Arizona
Tucson, AZ 85721
USA

Gary E. Oertli
Molecular and Cellular Biology
Unviersity of Washington
Seattle, WA 98195-7275
USA

Itzhak Ofek
Department of Human Microbiology
Tel Aviv University
69978 Ramat Aviv
Israel

Bernard Ollivier
Laboratoire ORSTOM de Microbiologie des Anaérobies
Université de Provence
CESB-ESIL
13288 Marseille
France

Scott L. O'Neill
Department of Epidemiology and Public Health
Yale University School of Medicine
New Haven, CT 06520-8034
USA

Aharon Oren
Division of Microbial and Molecular Ecology
The Institute of Life Sciences
and
Moshe Shilo Minerva Center for Marine Biogeochemistry
The Hebrew University of Jerusalem
91904 Jerusalem
Israel

Jani L. O'Rourke
School of Microbiology and Immunology
University of New South Wales
Sydney, NSW 2052
Australia

Jörg Overmann
Bereich Mikrobiologie
Department Biologie I
Ludwig-Maximilians-Universität München
D-80638 München
Germany

Norman R. Pace
Department of Molecular, Cellular and Developmental Biology
Unversity of Colorado
Boulder, CO 80309-0347
USA

Norberto J. Palleroni
Rutgers University
Department of Biochemistry and Microbiology
New Brunswick 08901-8525
New Jersey
USA

Bruce Panilaitis
Department of Chemcial and Biomedical Engineering
Tufts University
Medford, MA 02155
USA

Nickolas Panopoulos
Department of Plant Pathology
University of California-Berkeley
Berkeley, CA 94720
USA

Yong-Ha Park
Korean Collection for Type Cultures
Korea Research Institute of Bioscience & Biotechnology
Taejon 305-600
Korea

Matthew R. Parsek
University of Iowa
Iowa City, IA 52242
USA

Bruce J. Paster
Department of Molecular Genetics
The Forsyth Institute
Boston, MA 02115
USA

Jerome J. Perry
3125 Eton Road
Raleigh, NC 27608-1113
USA

Jeannette Pham
The CDS Users Group
Department of Microbiology
South Eastern Area Laboratory Services
The Prince of Wales Hospital Campus
Randwick NSW 2031
Australia

Harvey M. Pickrum
Proctor and Gamble Company
Miami Valley Laboratories
Cincinnatti, OH 45239
USA

Dietmar H. Pieper
Gesellschaft für Biotechnologische Forschung
Division of Microbiology
Braunschweig D-38124
Germany

Beverly K. Pierson
Biology Department
University of Puget Sound
Tacoma, WA 98416
USA

Mariagrazia Pizza
IRIS, Chiron SpA
53100 Siena
Italy

Jeanne S. Poindexter
Department of Biological Sciences
Barnard College/Columbia University
New York, NY 10027-6598
USA

Andreas Pommerening-Röser
Institut für Allgemeine Botanik
Abteilung Mikrobiologie
Universität Hamburg
D-22069 Hamburg
Germany

Michel R. Popoff
Unité des Toxines Microbiennes
Institut Pasteur
75724 Paris
France

Anton F. Post
Department of Plant and Environmental Sciences
Life Sciences Institute
Hebrew University
Givat Ram
91906 Jerusalem
Israel

Bruno Pot
Laboratorium voor Microbiologie en Microbiële Genetica
Rijksuniversiteit Ghent
B-9000 Ghent
Belgium

David Prangishvili
Department of Mikrobiology
Universitity of Regensburg
D-93053 Regensburg
Germany

Helmut Prauser
DSMZ-German Collection of Microorganisms and Cell Cultures GmbH
D-38124 Braunschweig
Germany

Michael Prentice
Bart's and the London School of Medicine and Dentistry
Department of Medical Microbiology
St. Bartholomew's Hospital
London EC1A 7BE
UK

Ulrike Purkhold
Lehrstuhl für Mikrobiologie
Technische Universität München
D-80290 Munich
Germany

Wim J. Quax
Department of Pharmaceutical Biology
University of Groningen
Groningen 9713AV
The Netherlands

Erika Teresa Quintana
School of Biology
Universtiy of Newcastle
Newcastle upon Tyne NE1 7RU
UK

Ralf Rabus
Max-Planck-Institut für Marine Mikrobiologie
D-28359 Bremen
Germany

Reinhard Rachel
Lehrstuhl für Mikrobiologie
Universität Regensburg
D-93053 Regensburg
Germany

A. N. Rai
Biochemistry Department
North-Eastern Hill University
Shillong 793022
India

Frederick A. Rainey
Department of Biological Sciences
Louisiana State University
Baton Rouge, LA 70803
USA

Juan-Luis Ramos
Estación Experimental del Zaidin
Department of Biochemistry and Molecular and Cell Biology of Plants
Granada E-18008
Spain

Rino Rappuoli
IRIS Chiron Biocine Immunobiologie
Research Institute Siena
53100 Siena
Italy

Shmuel Razin
Department of Membrane and Ultrastructure Research
The Hebrew University-Hadassah Medical School
Jerusalem 91120

Annette C. Reboli
Department of Medicine
Hahneman University Hospital
Philadelphia, PA 19102
USA

David W. Reed
Biotechnology Department
Idaho National Engineering and
Environmental Laboratory (INEEL)
Idaho Falls, ID 83415-2203
USA

Hans Reichenbach
GBF
D-3300 Braunschweig
Germany

Barbara Reinhold-Hurek
Laboratory of General Microbiology
Universität Bremen
Laboratorium für Allgemeine Mikrobiologie
D-28334 Bremen
Germany

Markus Riegler
Integrative Biology School
University of Queensland
Australia

Monica Riley
Marine Biological Lab
Woods Hole, MA 02543
USA

Lesley A. Robertson
Department of Biotechnology
Delft University of Technology
2628 BC Delft
The Netherlands

Edson R. Rocha
Department of Microbiology and Immunology
East Carolina University
Greenville, NC 27858-4354
USA

Palmer Rogers
Department of Microbiology
University of Minnesota Medical School
Minneapolis, MN 55455
USA

Lyudmila A. Romanenko
Senior Researcher in Biology
Laboratory of Microbiology
Pacific Institute of Bioorganic Chemistry of the
Far-Eastern Branch of the Russian Academy
of Sciences
Vladivostoku, 159
Russia

M. F. Romine
Pacific Northwest National Laboratory
Richland, WA 99352
USA

Eliora Z. Ron
Department of Molecular Microbiology and
Biotechnology
The George S. Wise Faculty of Life Sciences
Tel Aviv University
Ramat Aviv
69978 Tel Aviv
Israel

Julian I. Rood
Australian Bacterial Pathogenesis Program
Department of Microbiology
Monash University
Victoria 3800
Australia

Eugene Rosenberg
Department of Molecular Microbiology &
Biotechnology
Tel Aviv University
Ramat Aviv
69978 Tel Aviv
Israel

Frank Rosenzweig
Division of Biological Sciences
University of Montana
Missoula, MT 59812-4824
USA

Ornella Rossetto
Centro CNR Biomembrane and Dipartimento
di Scienze Biomediche
35100 Padova
Italy

Michael J. Sadowsky
Department of Soil, Water, and Climate
University of Minnesota
Minneapolis, MN 55455
USA

Hermann Sahm
Institut Biotechnologie
Forschungszentrum Jülich
D-52425 Jülich
Germany

Joseph W. St. Gemer, III
Department of Molecular Microbiology
Washington University School of Medicine
St. Louis, MO 63110
USA

James E. Samuel
Department of Medical Microbiology and Immunology
College of Medicine
Texas A&M University System Health Science Center
College Station, TX, 77843-1114
USA

Philippe J. Sansonetti
Unité de Pathogénie
Microbienne Moléculaire
Institut Pasteur
75724 Paris
France

Scott R. Santos
Department of Biochemistry & Molecular Biophysics
University of Arizona
Tucson, AZ 85721
USA

Beatrice Saviola
Departments of Molecular Microbiology and Immunology
Johns Hopkins School of Hygiene and Public Health
Baltimore, MD 21205-2105
USA

Klaus P. Schaal
Institut für Medizinische
Mikrobiologie und Immunologie
Universität Bonn
D-53105 Bonn
Germany

David B. Schauer
Biological Engineering Division and Division of Comparative Medicine
Massachusetts Institute of Technology
Cambridge, MA 02139
USA

Siegfried Scherer
Department für Biowißenschaftliche Grundlagen
Wißenschaftszentrum Weihenstephan
Technische Universität München
D-85354 Freising, Germany

Bernhard Schink
Fakultät für Biologie der Universität Konstanz
D-78434 Konstanz
Germany

Hans G. Schlegel
Institut für Mikrobiologie der Gessellschaft für Strahlen- und Umweltforschung mbH
Göttingen
Germany

Karl-Heinz Schleifer
Lehrstruhl für Mikrobiologie
Technische Universität München
D-85354 Freising
Germany

Heinz Schlesner
Institut für Allgemeine Mikrobiologie
Christian Albrechts Universität
D-24118 Kiel
Germany

Michael Schmid
GSF-Forschungszentrum für Umwelt und Gesundheit GmbH
Institut für Bodenökologie
D-85764 Neuherberg
Germany

Jean M. Schmidt
Department of Botany and Microbiology
Arizona State University
Tempe, AZ 85287
USA

Karin Schmidt
Institut für Mikrobiologie
Georg-August-Universität
D-3400 Göttingen
Germany

Ruth A. Schmitz
University of Göttingen
D-3400 Göttingen
Germany

Hildgund Schrempf
FB Biologie/Chemie
Universität Osnabrück
49069 Osnabrück
Germany

Milton N. Schroth
Department of Plant Pathology
University of California-Berkeley
Berkeley, CA 94720
USA

Heide N. Schulz
Institute for Microbiology
University of Hannover
D-30167 Hannover
Germany

Peter Schumann
DSMZ-German Collection of Microorganisms and Cell Cultures GmbH
D-38124 Braunschweig
Germany

Arthur Schüßler
Institut Botany
64287 Darmstadt
Germany

Edward Schwartz
Institut für Biologie/Mikrobiologie
Homboldt-Universität zu Berlin
D-10115 Berlin
Germany

James Scott
Geophysical Laboratory
Carnegie Institution of Washington
Washington, DC 20015
USA

Margrethe Haugge Serres
Marine Biological Lab
Woods Hole, MA 02543
USA

James P. Shapleigh
Department of Microbiology
Cornell University
Wing Hall
Ithaca, NY 14853-8101
USA

Nathan Sharon
The Weizmann Institute of Science
Department of Biological Chemistry
IL-76100 Rehovoth
Israel

Lawrence J. Shimkets
Department of Microbiology
The University of Georgia
Athens, GA 30602-2605
USA

Thomas M. Shinnick
Center for Infectious Diseases
Centers for Disease Control
Atlanta, GA 30333
USA

Yuval Shoham
Department of Food Engineering and Biotechnology
Technion—Israel Institute of Technology
Haifa 32000
Israel

Jörg Simon
Johann Wolfgang Goethe-Universität Frankfurt
Campus Riedberg
Institute of Molecular Biosciences
Molecular Microbiology and Bioenergetics
D-60439 Frankfurt
Germany

Michel Simonet
Départment de Pathogenèse des Maladies Infectieuses et Parasitaires
Institut de Biologie de Lille
59021 Lille
France

Mikael Skurnik
Department of Medical Biochemistry
University of Turku
20520 Turku
Finland

James M. Slauch
Department of Microbiology
College of Medicine
University of Illinois
and
Chemical and Life Sciences Laboratory
Urbana, IL 61801
USA

Ralph A. Slepecky
Department of Biology
Syracuse University
Syracuse, NY 13244
USA

C. Jeffrey Smith
Department of Microbiology and Immunology
East Carolina University
Greenville, NC 27858-4354
USA

Martin Sobierj
Department of Biology
Environmental Science and Engineering
Oregon Graduate Institute of Science and Technology
Portland State University
Portland, OR 97291-1000
USA

Pamela A. Sokol
Department of Microbiology and Infectious Diseases
University of Calgary Health Science Center
Calgary T2N 4N1
Canada

Jay V. Solnick
Department of Interanl Medicine (Infectious Diseases) and Medical
Microbiology and Immunology
University of California, Davis
School of Medicine
Davis, CA 95616
USA

Dimitry Yu. Sorokin
Department of Biotechnology
Delft University of Technology
2628 BC Delft
The Netherlands
and
S.N. Winogradsky Institute of Microbiology
117811 Moscow
Russia

Georg A. Sprenger
Institut Biotechnologie
Forschungszentrum Jülich
D-52425 Jülich
Germany

Stefan Spring
Deutsche Sammlung von Mikroorganismen und Zellkulturen
D-38124 Braunschweig
Germany

Erko Stackebrandt
Deutsche Sammlung von Mikroorganismen und Zellkulturen
D-38124 Braunschweig
Germany

David A. Stahl
University of Washington
Seattle, WA
USA

Donald P. Stahly
Department of Microbiology
University of Iowa
Iowa City, IA 52242
USA

James T. Staley
Department of Microbiology
University of Washington
Seattle, WA 98105
USA

Alfons J.M. Stams
Laboratorium voor Microbiologie
Wageningen University
NL-6703 CT Wageningen
The Netherlands

Thaddeus B. Stanton
PHFSED Research Unit
National Animal Disease Center
USDA-ARS
Ames, IA 50010
USA

Daniel C. Stein
Department of Cell Biology and Molecular Genetics
University of Maryland
College Park, MD 20742
USA

Reinhard Sterner
Universitaet Regensburg
Institut fuer Biophysik und Physikalische Biochemie
D-93053 Regensburg
Germany

Karl O. Stetter
Lehrstuhl für Mikrobiologie
Universität Regensburg
D-93053 Regensburg
Germany

Frank J. Stewart
Department of Organic and Evolutionary Biology
Harvard University
Cambridge, MA 02138
USA

Andreas Stolz
Institut für Mikrobiologie
Universität Stuttgart
70569 Stuttgart
Germany

Daphne L. Stoner
Idaho National Engineering Laboratory
EG & G Idaho
Idaho Falls, ID 83415
USA

Paul Stoodley
Center for Biofilm Engineering
Montana State University
Bozeman, MT 59717-3980
USA

James R. Swafford
Department of Botany and Microbiology
Arizona State University
Tempe, AZ 85287
USA

Jean Swings
Laboratorium voor Microbiologie
Department of Biochemistry
Physiology and Microbiology
BCCM/LMG Bacteria Collection
Universiteit Gent
Gent
Belgium

Mariko Takeuchi
Institute for Fermentation
Osaka 532-8686
Japan

Ralph Tanner
University of Oklahoma
Norman, OK, 73019-0390
USA

Andreas Teske
Department of Marine Sciences
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599
USA

Michael Teuber
ETH-Zentrum
Lab Food Microbiology
CH-8092 Zürich
Switzerland

Gabriele Timmermann
Institut für Allgemeine Botanik
Abteilung Mikrobiologie
Universität Hamburg
D-22069 Hamburg
Germany

Brian J. Tindall
Deutsche Sammlung von Mikroorganismen und Zellkulturen
Braunschweig D-38124
Germany

Kevin Towner
Consultant Clinical Scientist
Public Health Laboratory
University Hospital
Nottingham NG7 2UH
UK

Hans G. Trüper
Institut für Mikrobiologie und Biotechnologie
D-53115 Bonn
Germany

Elaine Tuomanen
Department of Infectious Diseases
St. Jude Children's Research Hospital
Memphis, TN 38105-2394
USA

Francisco A. Uzal
California Animal Health and Food Safety Laboratory
University of California, Davis
San Bernardino, CA 92408
USA

Peter Van damme
Laboraroorium voor Microbiologie
Faculteit Wetenschappen
Universiteit Gent
B-9000 Gent
Belgium

Antonio Ventosa
Department of Microbiology and Parasitology
Faculty of Pharmacy
University of Sevilla
41012 Sevilla
Spain

Gernot Vobis
Centro Regional Universitario Bariloche
Universidad Nacional de Comahue
Barioloche 8400, Rio Negro
Argentina

Alexander von Graevenitz
Department of Medical Microbiology
University of Zürich
GH-8028 Zürich
Switzerland

Günther Wächtershäuser
80331 Munich
Germany

Lawrence P. Wackett
Department of Biochemistry, Molecular Biology
and
Biophysics and Biological Process Technology Institute
University of Minnesota
St. Paul, MN, 55108-1030
USA

William G. Wade
Department of Microbiology
Guy's Campus
London, SE1 9RT
UK

Michael Wagner
Lehrstuhl für Mikrobielle Ökologie
Institut für Ökologie und Naturschutz
Universität Wien
A-1090 Vienna
Austria

David H. Walker
Department of Pathology
University of Texas Medical Branch
Galveston, TX 77555-0609
USA

Naomi Ward
The Institute for Genomic Research
Rockville, MD 20850
USA

Trudy M. Wassenaar
Molecular Microbiology and Genomics Consultants
55576 Zotzenheim
Germany

John B. Waterbury
Woods Hole Oceanographic Institution
Woods Hole, MA 02543
USA

Georges Wauters
Université Catholique de Louvain
Faculté de Médecine
Unité de Microbiologie
B-1200 Bruxelles
Belgium

Astri Wayadande
Department of Entomology and Plant Pathology
Oklahoma State University
Stillwater, OK
USA

Alison Weiss
Molecular Genetics, Biology and Microbiology
University of Cincinnati
Cincinnati, OH 45267
USA

Rodney A. Welch
Medical Microbiology and Immunology
University of Wisconsin
Madison, WI 53706-1532
USA

William B. Whitman
Department of Microbiology
University of Georgia
Athens, GA 30605-2605
USA

Friedrich Widdel
Max-Planck-Institut für Marine Mikrobiologie
D-28359 Bremen
Germany

Jürgen Wiegel
University of Georgia
Department of Microbiology
Athens, GA 30602
USA

Robert A. Whiley
Queen Mary, University of London
London E1 4NS
UK

Tracy Whilkins
TechLab, Inc.
Corporate Research Center
Blacksburg VA 24060-6364
USA

Anne Willems
Laboratorium voor Mikrobiologie
Universiteit Gent
B-9000 Gent
Belgium

Carl R. Woese
Department of Microbiology
University of Illinois
Urbana, IL 61801
USA

Ralph S. Wolfe
Department of Microbiology
University of Illinois
Urbana, IL 61801

Ann P. Wood
Division of Life Sciences
King's College London
London WC2R 2LS
UK

Donald E. Woods
Department of Microbiology and Infectious Diseases
University of Calgary Health Science Center
Calgary T2N 4N1
Canada

B. W. Wren
Department of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine
London WC1E 7HT
UK

Timothy L. Yahr
University of Iowa
Iowa City, IA 52242
USA

Atteyet F. Yassin
Institut für Medizinische
Mikrobiologie und Immunologie
Universität Bonn
D-53105 Bonn
Germany

Jung-Hoon Yoon
Korean Collection for Type Cultures
Korea Research Institute of Bioscience and Biotechnology
Yuson, Taejon 305-600
Korea

Allan A. Yousten
Biology Department
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061
USA

Xue-Jie Yu
University of Texas Medical Branch
Galveston, TX
USA

Vladimir V. Yurkov
Department of Microbiology
University of Manitoba
Winnipeg R3T 2N2
Canada

Georgi A. Zavarzin
Institute of Microbiology
Academy of Sciences of the USSR
117312 Moscow
Russia

Mary Jo Zidwick
Cargill Biotechnology Development Center
Freshwater Building
Minneapolis, MN 55440
USA

Stephen H. Zinder
Department of Microbiology
Cornell University
272 Wing Hall
Ithaca, NY 14853
USA

Prokaryotes (2006) 2:3–15
DOI: 10.1007/0-387-30742-7_1

CHAPTER 1.1

Planktonic Versus Sessile Life of Prokaryotes

KEVIN C. MARSHALL

Because of the extremely small size of most prokaryotic organisms, the limits on what is meant by the terms planktonic and sessile require definition. According to the *Oxford English Dictionary*, planktonic refers to "drifting or floating organic life found at various depths in the ocean or fresh water." At the micrometer level, a planktonic habitat for prokaryotes can also encompass water films around soil particles, saliva in the mouth, fluids in the intestinal lumen, serum in blood vessels, and urine in the bladder and urinary tract. Sessile, on the other hand, means "immediately attached, without a footstalk." Again, one can extend this definition to include those prokaryotes directly adhering to surfaces, those attaching by means of a holdfast at the end of a prostheca (e.g., *Caulobacter*), those embedded in biofilms developing as a result of extracellular polymer production by bacteria colonizing surfaces, and those colonizing mucus excreted by higher organisms (as in the gastrointestinal tract and the mucigel of plant roots).

Most microbiologists, oriented by their training to the study of pure cultures, regard suspension culture as the normal state for growth of these organisms. This is particularly true for research into the physiology and biochemistry of bacteria, whereby homogeneous suspensions of bacteria are readily harvested and manipulated for experimental purposes. The reality of prokaryotic life in natural habitats is that many organisms spend part or all of their life spans attached to surfaces (Marshall, 1976). However, recently there has been a veritable explosion in research devoted to understanding the behavior of bacteria at surfaces (Beachey, 1980; Bitton and Marshall, 1980; Marshall, 1984; Savage and Fletcher, 1985).

Many questions arise regarding the association of bacteria with surfaces. It is my aim in this chapter to consider the current state of knowledge concerning the following questions: How do prokaryotes adhere to surfaces? Is there a single, all-embracing mechanism or a range of mechanisms of adhesion in different organisms? Are some prokaryotes especially adapted to a sessile existence? Are particular organisms homogeneous in their adhesive characteristics or are they variable in their response to surfaces? Once attached to a surface, do prokaryotes always remain in a sessile state or do they return to the planktonic state at some stage? Do prokaryotes gain any real advantage from being associated with surfaces? Are certain prokaryotes specifically adapted to the colonization of excreted mucous layers? Are sessile bacteria in a different physiological state from planktonic organisms; that is, do prokaryotes exhibit a physiological response to contact with a surface? If they show such responses, what physicochemical factors are responsible for inducing the responses?

Mechanisms of Adhesion to Surfaces

Full details of proposed mechanisms of adhesion of prokaryotes to solid surfaces have been presented elsewhere (Marshall, 1985, 1986a) so only a brief outline will be presented in this paper.

Transport Processes

Water currents induced by temperature and gravity (fluid dynamic forces) provide the major mechanism for the transport of planktonic bacteria over large distances. When bacteria and other particles in flowing water are transported to the region of the boundary layer near a solid surface, a lift force directs the bacteria toward the surface where fluid frictional forces slow them down (Characklis, 1981a) and deposit them in the vicinity of the surface.

Sedimentation is of significance only when bacteria are aggregated together or are attached to particles. Individual bacteria behave essentially as colloidal particles (Marshall, 1976) and tend to remain in suspension. Nutrient gradients may become established across the boundary layer near some surfaces and these may provide opportunities for chemotactic responses towards the surfaces by motile bacteria. Brownian motion can account for random movement of very small bacteria within the quiescent water of a boundary layer near a surface (Marshall, 1976).

Long-Range Forces

Bacteria in the vicinity of a solid-liquid interface frequently show an instantaneous but reversible attraction to the interface (Marshall et al., 1971a), and an attempt has been made to explain this reversible attraction by means of the colloid stability (DLVO named for the originators—Derjaguin, Landau, Verwey, and Overbeek) theory. That this attraction is reversible is shown by the fact that the bacteria can be removed from the solid surface by gentle shearing forces. The DLVO theory accounts, at least in part, for the attraction of a negatively charged bacterium to a negatively charged substratum surface at the "secondary attraction minimum" resulting from the interaction between London-van der Waals attraction forces and electrical repulsion forces in the overlapping double layers of cations surrounding the negatively charged surfaces. In terms of the DLVO theory, a bacterial cell would be held at a distance of some 10 nm from the surface by repulsion forces.

Problems in applying DLVO theory to biological systems have been raised by Pethica (1980) and Rutter and Vincent (1980), especially when the complexity of the bacterial cell envelope and the extracellular components are taken into account. However, Busscher and Weerkamp (1987) have argued strongly in favor of such long-range forces in the initial attraction of bacteria to surfaces.

Short-Range Forces

Certain bacteria irreversibly attach to surfaces very rapidly (Fletcher, 1980), whereas other bacteria require a significant time of exposure to the surface before becoming firmly attached (Marshall et al., 1971a). Irreversible attraction is shown by the fact that the bacteria cannot be removed by moderate shear forces. What is the mechanism of this firm adhesion of bacteria to surfaces? Early observations indicated that polymer bridging by extracellular components of cells to the substratum surface (Fig. 1) resulted in firm adhesion (Marshall and Cruickshank, 1973; Fletcher and Floodgate, 1973), and these observations have been confirmed for many systems (Corpe, 1980; Costerton et al., 1981). These extracellular polymers have a small radius of curvature and can overcome any repulsion barrier near a surface and, thus, can bind the cell to the surface using a variety of short-range forces. These forces include: 1) chemical bonds (electrostatic, covalent, and hydrogen); 2) dipole interactions (dipole-dipole, dipole-induced dipole, and ion-dipole); and 3) hydrophobic interactions (Rutter and Vincent, 1980).

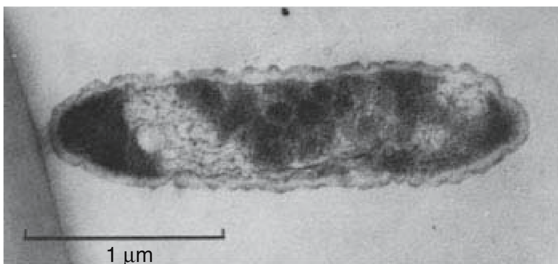

Fig. 1. Perpendicular adhesion of a marine bacterium to a solid plastic surface. The extracellular polymeric substances bridging between the cell and the surface are present only at the adhesive pole of the cell. (Courtesy of R. H. Cruickshank.)

Adhesion to surfaces in nature is generally considered to be *nonspecific*. That is, the bacteria adhere to a wide variety of different inanimate, and possibly animate, surfaces with varying degrees of adhesive strength. Bridging polymers involved in most cases of nonspecific adhesion are either extracellular polysaccharides, proteins, or glycoproteins. The precise mechanisms whereby such polymers interact with a range of substratum surfaces is not known, but it almost certainly involves various combinations of the short-range forces listed above.

Specific adhesion involves lectin-receptor-type mechanisms, in which a proteinaceous substance (lectin) on the bacterial surface reacts with a complementary carbohydrate receptor on another cell type (Switalski et al., 1989). The best-described examples of specific adhesion involve the attachment of pathogenic bacteria to the host cell surfaces they infect. However, specific attachment of bacteria to the heterocysts of the cyanobacterium *Anabaena* has been described (Lupton and Marshall, 1981).

Thermodynamic Approach to Bacterial Adhesion

Various workers have attempted to relate the extent of bacterial adhesion to the variation in surface free energy of the substratum, with very variable results (Dexter et al., 1975; Fletcher and Loeb, 1979). More detailed studies revealed that, in addition to the substratum-surface free energy, it was necessary to consider the bacterium-surface free energy and the surface tension of the liquid (Absolom et al., 1983; Pringle and Fletcher, 1983). The change in free energy associated with bacterial adhesion (ΔF_{adh}) is given by:

$$\Delta F_{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL}$$

where γ_{BS}, γ_{BL}, and γ_{SL} are the bacterium-substratum, bacterium-liquid, and substratum-liquid interfacial tensions, respectively. Bacterial adhesion is favored if the process results in a free energy decrease. In general, Absolom et al. (1983) found good agreement between bacterial adhesion to a variety of substrata and the adhe-

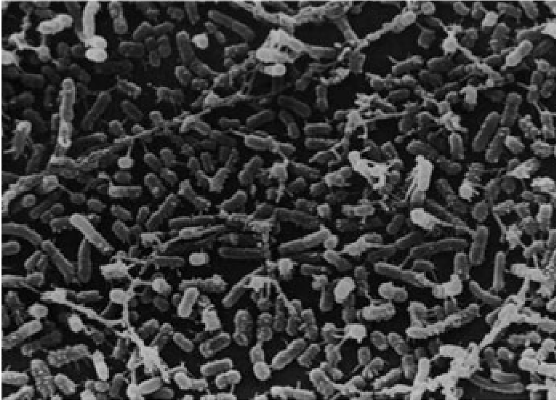

Fig. 2. Colonization of a glass surface, rendered hydrophobic by treatment with silane, by a marine bacterium after 16 h exposure. The condensed extracellular polymeric substances are clearly visible, as a result of drying on a cold stage. (Courtesy of T. Neu.)

sion behavior predicted by the thermodynamic model.

Detachment of Bacteria from Surfaces

Not all cells remain adherent at the surface. Mechanisms of detachment include fluid shear forces (Marshall et al., 1971a), changes in surface free energy of the substratum (Busscher et al., 1986) or the organism (Rosenberg et al., 1983; Fattom and Shilo, 1984), reproductive mechanisms (Power and Marshall, 1988), and enzymatic degradation of adhesive structures. In most cases, however, the majority of adhering bacteria remain at the surface, where they are capable of growth, reproduction (Fig. 2) (Lawrence and Caldwell, 1987; Power and Marshall, 1988; Szewzyk and Schink, 1988), and even biofilm formation. A *biofilm* consists of cells immobilized at a substratum surface and frequently embedded in an organic polymer matrix of microbial origin (Characklis and Marshall, 1990). Other practical aspects of bacterial detachment from surfaces will be considered in later sections.

Occurrence of Sessile Prokaryotes

Microbial Succession at Surfaces

Early reports indicated that very small bacteria were the primary colonizers of surfaces immersed in seawater and were succeeded by conventional rod-shaped and, somewhat later, by prosthecate bacteria (Marshall et al., 1971b). It was realized that the initial colonizing organisms were starvation-survival forms (Morita, 1982) that eventually produced cellular growth at surfaces and thus gave rise to rod-shaped forms (Dawson et al., 1981; Power and Marshall, 1988). Early colonizing organisms tend to be Gram-negative bacteria, particularly species of *Pseudomonas*, *Flavobacterium*, and *Achromobacter*, followed later by prosthecate bacteria (Corpe, 1973). Gram-positive bacteria have rarely been recorded on surfaces in aquatic habitats, although there have been recent reports of significant numbers of Gram-positive bacteria on surfaces associated with groundwater (Kölbel-Boelke and Hirsch, 1989) and on the seagrass *Zostera capricorni* (Angles, 1988). The numbers, overall biomass, and diversity of attached microorganisms increased with increasing time of immersion of a surface (Jordon and Staley, 1976). Scanning electron microscopic studies also have revealed a progression from rod-shaped primary colonizers, to prosthecate forms, and then to a complex biofilm whose composition varies with the nature of the exposed surface and with time (Gerchakov et al., 1977; Marszalek et al., 1979; Dempsey, 1981). Even in illuminated waters, microalgae are not primary colonizers of surfaces (Marshall et al., 1971b; Corpe, 1973; Jordon and Staley, 1976), but extensive development of diatoms, fungi, and protozoa has been observed following bacterial biofilm formation (Gerchakov et al., 1977; Marszalek et al., 1979).

Biologically inert substrata, such as stainless steel or glass, were colonized rapidly following immersion in seawater and produced a complex, two-tiered, microfouling layer (Gerchakov et al., 1977; Marszalek et al., 1979; Dempsey, 1981). The first stage of colonization consisted mainly of bacteria followed by nonmotile diatoms and fungi, whereas the second stage, which appeared after a 5-week exposure, consisted of large, colonial, motile diatoms, other diatoms, flagellates, and ciliates. On the other hand inhibitory substrata, such as copper-nickel alloys or brass, were slowly fouled by bacteria capable of secreting mucoid extracellular polymeric substances (EPS). Such substrata eventually developed a much less diverse biofilm community than inert ones.

Sequential establishment of sessile populations also occurs in freshwater streams (Geesey et al., 1977, 1978) and lakes (Paerl, 1980); in soils where the complexity and variability of the solid matrix makes adequate study difficult (Marshall, 1975; Stotzky, 1986); in the oral cavity (Bowden et al., 1979; Newman, 1980); in the gastrointestinal tract, where the normal sessile biota plays an important role in preventing colonization by bacterial pathogens (Lee, 1980, 1985; Savage, 1980, 1984); and in the colonization of prosthetic devices employed in human patients (Gristina, 1987).

Biofilm Formation

The combined effects of continuous adhesion and both growth and reproduction at surfaces

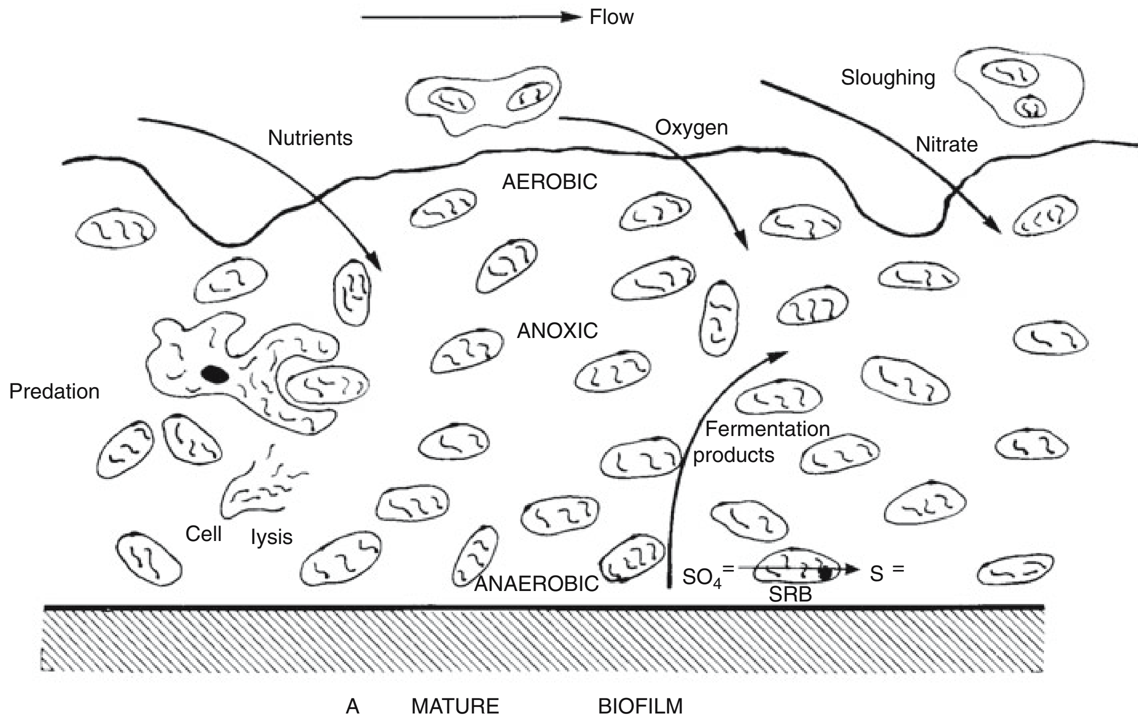

Fig. 3. Diagram of a section through a well-developed biofilm, showing bacteria embedded in an EPS matrix and the direction of decreasing gradients (arrows) of nutrients, oxygen, nitrate, and fermentation products. A predatory amoeba within the biofilm is shown at the left. SRB = sulfate reducing bacterium.

eventually gives rise to a macroscopic slime, or biofilm (Fig. 3).

Biofilms are of considerable nuisance on artificial structures, such as ship hulls, hydroelectric pipelines, water reticulation systems, heat exchangers, oil rigs, and floating oceanographic equipment, but find useful applications in wastewater trickling-filter plants and other fixed-film systems, as well as in fluidized-bed fermenters.

The development of a biofilm on a surface subjected to high shear rates may be described by a sigmoid-shaped curve, where the phase of biomass increase is a function of growth of attached bacteria along with further accretion of cells to the developing biofilm. The plateau of the curve represents the point at which the film penetrates the boundary (or viscous) sublayer (Characklis, 1981b). The final biofilm thickness is dependent on the magnitude of the fluid shear rate. Any protrusion of film irregularities above the viscous sublayer creates turbulence in the water flowing past the biofilm surface leading to frictional flow resistance.

The colonization of mucous excreted by higher organisms (e.g.) the mucous blanket of the animal gastrointestinal tract (Lee, 1985); and the mucigel of plant roots (Rovira et al., 1979), leads to a partial or complete immobilization of cells in the mucous adjacent to the organism's tissue. The final product in this instance bears a superficial resemblance to a biofilm but its mode of origin is entirely different. Certain organisms, particularly spiral bacteria (Phillips and Lee, 1983), appear to have a selective advantage in penetrating and colonizing this viscous habitat.

Methods of Studying Sessile Prokaryotes

Because of the inherent difficulty in directly observing the behavior of microorganisms at surfaces, a wide range of semidirect and indirect techniques have been employed to study adhesion, growth, biofilm development, and detachment from surfaces. Because of the different techniques needed for different surfaces and ecosystems, no attempt will be made here to give detailed instructions for the many techniques available but, rather, references to the descriptions of the original techniques will be provided.

Microscopy

Many of the applications of various forms of microscopy in the study of sessile bacteria have been reviewed (Marshall, 1986b). Most studies involve the use of transmitted or incident light microscopy, or of transmission (TEM) or scanning (SEM) electron microscopy. For transmit-

ted light microscopy, the use of transparent substrata (glass, mica, cellophane, polystyrene, etc.) as test surfaces is essential. Epifluorescence microscopy is necessary for translucent and opaque substrata (Zvyagintsev, 1962; Hobbie et al., 1977). Sessile bacteria may be observed by washing the exposed substratum to remove debris and loosely attached cells and then either staining, with conventional bacteriological stains or fluorescent dyes, or viewing directly with phase-contrast optics. The advantages and disadvantages of such techniques have been presented by Marshall (1986b).

Novel techniques involving light microscopy include the use of submerged microscopy (Staley, 1971), capillary microscopy (Perfil'ev and Gabe, 1969), computer-enhanced image analysis (Caldwell and Germida, 1985), interference reflection microscopy (Fletcher, 1988), dialysis microculture (Duxbury, 1977), marked slides (Bott and Brock, 1970), soil films (Harris, 1972), transparent sections in tubular reactors to study biofilm development (Characklis, 1980), and light section microscopy to measure biofilm thickness (Loeb, 1980).

Other Methods of Study

During the early stages of colonization of surfaces, and particularly if glass, plastic, metal, or wooden slides are immersed in an aqueous phase, bacteria adhering firmly to the surface may be cultured by washing the slides or coupons to remove loosely adhering organisms and then smearing the slide or coupon over the surface of a suitable agar plate (Marshall et al., 1971a). If a distinct biofilm has formed on a surface, the biofilm may be scraped from the surface, suspended in a suitable diluent, homogenized, a dilution series prepared, and aliquots of each dilution plated on an appropriate agar medium. Such methods suffer from the normal problems of selectiveness of the medium employed, and it is likely that some colonizing species (e.g., *Caulobacter*, *Hyphomicrobium*) are never obtained by such techniques. Often the use of special selective media is required in order to isolate particular organisms that may be obvious microscopically. In some cases, it may be necessary to resort to micromanipulation techniques to separate slow-growing or sensitive organisms from more aggressive or resistant species. The simple micromanipulation system devised by Skerman (1968) is especially recommended for this purpose.

A variety of other methods have been adapted to estimate numbers of microorganisms or the total biomass found in a sessile state at surfaces. These include: measurement of radioactivity following the uptake of labeled substrates (Brock, 1971; Lupton and Marshall, 1981), autoradiography (Fletcher, 1979; Bright and Fletcher, 1983), ATP determinations for total biomass (La Motta, 1976), muramic acid determinations for bacterial biomass (Moriarty, 1977), bacterial growth rates using thymidine incorporation (Moriarty, 1986), and determination of bacterial types at surfaces by phospholipid fatty acid signature analysis (Guckert et al., 1985) and by 16S rRNA sequence analysis (Pace et al., 1986; Weller and Ward, 1989). Other techniques that may prove valuable in analyzing biofilm composition and function include the use of Fourier transform infrared spectrophotometry (Nichols et al., 1985) and the use of microelectrodes to measure various gradients with depth of biofilms (Revsbech and Jørgensen, 1986) (Fig. 3).

Adaptation to the Sessile State

Are certain prokaryotes uniquely adapted to a sessile form of life? The answer to this question is not simple because of the very wide range of bacteria that can be found on various surfaces. Several examples of different modes of sessile behavior will be considered in order to illustrate the complexity that may be encountered in natural habitats.

Although many bacteria are capable of adhering to a wide variety of surfaces (nonspecific adhesion), the extent of adhesion on the various surfaces varies considerably. Some bacteria adhere best to hydrophobic surfaces (Fletcher and Loeb, 1979), some adhere best to hydrophilic surfaces (Dexter et al., 1975), whereas others adhere best to surfaces of more intermediate surface-free-energy values (Pringle and Fletcher, 1983). The conditions under which the bacteria are grown also modify the adhesive ability of various bacteria on a range of different surfaces (McEldowney and Fletcher, 1986).

Many bacteria that require relatively high nutrient concentrations (copiotrophic bacteria) exist planktonically in oligotrophic waters in a state of starvation. These starvation-survival forms are characterized by a significant reduction in size and by lower endogenous respiration and heat output, and are often more adhesive than actively growing cells (Morita, 1982; Dawson et al., 1981; Humphrey and Marshall, 1984). Adhesion to surfaces by these starvation-survival forms provides access to nutrients accumulated at the surfaces. The starved bacteria are able to scavenge these nutrients and metabolize them (Kefford et al. 1982; Kjelleberg et al., 1983), thereby leading to cellular growth and reproduction (Kjelleberg et al., 1982; Power and Marshall, 1988; Szewzyk and Schink, 1988). In

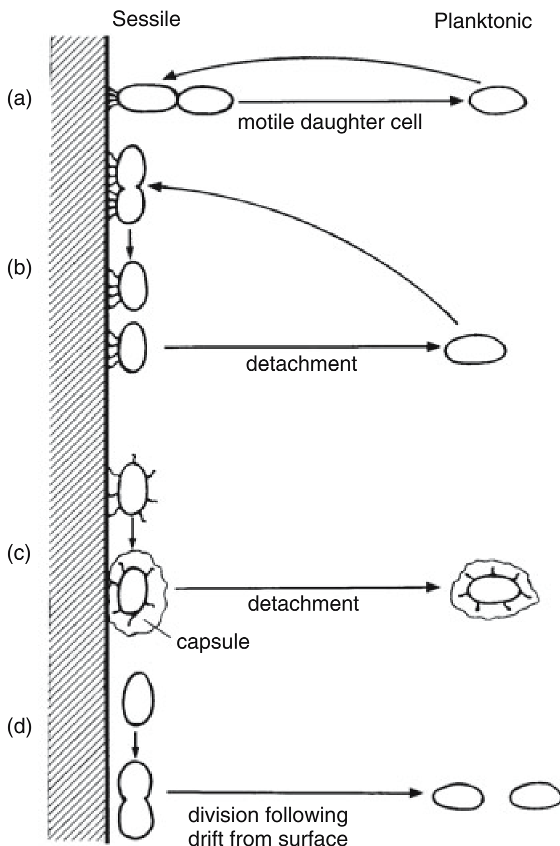

Fig. 4. Four mechanisms for alternating between the planktonic and sessile states: (a) a perpendicularly attached mother cell releases a motile daughter cell, as in *Vibrio* sp. DWI; (b) division of a cell adhering in a face-to-face manner, and release of a cell on utilization of a bound hydrophobic substrate, as in *Pseudomonas* sp. JD8; (c) detachment of a fimbrial-attached organism following the production of a hydrophilic capsule, as in *Acinetobacter calcoaceticus*; and (d) growth of a reversibly adhering organism at a surface and completion of the division phase following drift of the cell from the surface, as in *Vibrio* sp. MH3.

many marine environments, it appears that such small, starved bacteria are the primary colonizers of freshly immersed surfaces (Marshall et al., 1971b).

Some copiotrophic bacteria seem unable to adhere firmly to surfaces, yet, under oligotrophic conditions, any starvation-survival forms approaching a surface are able to metabolize surface-bound substrates (Hermansson and Marshall, 1985) and exhibit both cellular growth and reproduction (Power and Marshall, 1988). Thus, nonadhesive bacteria do exist in the planktonic state but it is still possible for such organisms to benefit from association with surfaces.

A particularly effective adaptation to the sessile state is the ability of many bacteria in nature to adhere in an orientation perpendicular to the surface (Fig. 4a; see also Fig. 1). Such prokaryotes appear to have either a specialized holdfast (*Caulobacter*) or a particularly adhesive portion at one pole of the cell (*Hyphomicrobium*, *Flexibacter*, and *Leucothrix*). Such an orientation allows a very efficient contact both with the solid and the aqueous phases, as well as providing an effective means of releasing daughter cells into the planktonic state. An examination of this mode of orientation at solid surfaces revealed that both *Hyphomicrobium* and *Flexibacter* exhibited the same perpendicular orientation at air-water and oil-water interfaces (Marshall and Cruickshank, 1973). It was postulated that the pole of the cell approaching the interface was hydrophobic while the bulk of the cell was hydrophilic, and the hydrophobic pole was rejected from the water phase and aligned at the nonaqueous phase, regardless of whether it was solid, air, or oil (Marshall and Cruickshank, 1973).

Some bacteria are adapted to growth at surfaces, yet possess various mechanisms to ensure that some cells return to the planktonic state. For instance, cells of the marine species *Vibrio* DW1 adhered to a surface in a perpendicular manner (Fig. 4a) and, following cellular growth of the starved cells to normal size, motile daughter cells were released at regular intervals (approximately 57 min) from the attached mother cells (Kjelleberg et al., 1982). Cells of the marine *Pseudomonas* sp. JD8 adhered in a face-to-face manner (Fig. 4b) and, following cellular growth and one division cycle the daughter cells slowly (about 0.15 μg/min) began to migrate away from each other while still adhering to the surface. After subsequent division cycles, similar migration patterns were observed but, eventually, some of the daughter cells detached from the surface (Power and Marshall, 1988). This slow migration was explained in terms of the cells being initially irreversibly attached to the hydrophobic stearic-acid-covered surface but, upon utilization of the fatty acid in the microenvironment around the cell, the cells became reversibly attached to the underlying hydrophilic substratum (Busscher et al., 1986) and were capable of some form of movement. As soon as the cells moved a short distance, however, they encountered more hydrophobic stearic acid and adhered irreversibly again until that substrate was utilized, and the cycle was repeated. When the bound substrate was essentially exhausted, cells detached from the underlying hydrophilic surface (Power and Marshall, 1988). Even the nonadhesive *Vibrio* MH3 (Fig. 4d) was able to grow from the small starvation-survival form to normal size and then begin the division cycle when exposed to surface-bound stearic acid (Power and Marshall, 1988). The dividing cells drifted away from the surface and completed the division cycle in the planktonic state.

An interesting adaptation ensuring reversibility of the sessile state has been described in *Acinetobacter calcoaceticus*, which adheres

reversibly to epithelial cells and oil by means of thin fimbriae (Fig. 4c). The adhesion of this bacterium is reversed as a result of the production of an excessive amount of extracellular emulsan that surrounds and thus masks the adhesive properties of the fimbriae (Rosenberg et al., 1983). Another example of reversible adhesion has been described in the cyanobacterium *Phormidium*, which in its sessile state possesses a hydrophobic surface but under certain conditions produces a hydrophilic capsule, thus allowing the organism to revert to the planktonic state (Fattom and Shilo, 1984).

These studies emphasize the ability of some prokaryotes to take advantage of substrates adsorbed to surfaces, as well as revealing a variety of strategies for releasing daughter cells from the sessile to the planktonic state. As pointed out by Pedros-Alio and Brock (1983), a simple division into sessile and planktonic forms is overly simplistic. Different bacteria have a variety of mechanisms to attach at surfaces but they also possess a range of mechanisms for detachment in order to return to a planktonic existence.

Advantages of the Sessile State

Nutrient Availability

When a clean surface is immersed into a natural habitat, a molecular film rapidly forms on the surface as a result of adsorption of macromolecules and smaller hydrophobic molecules. This film serves to "condition" the surface, causing alterations in surface charge (Neihof and Loeb, 1974) and surface free energy (Baier, 1980). One of the most obvious advantages of the sessile state is the increased probability of access to nutrients accumulating at surfaces, particularly in flowing, oligotrophic conditions. ZoBell (1943) was the first to suggest that complex macromolecules adsorbed at surfaces would serve as concentrated sources of nutrients for organisms adhering at those surfaces. It was clearly demonstrated by Jannasch (1958) that the beneficial effect of surfaces in the presence of added complex nutrients only occurred at very low nutrient concentrations, where the level of nutrient in the aqueous phase was negligible and the nutrients had adsorbed to the surfaces.

Many investigators comparing the activities of bacteria in the sessile and planktonic states have employed simple soluble substrates such as glucose and amino acids (Azam and Hodson, 1977; Berman, 1975; Berman and Stiller, 1977; Campbell and Baker, 1978; Ferguson and Palumbo, 1979; Fletcher, 1979, 1986; Hanson and Wiebe, 1977; Kirchman and Mitchell, 1982; Pedros-Alio and Brock, 1983; Riemann, 1978). In natural habitats, and particularly in low nutrient situations, such soluble substrates would be rapidly utilized by planktonic bacteria and would rarely encounter a substratum surface. Similarily, many of these low-molecular-weight substrates cannot adsorb to surfaces and would not be expected to concentrate these. If the substrates do adsorb, their availability for bacterial utilization is often reduced substantially (Gordon and Milero, 1985). In many field studies, filtration has been used to separate sessile and attached bacteria, but filtration can lead to problems in that: 1) shear forces involved in filtration are sufficient to remove some reversibly attached bacteria that are feeding at surfaces (Hermansson and Marshall, 1985); and 2) such reversibly attached bacteria may have fed, grown, and reproduced at the surface and then returned to the aqueous phase at some time prior to filtration (Power and Marshall, 1988).

A more logical method of studying the activity of bacteria at surfaces is to provide substrates such as macromolecules or lower molecular weight hydrophobic molecules that are likely to adsorb at surfaces. Using surface-bound stearic acid as a model substrate, Kefford et al. (1982) and Kjelleberg et al. (1983) clearly demonstrated that a range of bacteria were capable of scavenging ^{14}C-labeled stearic acid from a surface. In particular, a reversibly adhering *Leptospira* species rapidly utilized the labeled fatty acid, and ^{14}C-labeled bacteria were readily recovered from the planktonic state. A similar result was obtained with the nonadhesive marine *Vibrio* MH3 (Hermansson and Marshall, 1985), a result that emphasizes the fact that bacteria do not need to firmly adhere to surfaces in order to utilize substrates adsorbed at the surface. Subsequent studies have shown that starved bacteria adhering to surfaces where nutrients have accumulated not only metabolize the nutrients but are capable of cellular growth and reproduction (Kjelleberg et al., 1982; Power and Marshall, 1988; see also Fig. 4 a–d).

Protection from Harmful Factors

Sessile bacteria appear to be more resistant to the inhibitory effects of antibacterial agents, such as antibiotics, chlorine, and heavy metals (Costerton et al., 1981). In relatively thick biofilms, this apparent resistance may be the result of the reaction of the agents with the outer layers of cells and, in the case of chlorine and heavy metals, reaction with the extracellular polymer that makes up the matrix of the biofilm. There is increasing evidence, however, that bacteria attached to surfaces are inherently more resistant to certain antibacterial agents than are planktonic forms, but the mechanism of this increased resistance is not understood. Bacteria below the biofilm-water interface are also pro-

tected from external grazing by protozoa and metazoa. In addition, association of prokaryotes with various sizes of particles or colloidal clays can provide a degree of protection from parasitism by bacteriophage and *Bdellovibrio*, as well as from predation by amoebae and the lytic effects of certain gliding bacteria (Roper and Marshall, 1974, 1978).

Disadvantages of the Sessile State

Sedimentation

Although bacteria attached to particle surfaces may gain an advantage by utilization of adsorbed nutrients or by the dissolution of organic particles, such bacteria would sink to the sediments and would be unable to colonize new particle surfaces if mechanisms did not exist for their release or the release of daughter cells from the particle surfaces. As seen above, such mechanisms are common among sessile forms of bacteria (Fig. 4). It is precisely these phenomena of bacterial attachment, nutrient utilization, and recycling, and detachment that are continually occurring within "marine snow" in the pelagic zone of oceans (Alldredge, 1989).

Grazing

Zooplankton are capable of ingesting planktonic bacteria but detritus feeders have been found to consume the bacteria growing on detritus particles rather than ingest the particles themselves (Fenchel and Jørgensen, 1977). Fenchel (1986) reported that the flagellate *Bodo* sp. spends about 45 sec ingesting a bacterium from a surface, during which time the flagellate does not move. *Bodo* normally slides over the substratum at a velocity of 3.5 µm/sec and only detects and ingests bacteria lying in a 1.0 µm wide band along the path of the flagellate. Zooplankton grazing on biofilm surfaces, however, may play a useful role in maintaining the bacteria near the biofilm surface in an active state of growth. Amoebae have been observed grazing well within the matrix of a biofilm (Mack et al., 1975) (see Fig. 3).

Gradients

Decreasing gradients of nutrient and oxygen availability develop with increasing depth of a biofilm (Fig. 3) (Christensen and Characklis, 1990). Such gradients form as a result of diffusional resistance within the biofilm and of utilization of the nutrients and oxygen by microorganisms within the biofilm. Consequently, aerobic organisms near the biofilm-water-interface tend to be actively growing and create anoxic conditions at greater depths within the biofilm. If nitrate is present then some microorganisms at depth in the biofilm are capable of using the nitrate as an alternative to oxygen as an electron acceptor. Other aerobic organisms tend to be inactive, or even lyse, within the anoxic zone, whereas strict anaerobes and fermentative bacteria may be active in such sites. In biofilms developed on metallic surfaces, the activity of sulfate reducing bacteria (SRB) have been implicated in corrosion processes (Little et al., 1990).

Physiological Responses by Bacteria at Surfaces

Observed Responses

Probably the most obvious physiological response observed in bacteria associated with surfaces is cellular growth and, in some instances, reproduction (Jannasch, 1958; Bott and Brock, 1970; Kjelleberg et al., 1982; Pedros-Alio and Brock, 1983; Power and Marshall, 1988). Another possible response in bacteria to the physical presence of a surface is the time-dependent appearance of firm adhesion, which may indicate the induction of suitable bridging polymer production by the surface-associated bacteria (Marshall et al., 1971a).

The best documented response to a surface is the change observed in certain marine vibrios from a single, sheathed, polar flagellum in the planktonic stage to the production of multiple, lateral flagella when plated on an agar surface (Golten and Scheffers, 1975; de Boer et al., 1975; Belas and Colwell, 1982). Other reported responses include a reduction in size and an increase in endogenous respiration and in heat output by starving marine bacteria at interfaces in the absence of exogenous nutrients (Kjelleberg et al., 1982, 1983; Humphrey et al., 1983; Humphrey and Marshall, 1984). Also, attached bacteria show an increase in resistance to antibacterial substances (Costerton et al., 1981).

Control of Responses

Silverman et al. (1984) have described two possible control mechanisms regulating bacterial responses at surfaces, namely, "responsive" and "variable" control (Fig. 5). Essentially, responsive control involves information processing, whereby the bacterium senses some environmental signal and responds accordingly. In the case of *Vibrio parahaemolyticus*, the response to a shift from an aqueous medium to an agar surface is to deregulate lateral flagella production

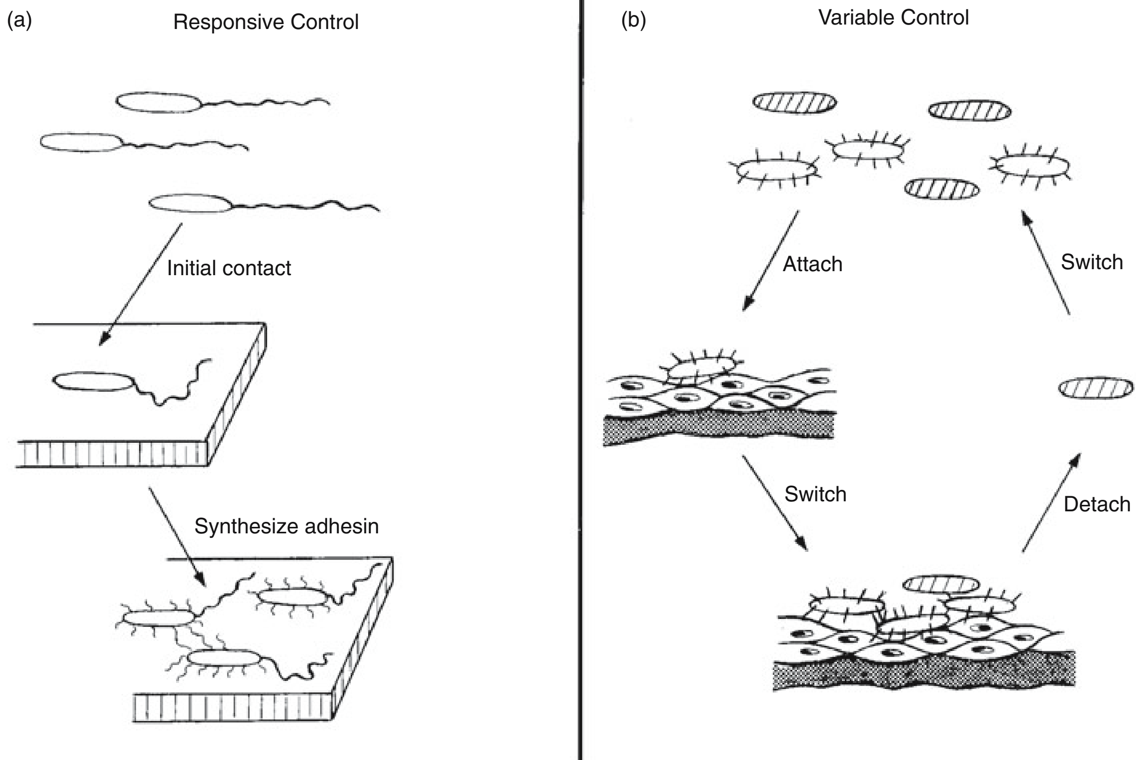

Fig. 5. Strategies for responsive and for variable control of adhesive substance expression. (a) Responsive control, as shown by a shift from polar to lateral flagella in *Vibrio parahaemolyticus*. (b) Variable control, in which a fraction of the cells are preadapted to the fimbriated state, and attach to epithelial cells. Nonfimbriated variants detach and return to the aqueous phase. (From Silverman et al., 1984.)

(Fig. 5a). In the case of variable control, a fraction of the cells are preadapted, for example, to adhere to a particular surface, and individuals within the population are constantly switching among a variety of forms. For instance, a portion of the population may produce fimbriae and attach to epithelial cells (Fig. 5b). Nonadhesive variants of these cells arise and detach to return to the aqueous phase. Such phase variation in certain salmonellae results from a rearrangement of the DNA structure involving the inversion of part of the molecule containing a transcriptional control element.

Physicochemical Triggering of Responses

Using *lux* gene fusion mutants, Belas et al. (1986) studied the responsive control of lateral gene expression when *Vibrio parahaemolyticus* was transferred from liquid to agar medium. They were able to show conclusively that the physicochemical factor triggering lateral flagella production was increased viscosity. Whether this surface effect was entirely the result of viscosity or whether it was also related to a reduction in water activity has not been tested.

Another important factor at surfaces that would result in metabolic, as well as cellular growth and reproduction responses, is the adsorption of organic nutrients at surfaces (Kefford et al., 1982; Kjelleberg et al., 1981; Hermansson and Marshall, 1985; Power and Marshall, 1988). Enhanced phosphorus uptake by attached bacteria has also been reported by Paerl and Merkel (1982). A further situation involving possible adsorption phenomena at surfaces is the finding by Humphrey and Marshall (1984) that changes in size, endogenous respiration, and heat output in starving marine bacteria at surfaces could be reproduced in the presence of surfactants and even when no surface was present. Many bacteria in nature produce surfactants, and these surfactants could adsorb to surfaces where they might trigger various responses in other bacteria adhering to the surfaces.

Other possible explanations for the triggering of physiological responses in bacteria at surfaces include alterations in the proton motive force on the face of the cell nearest the surface (Ellwood et al., 1982) and possible cell deformation near a surface (Fletcher, 1984).

Conclusions

Although the sessile state is very common in bacteria in natural habitats, it is not a state limited to particular groups of organisms. All sessile bacteria are derived from the planktonic state and, in addition to active growth and metabolism at surfaces, these sessile organisms have also evolved a variety of methods to ensure that representatives of the population can return to the planktonic state. Such mechanisms include direct release of daughter cells, changes in the hydrophobicity of the sessile cells or of the substratum surface, exclusively reversible adhesion (subject to removal by gentle shear forces), and, possibly, enzymatic degradation of adhesive bridging polymers.

Planktonic bacteria, on the other hand, possess a wide range of mechanisms whereby they can adhere to a variety of surfaces. In some instances these bacteria possess preformed adhesive polymers, whereas in other cases the bacteria appear to produce appropriate polymers following association with the surface. There is increasing evidence for responsive control of a number of physiological functions evident only at surfaces, but more detailed investigations are required to elucidate the nature of these physicochemical triggering mechanisms.

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Prokaryotes (2006) 2:16–31
DOI: 10.1007/0-387-30742-7_2

CHAPTER 1.2

Bacterial Adhesion

ITZHAK OFEK, NATHAN SHARON AND SOMAN N. ABRAHAM

Introduction

It is now well established that to initiate infection at a particular site bacteria must adhere to host cells or to layers covering these cells (Ofek and Doyle, 1994f). The mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts are the most common portals by which infectious bacteria enter the deeper tissues of a mammalian host. Thus, adhesion to the epithelial cells of these mucosal surfaces and then colonization of the mucosal tissue are considered the first stages on the infectious process. In numerous cases the adhesion is mediated by special protein molecules (known as adhesins) associated with proteinaceous organelles (known as fimbriae or pili). These adhesins, which are on the surface of the infectious bacteria, combine with complementary structures on the mucosal surfaces. Adhesion to mucosal surfaces offers the infectious agent a number of advantages. It allows the bacteria to firmly attach and thereby resist dislocation by the hydrokinetic forces that typically act on these surfaces. And it gives better access to nutrients as well as more protection from deleterious effects of antimicrobial agents in the surrounding milieu (Zafriri et al., 1987).

Although adhesion is an important determinant of mucosal colonization, especially with respect to the animal and tissue tropism of the invading organism, several critical post-adhesion events are required for bacterial colonization. Triggered by the adhesion of the bacteria to their complementary receptors, these events include upregulation of virulence factor expression in the bacteria on the one hand and induction of physiological changes in the host cells on the other. Among the latter are cell proliferation, increased mucus secretion, endocytosis of adherent bacteria, and release of pro- and anti-inflammatory mediators by mucosal and sub-mucosal cells.

In the present article, we review the current state of knowledge of bacterial adhesins and their mucosal cell receptors. We then discuss selected post-adhesion events and describe how they influence mucosal colonization and subsequent symptomatic infection. Finally, we show how the knowledge gained provides a basis for the development of anti-adhesion agents that can block and even reverse bacterial colonization of mucosal surfaces before tissue damage.

Bacterial Adhesins and Their Cognate Receptors

Types of Adhesin-Receptor Interactions

The adhesive interactions of over 100 bacterial pathogens of humans and farm animals have been studied (Ofek and Doyle, 1994a; Karlsson, 1995). Based on these studies, three main types of adhesin-receptor interactions can be distinguished (Table 1). The first type, probably shared by the majority of bacterial pathogens is due to lectin-carbohydrate recognition. Many of the bacterial adhesins are lectins, a class of sugar-binding proteins that link the bacteria to carbohydrate moieties of glycoproteins or glycolipids on the mammalian host cell (Table 2). In some cases bacterial surface polysaccharides of either the capsule or the outermembrane lipopolysaccharides binds to cognate lectins on host cell's (e.g., macrophage) surface (Ofek et al., 1995). The second type involves recognition of a protein on the bacteria by a complementary protein on the mucosal surface. The third type, and the one least well characterized, involves binding interactions between hydrophobic moieties of proteins on one cell with lipids ion the other cell, or between lipids on either cell (Ofek and Doyle, 1994b).

Gents differ only in a single hydroxyl group, present on the acyl of the 4-NH group in the piglet-associated compound but absent in the pig-associated compound as well as in the glycolipids of humans. The subtle age-related change in the glycolipids may explain why *E. coli* K99 can cause diarrhea in piglets, but not in adult pigs or humans.

Multiple Adhesins

A number of common themes have emerged regarding the interactions between bacteria and

Table 1. Molecular features of adhesin-receptor interactions in bacterial adhesion to host cells.

Type of interaction	Bacterial ligand (and example)	Receptor on host cell (and example)	References
Lectin-carbohydrate	Lectin (type 1 fimbriae)	Glycoprotein (uroplakin on bladder cells)	Wu et al., 1996
	Polysaccharide (Klebsiella capsule)	Lectin (mannose receptor of macrophages)	Ofek et al., 1995
Protein-protein	Fibronectin binding proteins (F protein of *S. pyogenes*)	Fibronectin (fibronectin on respiratory cells)	Hanski et al., 1992; 1996
Hydrophobin-protein	Glycolipid (lipoteichoic acid of *S. pyogenes*)	Lipid receptors? (lipid-binding region of fibronectin on epithelial cells)	Courtney et al., 1990 Hasty et al., 1992
	Lipid binding proteins (surface protein of *Campylobacter* spp.)	Membrane lipids (phospholipids and sphingolipids of cells)	Szymanski et al., 1996 Sylvester et al., 1996

mucosal cells. The most notable is the concept that pathogenic bacteria attach to mucosal cells typically through multiple adhesive interactions. Thus, a bacterial cell may express several adhesin moieties, each one specific for a distinct receptor molecule on the epithelial cell surface (for examples see Table 4). Interactions may be mediated by multiple bacterial adhesins that are structurally similar but may exhibit different binding specificities such as the type 1 and P fimbriae of uropathogenic *E. coli* (Table 3). Alternatively, adhesins may be structurally and chemically dissimilar, as is the case with the lipoteichoic acid (LTA) and proteinaceous adhesins of *Streptococcus sanguis*. Some pathogens (e.g., *Neisseria gonorrhoeae*) produce two surface lectins, each specific for distinct carbohydrate structures, one found in glycolipids and the other in glycoproteins.

In many instances, different sub-populations of a bacterial clone express these distinct adhesins. By generating several phenotypic variants each expressing adhesins of distinct specificities, a given bacterial clone will increase the reper-

Table 2. Examples of carbohydrates as attachment sites for bacteria colonizing mucosal surfaces.

Organism	Target tissue	Carbohydrate structure	Form[b]
E. coli type 1	Urinary tract	Manα3[Marα3(Manα6)	Glycoprotein
P	Urinary tract	Galα4Gal	Glycolipid
S	Neural	NeuAc(α2–3)Galβ3GalNAc	Glycolipid
CFA/1	Intestinal	NeuAc(α2–8)-	Glycoprotein
CS3	Intestinal	GalNAcβ4Gal	Glycoprotein[c]
K1	Endothelial	GlcNAc(β1–4)GlcNAc	Glycoprotein
K99	Intestinal	NeuGc(α2–3)Gal(β1–4)Glc	Glycolipid
H. pylori	Stomach	NeuAc(α2–3)Gal	Glycolipid
		Lewis-b blood group	Glycoprotein
		Glucose-fatty acid	Glycolipid
		Lactosyl ceramide	Glycolipid
N. gonorrkea	Genital	Gal(β1–4)Glcβ	Glycolipid
		NeuAc(α2–3)Gal(β1–4)GlcNAc	Glycoprotein
P. aeruginosa	Intestinal	Gal(β1–3)GlcNAc,	Glycoprotein
		Fucose	Glycoprotein
		Mannose	Glycoprotein
	Respiratory	GalNAc(β1–4)Gal	Glycolipid
H. influenza	Respiratory	GalNAcβ4Gal	Glycolipid
	Respiratory	GalNAcβ4Gal	Glycolipid
S. pneumoniae	Respiratory	GlcNAcβ3Gal	Glycoprotein
M. pneumoniae	Respiratory	NeuAc(α2–3)-Gal(β1–4)GlcNAc	Glycoprotein
S. suis	Respiratory	Gal(α1–4)Gal	Glycoprotein
K. pneumoniae	Respiratory & enterocytes	Gal(α1–4)Gal	Glycoprotein

[a]Based on Sharon and Lis, 1996; [b]Ofek and Doyle, 1994 and Karlsson, 1995; [c]Wenner et al., 1955.

Table 3. Types of receptor-adhesin relationship in bacterial adhesion to animal cells.

Type[a]	Receptor molecule	Animal cell	Adhesin molecule	Bacteria, (source)
A.				
	Dr blood group antigen	Erythrocytes	Dra fimbriae	*E. coli*, (UTI)
		Erythrocytes	AFA II	*E. coli*, (ETEC)
		Erythrocytes	F 1845 fimb	*E. coli*, (pigs)
B.				
	Fibronectin (NH_2 terminal)	Epithelial cell	Lipoteichoic acid	*S. pyogenes*
			Fibronectin binding protein	*S. aureus*
C.				
	Glycolipid (Galα1–4Gal)	Uroepithelial cell	P fimbriae, FsoG	*E. coli*, (pyelonephritis)
	Fibronectin		P fimbriae, FsoF/H	
D.				
	66 kDa Gp	Erythrocytes	Type 1 fimbriae	*E. coli*, (mannose sensitive)
	CD11/18 Gp	Neutrophils	Type 1 fimbriae	
	CD 48Gp[b]	Macrophages	Type 1 fimbriae	
	Uroplakin[c]	Uroepithelial cell	Type 1 fimbriae	

Adapted from Ofek and Doyle, 1994.

[a]A. Target host cell express one receptor molecule that contain three attachment sites fro three different adhesins produced by three clones of bacteria; B. Two bacterial species express two distinct adhesins that bind the bacteria to the same receptor molecule on target host cell; C. The same bacterial clone produce a fimbrial structure comprised of two subunits, each bind the bacteria to distinct receptor on host target cell; D. The same adhesin bind the bacteria to similar attachment sites contained in different receptor molecules (isoreceptors) expressed by various host target cells.

[b]Baorto et al., 1997; [c]Wu et al., 1996.

Gp, Glycoprotein.

toire of its target tissues and perhaps also acquire antigenic variability that will enhance its ability to withstand the multifaceted defenses of the host (Ofek and Doyle, 1994b). This notion is exemplified by pyelonephritic isolates of *E. coli* which express either P fimbrial or type-1 fimbrial adhesin at any given time. Because transmission from one host to another is via the feacal-oral route, it was postulated that the pyelonephritogenic isolates may need the type 1 fimbriae mainly to transiently colonize the upper respiratory tract. Such colonies might then provide a constant source of bacteria entering the stomach and thus increase the chances for the incoming bacteria to colonize the intestine (Bloch et al., 1992). Once in the urinary tract, the bacteria seem to need the P fimbrial adhesins to adhere to the urinary tissues (Roberts et al., 1994; Winberg et al., 1995). In fact, the diverse types of fimbrial adhesins carried by various enterobacteria may determine by virtue of their distinct receptor specificity which of the unique niches along the intestine are colonized (Edwards and Puente, 1998).

In those instances where multiple adhesins are expressed simultaneously on the same organism, each adhesin appears to complement the other functionally. For instance, the cell surface LTA and the M protein co-expressed on the surface of *Streptococcus pyogenes* have both been implicated in mediating bacterial binding to Hep-2 cells (Hasty et al., 1992; Courtney et al., 1997). Adhesion of *S. pyogenes* appears to involve a two-step process. The first step is mediated by the interaction of LTA with fibronectin molecules on the host cells (Hasty et al., 1992) and the second

Table 4. Selected bacterial clones expressing multiple adhesins.

Bacterial clone	Source of isolation	Adhesin	Characteristics
E. coli	Pyelonephritis	Type P	Fimbrial lectin
		Type 1	Fimbrial lectin
S. saprophyticus	Urinary	Gal-GlcNAc	Peripheral lectin
		Lipoteichoic acid	Fibrillar hydrophobin
N. gonorrhea	Urogenital	Pilus	Pilin adhesin
		Opa protein	Outermembrane
S. sanguis	Dental plaque hydrophobin	Protein	Peripheral
		Fimbriae	Fimbrial adhesin
		Protein	Peripheral lectin
		Lipoteichoic acid	Fibrillar hydrophobin

Adapted from Ofek and Doyle, 1994.

by binding of the M protein to an as yet unidentified receptor on these cells (Courtney et al., 1997).

Adhesins as Lectins

Table 2 presents a list of bacterial lectins, their molecular forms and their sugar specifities. Whenever known, their animal and organ specificities are also included. Methods are available for the detection and identification of sugar specificities (Goldhar, 1994, 1995; Sharon and Ofek, 1995). For further details the reader is referred to the review literature (Cassels and Wolf, 1995; Karlsson, 1995; Ofek and Doyle, 1994c).

The lectin-mediated adhesion can be inhibited both in vitro and in vivo by either simple or complex carbohydrates that compete with the binding of the lectins to host-cell glycoproteins or glycolipids. In general the affinity of simple sugars (e.g., mono- or disaccharides) to the adhesins or lectins is low, in the millimolar range. Affinity can be increased several orders of magnitude by suitable chemical derivatization (Firon et al., 1987). Increase also can be obtained by attachment of the mono- or disaccharides to polymeric carriers, to form multivalent ligands (Lindhorst et al., 1997; Sharon, 1996; Sharon and Lis, 1997).

Some bacterial lectins recognize not only terminal sugars but internal sequences as well. For example, the tip adhesin Pap G of P fimbriae recognizes internal Galα (1-4)-Gal sequences on cell surface glycolipids (Table 2). When the bacterial adhesin binds the pathogen to a cognate glycolipid, the ceramide group of the latter may contribute to the affinity of the interaction in some cases (e.g. *Helicobacter pylori*; Table 3).

The study of bacterial lectins or adhesins, especially when these molecules are associated with fimbriae that are multi-subunit structures, has been hampered by difficulties in obtaining lectins in pure soluble form. Recently, however, a major breakthrough was achieved by preparing fusion proteins from the ZZ polypeptide of staphylococcal protein A and the amino terminal region of either PapG I, PapGII or PapGIII (Hansson et al., 1995). The three fusion proteins exhibited distinct fine sugar specificities identical with those of the parent fimbriae. It is anticipated that many of the fimbrial lectins will be purified and their combining sites identified using fusion to stabilize the proteins and preserve their carbohydrate-binding activity.

Bacterial Glycoconjugates as Adhesins

Mammalian macrophages express lectins, which recognize complementary carbohydrate structures on bacterial surface and mediate nonopsonic phagocytosis of bacteria. Although phagocytosis, termed lectinophagocytosis, of a number of bacterial species was found to involve macrophage lectins, the surface glycoconjugates that mediate binding to the macrophage lectin have been identified for only a few bacteria. The mannose receptor of macrophages was found to recognize *K. pneumoniae* capsules that contain Manα2/3Man or Rhaα2/3Rha sequences and *Mycobacterium tuberculosis* that have arabinomannan on the surface (Athamna et al., 1991; Schlesinger et al., 1994). For comprehensive reviews on macrophage lectin and bacterial polysaccharide interaction in the infectious process, see Ofek et al. (1995), Ofek and Sharon (1988), Speert (1992, 1988); and Zwilling and Eisenstein (1994).

It was suggested that lipo-oligosaccharide/lipopolysaccharide (LOS/LPS) on the outer membrane of Gram-negative bacteria mediates adhesion to nonprofessional phagocytes (including mucosal cells) as well as to mucus constituents (Jacques, 1996; Nassif and Magdalene, 1995). The evidence for this effect is not conclusive and is based on the following observations: (1) epithelial cells bind less mutant strain (lacking the O side chain of LPS) than they do parental strains, and isolated LPS acts as inhibitor of the binding; (2) LPS isolated from *Vibrio mimicus* causes agglutination of rabbit erythrocytes (Alam et al., 1996); (3) the heptose-3-deoxy-D-manno-2-octulosonic acid disaccharide present in the inner core of LPS is recognized by a lectin-like molecule on the plasma membrane of rat hepatocytes (Parent, 1990); and (4) the binding and internalization of *Pseudomonas aeruginosa* by corneal epihtelial cells requires intact inner-core LPS with a terminal glucose residue (Zaidi et al., 1996). In a few cases interaction between a lectin on one bacterial cell and the lipo-oligosaccharide on another cell may mediate aggregation of the bacteria (Blake et al., 1995). The animal lectin galectin-3 was found to recognize bacterial lipopolysaccharides of Gram-negative bacteria (Mey et al., 1996). In no case, however, has there been definitive proof presented or identification made of a mucosal cell lectin that binds carbohydrates from pathogenic bacteria.

Adhesin-Receptor Relationship

The adhesins of a number of bacterial pathogens and their cognate receptor on the host cells has been characterized in a considerable number of pathogenic organisms (reviewed in Ofek and Doyle, 1994b; Sharon and Lis, 1997). Several general features are notable (Table 2). One receptor may contain more than one attachment site that is specific for two or more adhesins. This is illustrated by the Dr blood group glycoprotein, which acts as receptor on host cell membrane for

three different clones of *E. coli* each one produces a distinct adhesin that binds to a different region of the Dr group molecule (Ofek and Doyle, 1994e).

Another general feature is that two different pathogens, each expressing structurally distinct adhesins, can exhibit the same receptor specificity. This is the case with *Staphylococcus aureus* and *S. pyogenes*, both of which bind to the amino terminal region of fibronectin on mucosal cells. The adhesin on *S. aureus* is a fibronectin-binding protein, whereas that of *S. pyogenes* is lipoteichoic acid (Table 3). The finding that several different respiratory tract pathogens recognize the disaccharide GalNAcβ4Gal is yet another example of the above (Table 2). It has been suggested that the GalNAcβ4Gal sequence is preferentially accessible in glycolipids of the respiratory epithelium and this allows firm binding of a diverse group of respiratory pathogens bearing the suitable adhesins. In some cases, however, distinct adhesins share specificity but are carried by different bacteria that colonize different tissues and animal hosts, as is the case for the Galα(1-4)Gal-specific lectins of the uropathogenic P-fimbriated *E. coli*, the pig pathogen *Streptococcus suis* (Tikkanen et al., 1995), and the respiratory/enteropathogenic P-like fimbriated *K. pneumoniae* (Prondo-Mordarska et al., 1996).

Conversely, the same bacterial adhesin can bind to several distinct receptors on different cell types; such receptors are called isoreceptors. For instance several glycoproteins ranging in size from 110–45 kDa have been described as receptors for type 1 fimbriae on different cell types (Table 2). All these isoreceptor glycoproteins share a common oligomannose-containing attachment site for FimH, the adhesin subunit of type 1 fimbriae. Another situation is when an adhesin molecule contains multiple domains, each with distinct receptor specificity as is the case of the filamentous hemagglutinin adhesin of *Bordetella pertussis*. This hemagglutinin, which has been cloned and sequenced, contains at least three domains: (1) an arginine-glycine-aspartate (RGD)-containing sequence which binds the bacteria to a CR3 integrin present on pulmonary macrophages (Relman et al., 1989); (2) a carbohydrate-binding domains specific for galactose (Tuomannen et al., 1988) and (3) a carbohydrate-binding domain specific for sulfated sugars (Menozzi et al., 1994).

Interaction of Bacterial Adhesins with Extracellular Matrix

Mucosal cells are often covered by a layer referred to as extracellular matrix (ECM), which is a heterogeneous assembly of proteins, mainly glycosylated but to different extents. Included are structural glycoproteins that are typical constituents of the ECM such as collagens, elastin, fibronectin, fibrinogen, laminin, chondriotin sulfate proteoglycans and heparan sulfate proteoglycans. Many mucosal colonizers express adhesins that specifically recognize one or more of these substances. The same three categories of adhesin-receptor interactions, presented in Table 1, occur between bacteria and ECM components. They may be interactions between proteins only, between lipids and proteins, or between lectins and carbohydrates. A more thorough discussion of these ECM-bacteria interactions may be found in excellent reviews (Patti and Höök, 1994; Hasty et al., 1994; Patti et al., 1994; Wadstrom et al., 1994). Among the various ECM components, interactions with fibronectin have been studied the most at both the molecular and cellular levels. Because this multifunctional glycoprotein is found on the surface of many types of cells including mucosal ones, fibronectin probably acts as a receptor for bacterial adhesion and colonization. The adhesion of bacteria to extracellular matrix components other than fibronectin is becoming more appreciated. Examples of recent studies describing specific structures that mediate binding of bacteria to such beta-components are shown in Table 5. A remarkable feature is that many of the bacterial species studied express on their surfaces at least two proteins that bind a specific ECM component. Thus, *Helicobacter pylori* expresses a laminin-specific adhesin that may be either a 25 kDa sialic-acid-binding lectin, which recognizes sialyl residues of laminin, or a lipopolysaccharide which recognizes other, as yet unidentified, regions in laminin (Valkonen et al., 1994, 1997). Many studies have established fibronectin as an important receptor for *S. pyogenes* and other bacteria on mucosal surfaces (Ofek and Doyle, 1994e; Courtney et al., 1990). At least six different molecules on *S. pyogenes* surfaces were found to recognize fibronectin, including LTA, protein F/Sfb, a 28 kDa fibronectin-binding protein, glyceraldehyde-3-phosphate dehydrogenase, serum opacity factor and a 54 kDa fibronectin-binding protein (FBP54; reviewed in Hasty and Courtney, 1996). It is not clear whether all these fibronectin-binding entities mediate the adhesion of streptococci to mucosal surfaces.

Consequences of Bacterial Adhesion to Cells and Tissues

Recently it has been shown that adhesins not only enable colonization of mucosal surfaces but also elicits a variety of distinct responses in the

Table 5. Examples of bacterial adhesins mediating binding of the bacteria to ECM glycoproteins.

Bacteria	Bacterial adhesin	ECM component	References
Borrelia burgdorferi	19 and 20kDa proteins	Proteoglycan decorin	1, 1a
	Protein A (Osp A) and 70kDa protein	Plasminogen	2
H. influenzae	P2 and P5 outermembrane proteins	Respiratory mucin	3, 3a, 3b
N. gonorrhea	Opa protein	Proteoglycan	4
P. aeruginosa	57 and 59kDa outermembrane proteins	Laminin	5
	42–48 and 77–85kDa outermembrane proteins and Flagellar 65.9kDa FLi F (MS ring)	Respiratory mucins	6, 6a
Staphylococcus aureus	138 and 127 surface proteins	Nasal mucin	7
	Cna protein (55Kda domain)	Collagen	
	ClfA (clumping factor)	Fibrinogen	
	FnBPA and FnBPB	Fibronectin	
Mycobacterium bovis	28kDa protein	Heparan	8
E. coli	Gaf D protein of G fimbriae	Laminin	9
Bordetella pertussis	Filamentous hemagglutinin (N-terminal region of FHA)	Heparan	10
H. pylori	Lipopolysaccharide and 25kDa protein	Laminin	11, 11a
Listeria monocytogenes	ActA outermembrane protein	Heparan	12

Key to references:
1. Guo et al., 1995; 1a. Leong et al., 1995; 2. Hu et al., 1995; 3. Davis et al., 1995; 3a. Reddy et al., 1996; 3b. Kubiet and Ramphal, 1995; 4. Putten and Paul, 1995; 5. Plotkowski et al., 1996; 6. Scarfnman et al., 1996; 6a. Akora et al., 1996; 7. Shuter et al., 1996; Foster and Hook, 1998; 8. Menozzi et al., 1996; 9. Saarela et al., 1996; 10. Hannah et al., 1994; 11. Valkonen et al., 1994; 11a. Valkonen et al., 1997; 12. Alvarez-Domínguez et al., 1997.

host cells as well as in the bacteria which can markedly affect the course of the infectious process (reviewed in Finlay and Cossart, 1997). In this section, selected examples are presented to illustrate the above notion.

Induction of Bacterial Virulence Genes

The urinary tract is relatively refractory to bacterial colonization. In addition to resisting the constant hydrokinetic forces acting in this organ, a potential pathogen must multiply fast enough in urine to compensate for the diluting effects of the latter. Urine is a complex fluid containing a variety of excreted products but is growth limiting for bacteria, in part, because it is low in free iron. The intrinsic iron acquisition machinery of uropathogenic *E. coli* is activated upon complex formation between the PapG fimbrial adhesin with its Galα (1-4)Gal-containing globoseries receptor (Zhang and Normark, 1996). When P-fimbriated bacteria attached to immobilized receptor, transcriptional activation of a sensor-regulator protein, AirS, was detected. This sensor protein, located in the cytoplasmic membrane, belongs to the two-component family of signal transduction factors. The precise mechanism of AirS action is as yet not known. It is believed to regulate the bacterial iron acquisition system and iron-regulated membrane proteins to facilitate the translocation of iron into the bacterium. Uropathogenic *E. coli*, in which the *airS* gene was knocked out, lost its capacity to grow in urine. It would appear that uropathogenic bacteria can "sense" receptors (e.g. of the globoseries) in the urinary tract environment via PapG and respond by colonizing this body site. These findings point to an intriguing new function for bacterial P fimbriae, namely, that of a sensory organelle. The strategic location of PapG at the distal tips of the peritrichously arranged fimbriae probably facilitates this purported role. This finding is one of an increasing number of cases showing that bacterial pathogens are intrinsically capable of responding to cues from host cells following interactions between complementary cell-surface molecules (Cotter and Miller, 1996; Finlay and Cossart, 1997). In addition, these observations provide a molecular basis for earlier findings. Various bacteria obtain a growth advantage after attachment to host cells, as demonstrated for type 1 fimbriated *E. coli* and *N. gonorrhoeae*, which exhibit shorter lag periods when adhering to tissue culture cells (Zafriri et al., 1987; Bessen and Gotschlich, 1986).

Induction of Cytokine Release from Mucosal Cells

In addition to evoking responses in the adherent bacteria, the specific coupling of the bacterial adhesins with their receptors also elicits a range of mucosal cell responses (Bliska and Falkow, 1992). For example, adhesion of the P-fimbrial adhesin to its receptors on mouse uroepithelial cells elicits the release from these cells of several immunoregulatory cytokines including inter-

leukins (ILs)-1α-, β, -6 and -8 (Svanborg et al., 1996). It also triggers intracellular release of ceramides that may be derived from the globoseries receptor itself or from neighboring sphingomyelin molecules by the action of endogenous sphingomyelinases (Hedlund et al., 1996; Svanborg et al., 1996). Ceramide is known to be a critical second messenger in signal transduction processes capable of activating the Ser/Thr family of protein kinases and phosphatases and leading eventually to cytokine production. This bacterial adhesin- mediated mechanism of signaling is reminiscent of that utilized by immunoregulatory cytokines such as tumor necrosis factor alpha (TNFα) and IL-1 when evoking cellular responses (Svanborg et al., 1996). Thus bacterial adhesin appears to be functionally mimicking the host's immunoregulatory molecules. Although the type 1 fimbriae of uropathogenic *E. coli* also stimulate a cytokine response from uroepithelial cells, the array of cytokines released is different from those elicited by P fimbriae (Connell et al., 1996b). The transmembrane signaling pathway of cytokine release by type 1 fimbriae has not been investigated but its clarification could benefit from the recent identification of uroplakin as the putative FimH-receptor on epithelial cells (Wu et al., 1996). Adhesion of Gram-positive bacteria to epithelial cells may also cause release of cytokines from the cells. For instance, group A streptococci adherent to HEp-2 cells via both M protein and LTA adhesins cause release of IL-6 from the target cells (Courtney et al., 1997). Perhaps more interesting are the findings that interaction of bacteria with ECM constituents may also trigger signal transduction in the underlying host cells (Juliano and Haskill, 1993).

Induction of Cytokine Responses in Inflammatory Cells

The capacity of bacterial adhesins to elicit cytokine responses is not confined to mucosal cells. Lectinophagocytosis mediated by fimbriae such as type 1 fimbriae of *E. coli* or of type 2 fimbriae of *Actinomyces viscosus* is associated with stimulation of the phagocytic cells (Sandberg et al., 1988; Ofek et al., 1995). Indeed, type 1 fimbriae of uropathogenic *E. coli* are capable of binding to and eliciting immunoregulatory products from a wide range of inflammatory cells including macrophages, neutrophils, mast cells, and B and T lymphocytes in vitro (reviewed in Connell et al., 1996a). That these interactions may occur in vivo with significant physiologic effects is suggested by experiments in which mice injected intraperitoneally with type 1 fimbriated *E. coli* generated lysosomal β-N-acetylglucosaminidase and a large spike of TNFα in the peritoneal fluid (Bernhard et al., 1992; Malaviya et al., 1996). The fimbrial adhesin, FimH, plays a key role in this exposure because intraperitoneal challenge with a FimH-minus isogenic mutant resulted in only a limited TNFα response (Malaviya et al., 1996). The source of TNFα in the mouse peritoneum was determined to be mast cells because mice genetically deficient in these cells exhibited a limited TNFα response following intraperitoneal injection of type 1 fimbriae. Notably, this TNFα response was accompanied by a large influx of neutrophils into the peritoneum, consistent with the fact that TNFα is a potent neutrophil chemoattractant (Malaviya et al., 1996). Thus, one of the immediate outcomes of type 1 fimbriae-mediated activation of mast cells is recruitment of neutrophils to sites of bacterial challenge. Because mast cells are found preferentially in mucosal surfaces, the interaction of type 1 fimbriae of *E. coli* with such cells could contribute to the influx of neutrophils from surrounding blood vessels leading to the translocation of the bacteria through the epithelial barrier and subsequent entry into the lumen. The excessive transepithelial migration of neutrophils during infections may predispose this barrier to increased bacterial penetration (Finlay and Cossart, 1997) and raises the possibility that facets of the host's immune response may be co-opted by pathogenic bacteria to enhance their virulence.

Impact of Bacteria-Elicited Inflammatory Responses

Evaluating the physiologic effects of some of the adhesin-elicited cytokines at sites of bacterial infection is difficult because these effects are numerous and complex (Abraham and Malaviya, 1997; Henderson et al., 1996). Some of the responses evoked in the mucosa following the adherence of pathogenic bacteria include increased mucus secretion, proliferation of epithelial cells and recruitment and activation of a variety of phagocytic cells. All of these responses could potentially affect the early elimination of the pathogen (Abraham and Malaviya et al., 1997; Henderson et al., 1996). However, some of the adhesin-triggered secreted products of host cells may have severe pathophysiologic effects on the surrounding tissue, particularly when released in excess or at inopportune times (Abraham and Malaviya, 1997). Although direct evidence is still lacking, considerable circumstantial evidence supports the notion that the many proteases, oxygen radicals, and cytotoxic cytokines secreted after inflamatory cells are activated by type 1 fimbriated *E. coli* (Tewari et al., 1994; Malaviya et al., 1994, 1996) are detrimental to the host and foster bacterial pathogenesis. For example, the elastases, oxygen radicals and other

cytotoxic agents, released from neutrophils following their interaction with type 1 fimbriae of *E. coli* in the kidney, are major contributors to renal scarring (Steadman et al., 1988; Topley et al., 1989). Whether an inflammatory response favors the host or pathogen may depend on other prevailing factors including the host's immune status and the intrinsic virulent capabilities of the pathogen. The number of bacteria at the site of infection may be another critical factor in light of the recent findings that certain bacteria have "quorum sensing" ability (Passador et al., 1993; i.e., they sense their population density at a given site and, upon reaching a critical density, coordinately turn on the expression of a battery of new virulence factors.)

Bacterial Uptake by Phagocytes

In addition to inducing the release of pharmacologically active mediators from various host cells, bacterial adhesins also elicit the phagocytic uptake of bacteria under serum-free conditions (reviewed in Ofek et al., 1995). The process involves a number of molecular mechanisms; as mentioned this process has been termed lectinophagocytosis, in analogy to opsonophagocytosis (Ofek and Sharon, 1988; Ofek et al., 1995). The best-characterized system of lectinophagocytosis is that of bacteria carrying the mannose-specific type 1 fimbrial lectins. The fact that a bacterial adhesin that promotes bacterial colonization and infection may also promote ingestion by phagocytic cells would seem a paradox. Although earlier work showed that bacteria are occasionally killed by the phagocytes, new evidence has emerged to suggest that type 1 fimbriae-elicited bacterial phagocytosis by macrophages may actually benefit the bacterial population (Baorto et al., 1997). In vitro survival assays in macrophages revealed that, unlike *E. coli* phagocytized via opsonin-mediated processes, *E. coli* phagocytized via type 1 fimbriae survived much of the intracellular killing. It has been suggested that by associating with CD48, a glycosylphosphoinositol-linked moiety on the surface of macrophages, the bacteria gain access to a lipid processing pathway that bypasses the normal phagocytic killing mechanisms of the macrophages (Baorto et al., 1997). This finding provides a molecular basis for earlier observations showing that, compared to bacteria ingested via opsonophagocytosis, bacteria subjected to lectinophagocytosis are often markedly less sensitive to killing by phagocytes (reviewed in Ofek et al., 1995). It is noteworthy that lectinophagocytosis comes into play only at body sites where opsonizing is poor such as in the urinary mucosa.

Internalization by Nonphagocytic Cells

Contact between bacterial adhesins and complementary receptors on so called nonphagocytic cells can trigger internalization of adherent bacteria (reviewed by Finlay and Falkow, 1990, 1997; Marra and Isberg, 1996). This has been demonstrated with such classical intracellular pathogenic species as *Listeria*, *Yersinia*, *Shigella*, *Salmonella* and *Bartonella* (Table 7). These organisms enter and proliferate in nonphagocytic cells in vitro and in vivo. Probably because of the development of highly sensitive and reproducible techniques to measure bacterial entry into mammalian cells (Tang et al., 1993), several well-known "extracellular" pathogens have recently been reported to be capable of penetrating nonphagocytic cells (e.g., epithelial and endothelial cells) and of surviving for a limited period and, in some cases, even of proliferating intracellularly. Unlike the classical or professional intracellular pathogens, entry of the extracellular pathogens is usually limited to a subset of bacterial strains within the same species, probably because entry into requires the co-expresion of multiple components such as adhesins and constituents of the secretory system (De Vries et al., 1996). Furthermore, the capacity to enter nonphagocytic cells is not necessarily associated with virulence of the extracellular pathogen. For example, isolates from carrier-state or nonencapsulated strains of *S. pyogenes* can invade epithelial cells, whereas pharyngitis isolates (Sela, 1998) or virulent encapsulated strains (Schrager et al., 1996) invade poorly. Excluding the classical intracellular pathogens, the list of bacterial species capable of invading nonphagocytic cells includes *Actinobacillus actinomycetemcomitans* (Meyer et al., 1996), *Pseudomonas aeruginosa* (Fleiszig et al., 1995, 1996), *Burkholderia* (*Pseudomonas*) *cepacia* (Burns et al., 1996), *E. coli* (Meier et al., 1996; Jouve et al., 1997; Donnenberg et al., 1997; Goluszko et al., 1997), *K. pneumoniae* (Oelschlaeger and Tall, 1997), *N. gonorrhoeae* (Weel et al., 1991), *N. meningitidis* (Virji et al., 1993), *Porphyromonas gingivalis* (Weinberg et al., 1997), *Streptococcus agalactiae* (Hulse et al., 1993; Valentin-Weigand et al., 1997; Gibson et al., 1993), *S. aureus* (Vann et al., 1987; Hamill et al., 1986) and *S. pyogenes* (Greco et al., 1995; LaPenta et al., 1994). Conceivably, the ability to enter nonphagocytic cells is an integral part of the pathogenic process of many infectious bacteria. While the classical intracellular pathogens utilize this ability to spread from cell to cell and to penetrate into deep tissue, other pathogens may utilize this trait to temporarily hide from the host's immune cells or from antibiotics. Thus, bacteria surviving within nonphagocytic cells

Table 6. Inhibitors of bacterial lectin/adhesin as anti-adhesion drug for preventing infection in experimental animals.

Inhibitor	Bacteria	Animal	Site of infection
Mannose or its glycosides	*E. coli* type 1	Mice	Bladder
		Mice	Gut
	K pneumoniae type 1	Rats	Bladder
	Shigella flexneri type 1	Guinea pigs	Eye
Galα4Galβ containing oligosaccharide	*E. coli* type P	Mice	Urinary tract
		Monkeys	Urinary tract
Glycopeptides (from serum glycoproteins)	*E. coli* K99	Calves	Gut
Galactose, mannose and N-acetylglucosamine	*P. aeruginosa*	Human	Ear
Sialyl containing oligosaccharide	*H. pylori*	Piglet	Gut
GalNAcβ4Gal containing oligosaccharide	*S. pneumoniae*	Rabbit	Lung
N-Acetylglucosamine	*S. pneumoniae*	Mouse	Lung

Gal, galactose; GalNAc, N-acetylgalactosamine.
[a]Adapted from Sharon, 1996.

serve as a critical reservoir from which reinfection of the host can take place.

The mechanisms employed by various bacteria to gain access into nonphagocytic cells are diverse and often complex (see range of molecules implicated in bacterial invasion of host cells in Table 7). For example, *Yersinia enterolitica* employs a single cell surface protein, invasin, whose cognate receptors on the host cell membrane are β integrins (Isberg, 1996). When invasin binds with high affinity to β integrins, the close association between the integrins and cytoskeletal elements of the cell membrane triggers the bacterial uptake. Particles that are

Table 7. Examples of bacteria capable of invading nonphagocytic cells.

Bacteria (reference)	Surface constituents for adhesion	Surface constituents for entry[a]	Receptor for entry	Intracellular proliferation	Tissue damage[b]
Listeria monocytogenes (1)	?	InlA	E cadherin	+	+
		InlB			
		ActA	Proteoglycan		
Yersinia sp[c] (2)	Ail protein	Invasin[b]	Integrins	+	+
	YadA protein				
Salmonella sp (3)	?	Sip proteins	CD42	+	+
Shigella sp (4)	?	Ipa[b] proteins	Integrins	+	+
Bartonella sp. (5)	BFP	IalA	Glycolpid	+	+?
		IalB			
EPEC, STEC[d] (6)	BFP[e]	Intamin	Integrin, HP90	−	+
E. coli (7)	AfaIII	AfaE, AfaD proteins	?	−?	?
N. gonorrhea (8)	Pili	Opa A,C	CD66 family	−	+
			Vitronectin		
			Heparan sulfate		
S. pyogenes (9)	LTA	F protein	Fibronectin	−	−
		M protein			
K. pneumoniae (10)	Type 1 fimb.	?	GlcNAc[f]	NT	−

Key to references
(1) Gaillard et al., 1991; Mengaud et al., 1996; (2) Iseberg et al., 1987; Iseberg and Leong, 1990; Miller and Falkow, 1998; Saltman et al., 1996; Schulze-Koops et al., 1992; 1993; (3) Chen et al., 1996; Francis et al., 1993; (4) Watarai et al., 1995, Mennardi et al., 1996; Zychlinski and Sansonetti, 1997; (5) Minnick et al., 1996; (6) Donnenberg et al., 1992, 1997; Frankel et al., 1995, 1996; Rosenshine et al., 1996; Paton and Paton, 1998; (7) Jouve et al., 1997; (8) Weel et al., 1991, Makino et al., 1991, van Putten et al., 1995, Virji et al., 1996; Chen et al., 1997; Gomez-Durate et al., 1997; (9) LaPenta et al., 1994; Greco et al., 1995; Jadoun et al., 1997; Molinary et al., 1997; (10) Oelschlaeger and Tall, 1997; Fumagalli et al., 1997.
[a]The surface constituents required for entry usually can function as adhesins as well.
[b]Damage usually associated with inflammation resulting from the entry process (Shigella) or from direct damage of the cell membrane of the target host cells (e.g. *E. coli* and Salmonella).
[c]Enteropathogenic *Yersinia* species e.g. *Y. enterolytica* and *Y. pseudotuberculosis*.
[d]Enteropathogenic *E. coli*. Entry was documented only in tissue cell culture (Donnenber et al., 1990), but the intimin is required for intimate association and induction of the effacement/attaching lesion.
[e]Bundle forming pili.
[f]N-Acetylglucosamine containing glycoprotein on tissue culture cells.

coated with invasin proteins (or functionally relevant portions of the protein) and exposed to tissue culture cells are readily internalized by the cells. More complex modes of entry requiring specific secretion systems of the bacteria have been reported for certain enteropathogenic *E. coli* (Javris et al., 1995) and species of *Shigella* (Allaoui et al., 1993), *Salmonella* (Ginocchio et al., 1992) and *Bartonella* (Minnick et al., 1996). Perhaps the most remarkable of these systems involves enteropathogenic *E. coli* (EPEC) where the type III secretory system of the bacteria inserts into the host cell membrane a protein (HP90) that serves in turn as the receptor for the bacterial adhesin (Kenny et al., 1997; Nataro and Kaper, 1998). The process of internalization involve sequential interactions between EPEC and the host cell. The first step of adhesion occurs via bundle fimbria and is followed by intimate contact via a second adhesin termed intimin. The receptor on the host cell for the intimin is HP90, which is produced by the bacteria, phosphorylated, and then inserted into the host cell membrane by the type III secretory system of the bacteria. With the binding of intimin, the bacteria become internalized by the host cells. A similar mechanism was described for the internalization of pathogenic Neisseria by nonphagocytic cells (Dehio et al., 1998). In another recently reported mechanism, the bacteria after adhesion to their cognate receptor initiate a signaling cascade resulting in activation of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase, to allow entry of *N. gonorrhoeae* into nonphagocytic cells (Grassme et al., 1997). Finally, in some cases the molecular mechanism utilized by the bacteria to gain entry into nonphagocytic cells appears to be the same as that involved in the uptake of bacteria by phagocytes. A case in point is the specific interaction between CD66 on the mammalian cell surface and the *N. gonorrhoeae* Opa proteins that triggers the uptake of bacteria by both epithelial cells and polymorphonuclear cells (Gray-Owen et al., 1997; Virji et al., 1996; Chen et al., 1997; Sauter et al., 1993).

Concluding Remarks

Experiments in animals have proven that it is possible to prevent infections by blocking the adhesion of the pathogen to target tissue. These findings have stimulated the development of anti-adhesion drugs for preventing and treating microbial infections in humans (reviewed in Kahane and Ofek, 1996). New classes of these drugs are greatly needed because of the increasing incidence of pathogenic organisms resistant to conventional antibiotics. It is believed that strains with genotypic resistance to the anti-adhesion agents will spread much slower than strains resistant to conventional drugs, such as antibiotics aimed at killing the organisms. The reason is that both anti-adhesion-sensitive and -resistant strains are shed to continue transmission from host to host, whereas only antibiotic-resistant strains are transmitted following therapy.

Because lectin-mediated adhesion is a mechanism shared by many pathogens most investigators have focused their efforts to prevent bacterial infections on blocking the pathogen's lectins. The preferable target site is the mucosal surfaces where phagocytic cells are scarce and where most infections are initiated. A number of strategies have been suggested including enhancement of mucosal immunity by s-IgA anti-adhesin antibody induction, use of metabolic inhibitors of adhesin expression (e.g. sublethal concentration of antibiotics), and of dietary inhibitors, in particular receptor analogs (reviewed in Ofek and Doyle, 1994b; Kahane and Ofek, 1996). In the latter strategy, the lectin or adhesin is inhibited by sugars for which the lectin is specific (Table 6). This was first demonstrated in the late 1970s, when it was shown that methyl α-mannoside can protect mice against urinary tract infection by type 1 fimbriated *E. coli*; methyl α-glycoside which is not recognized by the bacteria, was not effective (Aronson et al., 1979). Subsequent studies by many other groups have proven beyond any doubt the drug potential of anti-adhesive compounds (Table 6; Beuth et al., 1995; Sharon, 1996; Ofek and Sharon, 1990; Zopf and Roth, 1996). Thus, derivatives of galabiose that inhibit the adhesion of P fimbriated *E. coli* to animal cells in vitro, prevented bacterial infections in the urinary tract of mice and monkeys. Antibodies against mannose-containing compounds present on epithelial cells prevented urinary tract infection in mice by type 1 fimbriated *E. coli*. in mice, and orally administered sialylated glycoproteins protected colostrum-deprived newborn calves against lethal doses of enterotoxigenic *E. coli* K99. In a clinical trial in humans, patients with otitis externa (a painful swelling with secretion from the external auditory canal) caused by *P. aeruginosa* were treated with a solution of galactose, mannose and N-acetylneuraminic acid (Beuth et al., 1996). The results were fully comparable to those obtained with conventional antibiotic treatment. An attractive candidate is oligosaccharides such as those found in human milk and other body fluids, that have been shown to inhibit the adhesion to cells and tissues of strains of *H. pylori* and *S. pneumoniae* (Zopf et al., 1996; Simon et al., 1997).

Human milk is a potential source of inhibitors of bacterial adhesion because it is rich in disac-

charides that may act as receptor analogs (Ashkenazi, 1996). However, other dietary constituents also may exhibit anti-adhesion activity and may be used to prevent bacterial infections. For example, cranberry juice contains at least two inhibitors of uropathogenic *E. coli* (Ofek et al., 1991) and according to one well documented report, it reduced the incidence of urinary tract infections in elderly women (Avorn, et al., 1994).

These findings illustrate the great potential of inhibitors of adhesion in the prevention and perhaps also treatment of bacterial infections. Moreover, they raise hopes for the development of anti-adhesive drugs for human use. The development of anti-adhesion therapy targeted at the microbial lectins has been hampered by the great difficulty in large-scale synthesis of the required inhibitory saccharides. An alternative is glycomimetics, compounds that structurally mimic the inhibitory carbohydrates, but which may be more readily obtainable. Eventually, a cocktail of inhibitors, or a polyvalent one, will have to be used, since many infectious agents express multiple specificities. The design of such drugs will certainly benefit from more detailed information about the specificity of the microbial surface lectins and the elucidation of the atomic structure of their combining sites, none of which is yet known.

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Prokaryotes (2006) 2:32–85
DOI: 10.1007/0-387-30742-7_3

CHAPTER 1.3

The Phototrophic Way of Life

JÖRG OVERMANN AND FERRAN GARCIA-PICHEL

Introduction

Photosynthesis is the utilization of radiant energy for the synthesis of complex organic molecules. The phototrophic way of life implies the capture of electromagnetic energy (see Light Absorption and Light Energy Transfer in Prokaryotes in this Chapter), its conversion into chemical energy (see Conversion of Light into Chemical Energy in this Chapter), and its use for cellular maintenance and growth (see Efficiency of Growth and Maintenance Energy Requirements in this Chapter). Photosynthesis may encompass the reduction of carbon dioxide into organic molecules, a mode of growth defined as photoautotrophy. The solar electromagnetic energy reaching the Earth's surface (160 $W \cdot m^{-2}$; see Light energy and the spectral distribution of radiation) surpasses the energy contributed by all other sources by four to five orders of magnitude (electric discharge, radioactivity, volcanism, or meteoritic impacts; ~0.0062 $W \cdot m^{-2}$ on primordial Earth; Mauzerall, 1992; present day geothermal energy ~0.0292 $W \cdot m^{-2}$; K. Nealson, personal communication).

At present the flux of electromagnetic energy supports a total primary production of 172.5×10^9 tons dry weight·year^{-1} (168 g $C \cdot m^{-2} \cdot year^{-1}$; Whittaker and Likens, 1975). If this global primary production is converted to energy units (39.9 kJ·g C^{-1}, assuming that all photosynthetic products are carbohydrate), 0.21 $W \cdot m^{-2}$ and thus 0.13% of the available solar energy flux are converted into chemical energy. Even at this low efficiency, the chemical energy stored in organic carbon still exceeds geothermal energy by at least one order of magnitude. As a consequence, photosynthesis directly or indirectly drives the biogeochemical cycles in all extant ecosystems of the planet. Even hydrothermal vent communities, which use inorganic electron donors of geothermal origin and assimilate CO_2 by chemolithoautotrophy (rather than photoautotrophy), still depend on the molecular O_2 generated by oxygenic phototrophs outside of these systems (Jannasch, 1989).

Several lines of evidence indicate that in the early stages of biosphere evolution, prokaryotic organisms were once responsible for the entire global photosynthetic carbon fixation. Today, terrestrial higher plants account for the vast majority of photosynthetic biomass; the chlorophyll bound in light-harvesting complex LHCII of green chloroplasts alone represents 50% of the total chlorophyll on Earth (Sidler, 1994). In contrast, the biomass of marine primary producers is very low (0.2% of the global value). However, the biomass turnover of marine photosynthetic microorganisms is some 700 times faster than that of terrestrial higher plants. Thus, marine photosynthetic organisms contribute significantly to total primary productivity ($55 \cdot 10^9$ tons dry weight·year^{-1}, or 44% of the global primary production). Because the biomass of cyanobacterial picoplankton (see Habitats of Phototrophic Prokaryotes in this Chapter) can amount to 67% of the oceanic plankton, and their photosynthesis up to 80% in the marine environment (Campbell et al., 1994; Goericke and Welschmeyer, 1993; Liu et al., 1997; Waterbury et al., 1986), prokaryotic primary production is still significant on a global scale. A single monophyletic group of marine unicellular cyanobacterial strains encompassing the genera *Prochloroccoccus* and *Synechococcus* with a global biomass in the order of a billion of metric tons (Garcia-Pichel, 1999) may be responsible for the fixation of as much as 10–25% of the global primary productivity. Additionally, prokaryotic (cyanobacterial) photosynthesis is still locally very important in other habitats such as cold (Friedmann, 1976) and hot deserts (Garcia-Pichel and Belnap, 1996) a nd hypertrophic lakes.

Today, the significance of anoxygenic photosynthesis for global carbon fixation is limited for two reasons. On the one hand, phototrophic sulfur bacteria (the dominant anoxygenic phototrophs in natural ecosystems) form dense accumulations only in certain lacustrine environments and in intertidal sandflats. The fraction of lakes and intertidal saltmarshes which harbor anoxygenic phototrophic bacteria is unknown, but these ecosystems altogether contribute only 4% to global primary production (Whittaker and Likens, 1975). In those lakes harboring pho-

totrophic sulfur bacteria, an average of 28.7% of the primary production is anoxygenic (Overmann, 1997). Consequently, the amount of CO_2 fixed by anoxygenic photosynthesis must contribute much less than 1% to global primary production. On the other hand, anoxygenic photosynthesis depends on reduced inorganic sulfur compounds which originate from the anaerobic degradation of or ganic carbon. Since this carbon was already fixed by oxygenic photosynthesis, the CO_2-fixation of anoxygenic phototrophic bacteria does not lead to a net increase in organic carbon available to higher trophic levels. The CO_2-assimilation by anoxygenic phototrophic bacteria has therefore been termed "secondary primary production" (Pfennig, 1978). Therefore, capture of light energy by anoxygenic photosynthesis merely compensates for the degradation of organic carbon in the anaerobic food chain. Geothermal sulfur springs are the only exception since their sulfide is of abiotic origin. However, because sulfur springs are rather scarce, anoxygenic photosynthetic carbon fixation of these ecosystems also appears to be of minor significance on a global scale.

The scientific interest in anoxygenic phototropic bacteria stems from 1) the simple molecular architecture and variety of their photosystems, which makes anoxygenic phototrophic bacteria suitable models for biochemical and biophysical study of photosynthetic mechanisms, 2) the considerable diversity of anoxygenic phototrophic bacteria, which has implications for reconstructing the evolution of photosynthesis, and 3) the changes in biogeochemical cycles of carbon and sulfur, which are mediated by the dense populations of phototrophic bacteria in natural ecosystems.

All known microorganisms use two functional principles (both mutually exclusive and represent two independent evolutionary developments) for the conversion of light into chemical energy. Chlorophyll-based systems are widespread among members of the domain Bacteria and consist of a light-harvesting antenna and reaction centers. In the latter, excitation energy is converted into a redox gradient across the membrane. In contrast, the retinal-based bacteriorhodopsin system is exclusively found in members of a monophyletic group within the domain Archaea. These prokaryotes lack an antenna system and use light energy for the direct translocation of protons across the cytoplasmic membrane. In both systems, photosynthetic energy conversion ultimately results in the formation of energy-rich chemical bonds of organic compounds.

The advent of modern genetic and biochemical methods has led to a considerable gain in knowledge of the molecular biology of phototrophic prokaryotes. At the same time, microbial ecologists have found these microorganisms of considerable interest and now frequently use molecular methods to investigate natural populations. The present chapter is limited to the discussion of phototrophic bacteria and attempts to link the physiology, ecology, and evolution of phototrophic bacteria to a molecular basis. Emphasis is laid on those molecular structures or functions that have evident adaptive value. This integrating view may provide a more solid foundation for understanding the biology of photosynthetic prokaryotes.

Taxonomy of Phototrophic Prokaryotes

The capacity for chlorophyll-based photosynthetic energy conversion is found in five of the 36 currently recognized bacterial lineages (Fig. 1; Hugenholtz et al., 1998): the *Chloroflexus* subgroup, the green sulfur bacteria, the *Proteobacteria*, the *Cyanobacteria*, and the *Heliobacteriaceae*. With the exception of the *Cyanobacteria*, phototrophic bacteria perform anoxygenic photosynthesis, which is not accompanied by photochemical cleavage of water and therefore does not lead to the formation of molecular oxygen. Based on their phenotypic characters, anoxygenic phototrophic bacteria had been divided previously into the five families Rhodospirillaceae, Chromatiaceae, Ectothiorhodospiraceae, Chlorobiaceae, and Chloroflexaceae (Trüper and Pfennig, 1981). However, 16S rRNA oligonucleotide cataloguing and 16S rRNA sequence comparisons have reveale d that the *Proteobacteria* and the *Chloroflexus*-subgroup both contain nonphototrophic representatives (Woese, 1987; Fig. 1). Therefore the use of light as an energy source for growth is not limited to phylogenetically coherent groups of bacteria. However, nonphototrophic representatives of the green sulfur bacterial and the cyanobacterial lineages have not been isolated to date.

Within the *Chloroflexus*-subgroup, three different species (*Chloroflexus aurantiacus*, *Chloroflexus aggregans* and *Heliothrix oregonensis*) of filamentous multicellular phototrophs have been described. All three are thermophilic and grow photoorganoheterotrophically. In addition four mesophilic species (*Oscillochloris chrysea*, *Oscillochloris trichoides*, *Chloronema giganteum*, *Chloronema spiroideum*) have been affiliated with the *Chloroflexus*-subgroup based on their multicellular filaments, gliding motility, and the presence of chlorosomes containing bacteriochlorophylls c or d (Pfennig and Trüper, 1989). The phylogenetic position of these latter bacteria

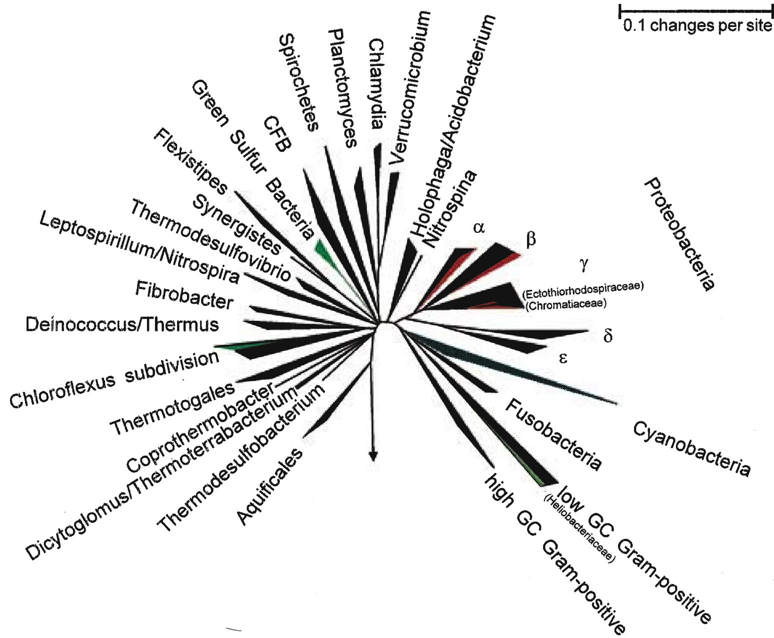


Fig. 1. Phylogenetic tree based on 16S rRNA sequences. All bacterial divisions containing culturable representatives were included in the analyses so that the phototrophic nature of the bacterial strains could be confirmed. Alignments were obtained with CLUSTAL W and pairwise distances calculated with the algorithm of Jukes and Cantor using the DNADIST program of PHYLIP 3.57c. The tree was constructed from evolutionary distances employing the least-squares algorithm of Fitch and Margoliash as implemented by the FITCH program of the package. The Archaeon *Methanopyrus kandleri* DSM 6324 was used as an outgroup to root the tree. (light green) Bacteria containing chlorosomes as light-harvesting antenna. (red) Bacteria containing antenna complexes within the cytoplasmic membrane and quinone/pheophytin-type reaction centers. (medium green) Gram-positive phototrophic bacteria with FeS-type reaction centers. (dark green) Bacteria containing the two types of reaction centers. Width of colored wedges indicates the phylogenetic divergence.

has not been investigated so far. With the exception of *Heliothrix oregonensis* all species mentioned contain chlorosomes as distinct light-harvesting structures (Fig. 2). Yet to be cultivated axenically, non-thermophilic "*Chloroflexus*-like" organisms are known from intertidal and hypersaline benthic environments (Pierson et al., 1994) and from cold freshwater sulfidic springs (F. Garcia-Pichel, unpublished observation). At least in the case of the hypersaline enrichments, the organisms are closely related to *Heliothrix* in terms of their 16S rRNA sequence (B.K. Pierson, personal communication to FGP). This, together with recent descriptions of *Oscillochloris trichoides* (Keppen et al., 1994) from freshwater sediments indicates a larger diversity and more widespread occurrence of the *Chloroflexaceae* and allied organisms than was previously recognized.

Green sulfur bacteria (see The Family Chlorobiaceae Volume 7) represent a coherent and isolated group within the domain Bacteria. They are strict photolithotrophs and contain chlorosomes (Fig. 3A). During the oxidation of sulfide, elemental sulfur is deposited extracellularly. Another typical feature of this group is the very limited physiological flexibility (see Docile Reaction). In the *Proteobacteria*, the α- and β-Proteobacteria comprise photosynthetic representatives (often also called the purple nonsulfur bacteria), which do not form separate phylogenetic clusters but are highly intermixed with various other phenotypes. Characteristically, members of these two groups exhibit a high metabolic versatility and are capable of photoorganotrophic, photolithoautotrophic and chemoorganotrophic growth. Photosynthetic pigments are bacteriochlorophyll *a* or b and a variety of carotenoids. Light-harvesting complexes, reaction centers, and the component s of the electron transport chain are located in intracellular membrane systems of species-specific architecture (Fig. 2; see Light Absorption and Light Energy Transfer in Prokaryotes in this Chapter).

Several members of the α-Proteobacteria are capable of bacteriochlorophyll *a* synthesis but cannot grow by anoxygenic photosynthesis. This physiological group has therefore been designated "aerobic anoxygenic phototrophic bacteria" (Shimada, 1995; Yurkov and Beatty, 1998), "aerobic phototrophic bacteria" (Shiba, 1989), or "quasi-photosynthetic bacteria" (Gest, 1993) and comprises a considerable number of species. So far, the marine genera *Erythrobacter* and

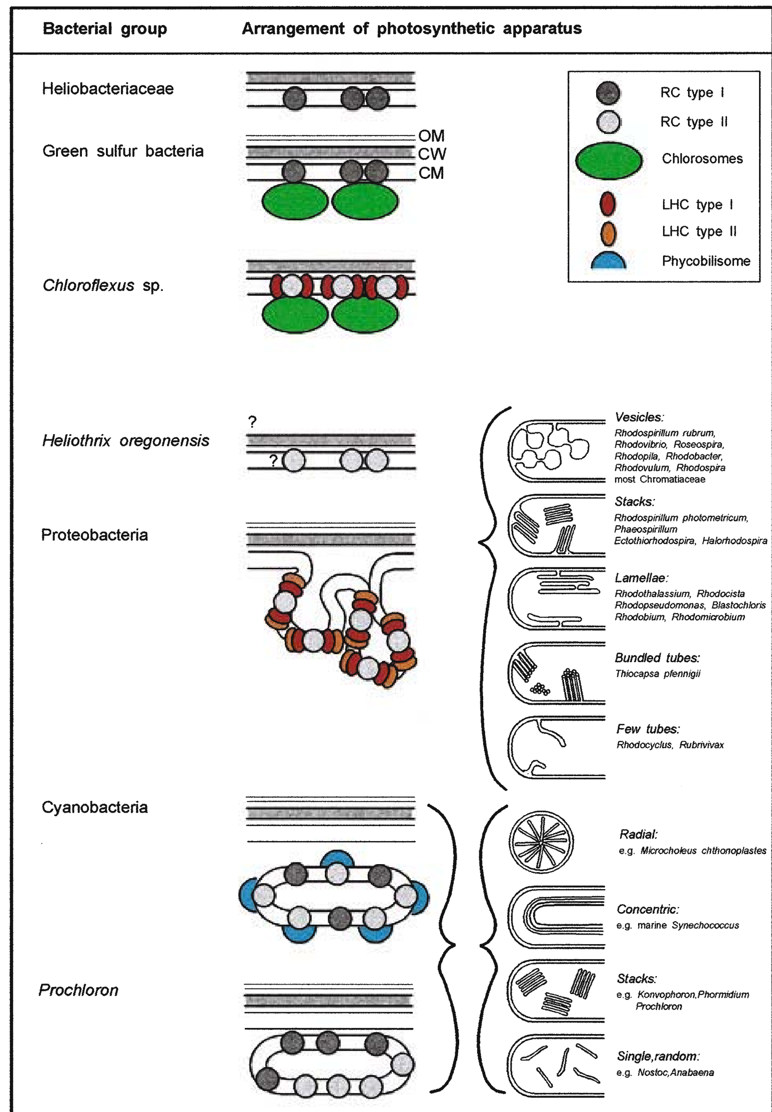


Fig. 2. Organization of the phototrophic apparatus in different groups of phototrophic bacteria. *OM* = outer membrane, *CW* = cell wall, *CM* = cytoplasmic membrane, *RC* = reaction center, *LHC* = light-harvesting complex. Question marks indicate that the organization of the cell envelope and the organization of the photosynthetic apparatus in *Heliothrix oregonensis* is not exactly known.

Roseobacter and the six freshwater genera *Acidiphilium*, *Erythromonas*, *Erythromicrobium*, *Porphyrobacter*, *Roseococcus*, *Sandarcinobacter* (Yurkov and Beatty, 1998) have been described. This group also includes some aerobic facultatively methylotrophic bacteria of the genus *Methylobacterium* and a *Rhizobium* (strain BTAi1; Evans et al., 1990; Shimada, 1995; Urakami and Komagata, 1984). The oxidation of organic carbon compounds is the principal source of metabolic energy. Photophosphorylation can be used as a supplementary source of energy, with a transient enhancement of aerobic growth following a shift from dark to illumination (Harashima et al., 1978; Shiba and Harashima, 1986). Aerobic bacteriochlorophyll-containing bacteria harbor a photosynthetic apparatus very similar to photosystem II of anoxygenic phototrophic Proteobacteria (Yurkov and Beatty, 1998). Photochemically acti ve reaction centers and light-harvesting complexes are present, as are the components of cyclic electron transport (e.g., a cytochrome *c* bound to the reaction center and soluble cytochrome c_2). In contrast to anoxygenic phototrophic bacteria, however, the aerobic phototrophic bacteria cannot grow autotrophically. Intracellular photosynthetic membrane systems as they are typical for anoxygenic phototrophic *Proteobacteria* are absent in most aerobic photosynthetic bacteria; *Rhizobium* BTAi1 being a possible exception (Fleischman et al., 1995). The presence of highly polar carotenoid sulfates and C_{30} carotenoid glycosides is a unique property of this group. All aerobic bacteriochlorophyll *a*-containing species group with the α-subclass of the Proteobacteria, but are more closely related to aerobic non-

bacteriochlorophyll-contain ing organisms than to anoxygenic phototrophs (Stackebrandt et al., 1996).

The γ-subclass comprises two families of phototrophic species, the *Chromatiaceae* and *Ectothiorhodospiraceae* (also called purple sulfur bacteria). *Chromatiaceae* accumulate sulfur globules within the cells and represent a conspicuous microscopic feature of these bacteria. With one notable exception (*Thiocapsa pfennigii*), the intracellular membrane system is of the vesicular type (Figs. 2 and 3B). In contrast, members of

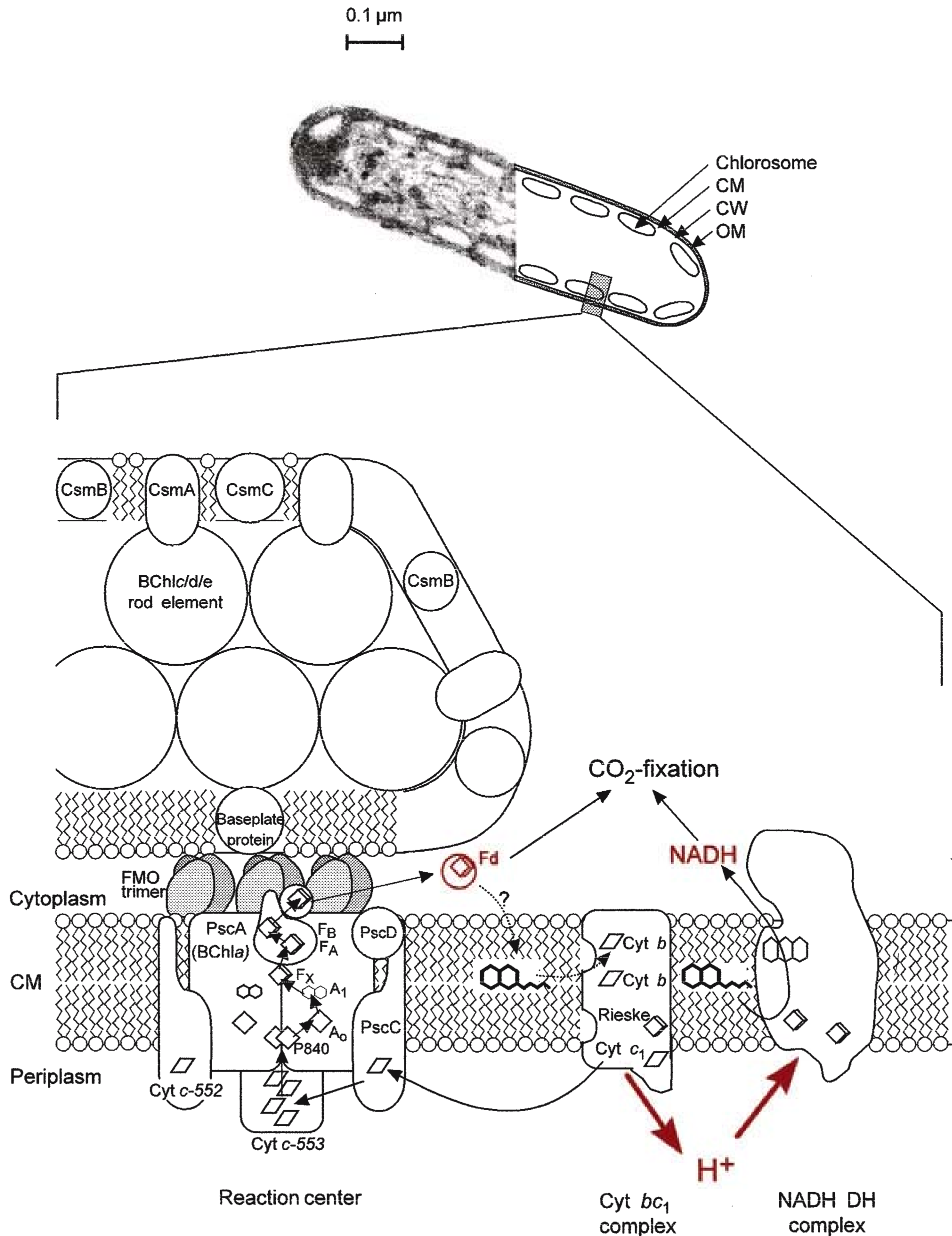


Fig. 3. Localization and organization of the photosynthetic apparatus in three major groups of phototrophic bacteria. Electron-donating enzyme systems, like flavocytochrome or sulfide quinone reductase, and ATP formation by the membrane-bound ATP synthase are not shown. A. Green sulfur bacteria (Chlorobiaceae). B. Purple nonsulfur bacteria and Chromatiaceae. C. Cyanobacteria. *OM* = outer membrane; *CW* = cell wall; *CM* = cytoplasmic membrane; *Cyt* = cytochrome; *P840* and *P870* reaction center special pair = primary electron donor; *B800, B850, B875* = bacteriochlorophyll molecules bound to light-harvesting complexes II and I; A_0 = primary electron acceptor in green sulfur bacteria = Chl *a*; A_1 = secondary electron acceptor in green sulfur bacteria = menaquinone; Q_A, Q_B = ubiquinone; F_X, F_A, F_B = FeS-clusters bound to the reaction center; *Fd* = ferredoxin; *FMO* = Fenna-Matthews-Olson protein; *FNR* = ferredoxin $NADP^+$ reductase; *PQ* = plastoquinone; *PC* = plastocyanin; *PS* = photosystem.

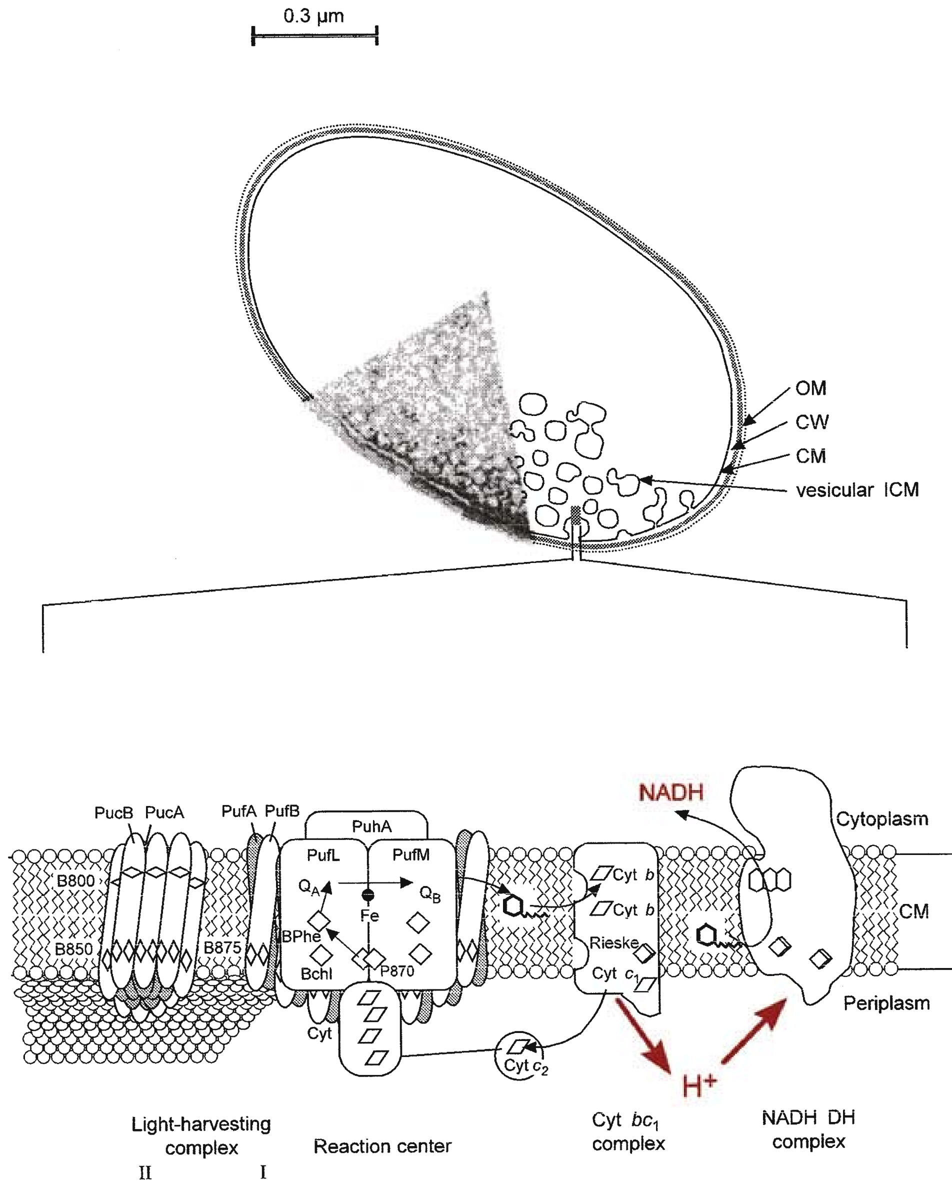


Fig. 3. *Continued*.

the *Ectothiorhodospiraceae* deposit elemental sulfur outside of the cells and contain lamellar intracellular membrane systems. Like their relatives of the α- and β-subclass of Proteobacteria, the purple sulfur bacteria contain bacteriochlorophylls *a* and *b*, and all components of the photosynthetic apparatus are located in the intracellular membrane.

No photosynthetic species have been described for the δ- or ε-subclass of the *Proteobacteria*.

Heliobacteriaceae differ from other anoxygenic phototrophic bacteria by their unique light-harvesting and reaction center pigment, bacteriochlorophyll g, and by their phylogenetic affiliation (Fig. 1). The first member of this group, *Heliobacterium chlorum* was described in 1983 by Gest and Favinger (Gest and Favinger, 1983b). Based on peptidoglycan structure studies (Beer-Romero et al., 1988), their high proportion of branched-chain fatty acids (Beck et al., 1990) and 16S rRNA sequencing, the *Heliobacteriaceae* belong to the Gram-positive low GC lineage. A close relatedness can also be deduced from the capability of *Heliobacterium modesticaldum* and *Heliobacterium gestii* to form endospores. However, a detailed phylogenetic analysis also indicated a close relatedness of

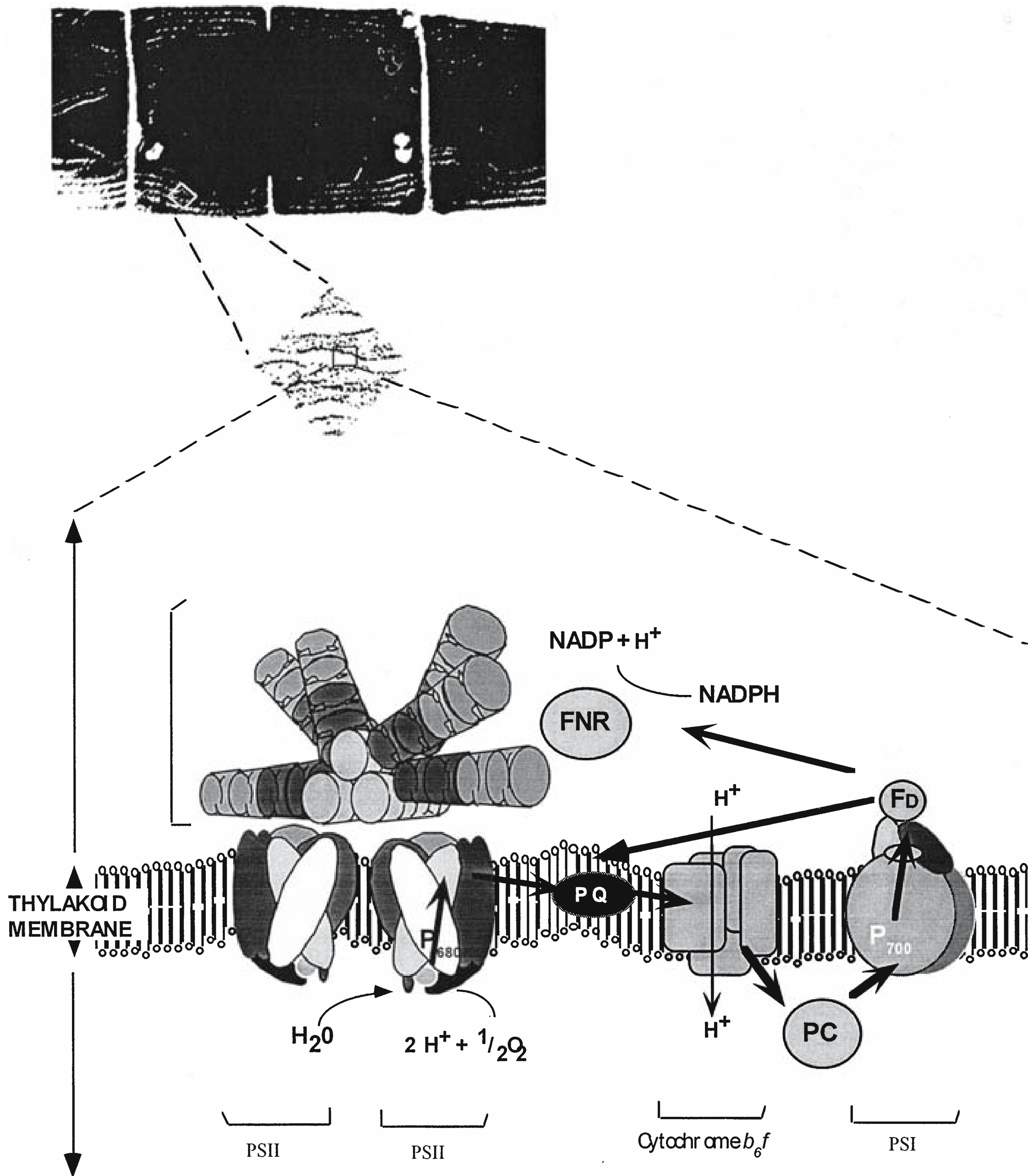


Fig. 3. *Continued*.

Heliobact eriaceae to the *Cyanobacteria* (Vermaas, 1994). *Heliobacteriaceae* do not contain distinct intracellular structures of the photosynthetic apparatus and the reaction centers are located in the cytoplasmic membrane. Bacteriochlorophyll g confers to the cells a near infrared absorption maximum at 788 nm, which is unique among photosynthetic organisms. The known species of *Heliobacteriaceae* all grow photoheterotrophically and are strict anaerobes.

Oxygenic photosynthesis is only found in members of a single bacterial lineage out of the five that contain phototrophs (Fig. 1). The *Cyanobacteria* by far comprise the largest number of isolated strains and described species (Table 1). The *Cyanobacteria* (= oxyphotobacteria) are defined by their ability to carry out oxygenic photosynthesis (water-oxidizing, oxygen-evolving, plant-like photosynthesis) based on the coordinated work of two photosystems (Fig. 3C). Phylogenetically, they constitute a coherent phylum that contains the plastids of all eukaryotic phototrophs. They all synthesize chlorophyll *a* as photosynthetic pigment, and

Table 1. Groups of photosynthetic prokaryotes and their characteristics.

Taxon		Preferred growth mode	Light harvesting	Photochemical reaction
Chloroflexus subdivision	(3)[a]	Anoxygenic photoorganoheterotroph(cls);	BChl *c*, car	Type II reaction center
		Aerobic chemoorganoheterotroph	—	—
Green sulfur bacteria	(15)	Anoxygenic photolithoautotroph	cls; BChl *cldle*, car	Type I reaction center
α-Proteobacteria	(31)	Anoxygenic photoorganoheterotroph	icm; BChl *alb*, car	Type II reaction center
		Aerobic chemoorganoheterotroph	—	—
α-Proteobacteria (aerobic photosynthetic)	(23)	Aerobic chemoorganoheterotroph	BChl *a*	Type II reaction center
β-Proteobacteria	(4)	Anoxygenic photoorganoheterotroph	icm; BChl *a*, car	Type II reaction center
		Aerobic chemoorganoheterotroph	—	—
Chromatiaceae Ectothiorhodospiraceae	(31) (9)	Anoxygenic photolithoautotroph	icm; BChl *alb*, car	Type II reaction center
Heliobacteriaceae	(5)	Anoxygenic photoorganoheterotroph	BChl *g*, car	Type I reaction center
Cyanobacteria	(>> 1000)	Oxygenic photolithoautotroph	thy; Chl *a* + PBS or Chl *b*, or Chl *d*; car	Type I + II reaction center
Prochloron, *Prochlorothrix*	(2)		thy; Chl *a/b*,car	
Prochlorococcus	(1)		thy; Chl a_2/b_2, car (PBS)	
Acaryochloris	(1)		thy; Chl*a,d*, car (PBS)	
Halobacteria	(3)	Aerobic chemoorganoheterotroph	Purple membrane; bacteriorhodopsin	Bacteriorhodopsin

[a]The numbers of photosynthetic species described for each taxon are given in parenthesis.
BChl = bacteriochlorophyll, car = carotenoids, Chl = chlorophyll, cls = chlorosomes, icm = intracellular membranes, PBS = phycobilisomes, thy = thylacoids.

most types contain phycobiliproteins as light-harvesting pigments. These multimeric proteinaceous structures are found on the cytoplasmic face of the intracellular thylakoid membranes and contain phycobilins as light-harvesting pigments. All *Cyanobacteria* are able to grow using CO_2 as the sole sou rce of carbon, which they fix using primarily the reductive pentose phosphate pathway (see Carbon Metabolism of Phototrophic Prokaryotes in this Chapter). Their chemoorganotrophic potential typically is restricted to the mobilization of reserve polymers (mainly starch but also polyhydroxyalkanoates) during dark periods, although some strains are known to grow chemoorganotrophically in the dark at the expense of external sugars. Owing to their ecological role, in many cases indistinguishable from that of eukaryotic microalgae, the cyanobacteria had been studied originally by botanists. The epithets "blue-green algae," "Cyanophyceae," "Cyanophyta," "Myxophyceae," and "Schizophyceae" all apply to the cyanobacteria. Two main taxonomic treatments of the *Cyanobacteria* exist, and are widely used, which divide them into major groups (orders) on the basis of morphological and life-history traits. The botanical system (Geitler, 1932 recognized 3 orders, 145 genera and some 1300 spe cies, but it has recently been modernized (Anagnostidis and Komárek, 1989, Komárek and Anagnostidis, 1989). The bacteriological system (Stanier, 1977; Rippka et al., 1979; Castenholz, 1989), relies on the study of cultured axenic strains. It recognizes five larger groups or orders, separated on the basis of morphological characters. Genetic (i.e., mol% GC, DNA-DNA hybridization) as well as physiological traits have been used to separate genera in problematic cases.

Previously, a separate group of organisms with equal rank to the cyanobacteria, the so-called "Prochlorophytes" (with two genera, *Prochloron*, a unicellular symbiont of marine invertebrates, and *Prochlorothrix*, a free-living filamentous form) had been recognized (Lewin, 1981). They were differentiated from cyanobacteria by their lack of phycobiliproteins (Fig. 2) and the presence of chlorophyll *b*. The recently recognized genus *Prochlorococcus* of marine picoplankters could be included here, even though the major chlorophylls in this genus are

divinyl-Chl *a* and divinyl-Chl *b*. Fourteen *Prochloron* isolates from different localities and hosts have been found to belong to a single species by DNA-DNA hybridization studies (Stam et al., 1985; Holtin et al., 1990). Some of the original distinctions leading to the separation of the Chl *b*-containing oxyphotobacteria from the cyanobacteria are questionable, since at least in one strain of *Prochloroccoccus marinus*, functional phycoerythrin (Lokstein et al., 1999), and genes encoding for phycobiliproteins have been detected (Lokstein et al., 1999). Additionally, phylogenetic analysis of 16S rRNA genes indicate that the three genera of Chl *b*-containing prokaryotes arose independently from each other and from the main plastid line (see Evolutionary Considerations in this Chapter), a result that is supported by the comparative sequence analysis of the respective Chl *a*/*b* binding proteins (Laroche et al., 1996; Vanders taay et al., 1998). Thus "Prochlorophytes" are just greenish cyanobacteria, and are not treated separately here. The recent discovery of Chl *d*-containing symbionts in ascidians (*Acaryochloris marina*, Miyashita et al., 1996) once again demonstrates the evolutionary diversification of light-harvesting capabilities among oxyphotobacteria (see Competition for Light in this Chapter). While the phylogenetic affiliation of *Acaryochloris marina* has not been presented as yet, ultrastructural and chemotaxonomic characters predict that *A. marina* belongs to the cyanobacterial radiation as well.

According to phylogenetic analysis of 16S rRNA sequences, the *Cyanobacteria* are a diverse phylum of organisms within the bacterial radiation, well separated from their closest relatives (Giovanonni, 1988; Wilmotte, 1995; Turner, 1887; Garcia-Pichel, 1999; Fig. 1). These analyses support clearly the endosymbiotic theory for the origin of plant chloroplasts, as they place plastids (from all eukaryotic algae and higher plants investigated) in a diverse, but monophyletic, deep-branching cluster (Nelissen et al., 1995). Phylogenetic reconstructions show that the present taxonomic treatments of the cyanobacteria diverge considerably from a natural system that reflects their evolutionary relationships. For example, separation of the orders *Chroococcales* and *Oscillatoriales* (Nelissen et al., 1995; Reeves, 1996), and perhaps also the *Pleurocapsales* (Turner, 1887; Garcia-Pichel et al., 1998) is not supported by phylogenetic analysis. The heterocystous cyanobacteria (comprising the two orders *Nostocales* and *Stigonematales*) form together a monophyletic group, with relatively low sequence divergence, as low as that presented by the single accepted genus *Spirulina* (Nübel, 1999). A grouping not corresponding to any official genus, the *Halothece* cluster, gathers unicellular strains of diverse morphology that are extremely tolerant to high salt and stem from hypersaline environments (Garcia-Pichel et al., 1998). A second grouping, bringing together very small unicellular ope n-ocean cyanobacteria (picoplankton) includes only marine picoplanktonic members of the genera *Synechococcus* and all *Prochlorococcus*. Several other statistically well-supported groups of strains that may or may not correspond to presently defined taxa can be distinguished. The botanical genus "*Microcystis*" of unicellular colonial freshwater plankton species is very well supported by phylogenetic reconstruction, as is the genus *Trichodesmium* of filamentous, nonheterocystous nitrogen-fixing species typical from oligotrophic marine plankton of the tropics. The picture that emerges from these studies is that sufficient knowledge of ecological and physiological characteristics can lead to a taxonomic system that is largely congruent to the 16S rRNA phylogeny.

A different principle of conversion of light energy into chemical energy is found in the Halobacteria. These archaea are largely confined to surface layers of hypersaline aquatic environments and grow predominantly by chemoorganoheterotrophy with amino or organic acids as electron donors and carbon substrates, generating ATP by respiration of molecular oxygen. In the absence of oxygen, several members are capable of fermentation or nitrate respiration. At limiting concentrations of oxygen, at least three of the described species of Halobacteria (*Halobacterium halobium*, *H. salinarium*, *H. sodomense*) synthesize bacteriorhodopsin (Oesterhelt and Stoeckenius, 1973), a chromoprotein containing a covalently bound retinal. Bacteriorhodopsin is incorporated in discrete patches in the cytoplasmic membrane ("purple membrane"). However, these prokaryotes have only a very limite d capability of light-dependent growth. Only slow growth and one to two cell doublings could be demonstrated experimentally (Hartmann et al., 1980; Oesterhelt and Krippahl, 1983). The fact that rhodopsin-based photosynthesis has been found only in the phylogenetically tight group of Halobacteria may indicate that, because of its lower efficiency, this type of light utilization is of selective advantage only under specific (and extreme) environmental conditions. Further information on the biochemistry, physiology and ecology of this group may be found in the chapters, *Introduction to the Classification of Archaea* and *The Family Halobacteriaceae*.

During the past years, culture-independent 16S rDNA-based methods have been used for the investigation of the composition of natural communities of phototrophic prokaryotes. These studies have provided evidence that more than

one genotype of *Chloroflexus* occur in one hot spring microbial mat and that four previously unkown sequences of cyanobacteria dominate in the same environment (Ferris et al., 1996; Ruff-Roberts et al., 1994; Weller et al., 1992). Similarly, nine different partial 16S rDNA sequences of *Chromatiaceae* and green sulfur bacteria, which differed from all sequences previously known, were retrieved from two lakes and one intertidal marine sediment (Coolen and Overmann, 1998; Overmann et al., 1999a).

However, 16S RNA signatures from natural populations were indistinguishable from those of cultured strains in the case of cyanobacteria with conspicuous morphologies, such as the cosmopolitan *Microcoleus chthonoplastes* (Garcia-Pichel et al., 1996) from intertidal and hypersaline microbial mats or *Microcoleus vaginatus* from desert soils (F. Garcia-Pichel, C. López-Cortés and U. Nübel, unpublished observations). In a similar manner, the 16S rRNA sequence of an isolated strain of *Amoebobacter purpureus* (*Chromatiaceae*) was found to be identical to the environmental sequence dominating in the chemocline of a meromictic salt lake (Coolen and Overmann, 1998; Overmann et al., 1999a). Obviously, the limited number of isolated and characterized bacterial strains rather than an alleged "nonculturability," at least in some cases, accounts for our inability to assign ecophysiological properties to certain 16S rRNA sequence types. This point is illustrated for extremely halotolerant unicellular cyanobacteria by the fact that only after a physiologically coherent group of strains was defined on the basis of newly characterized isolates (Garcia-Pichel et al., 1998) could the molecular signatures retrieved from field samples be assigned correctly.

It has to be concluded that 1) the numbers of species listed in Table 1 do not reflect the full phylogenetic breadth at least in the four groups of anoxygenic phototrophic prokaryotes as well as in morphologically simple *Cyanobacteria*, and 2) that the physiology and ecology of those species of phototrophic prokaryotes that are dominant in the natural environment in some cases may differ considerably from known type strains.

Habitats of Phototrophic Prokaryotes

Bacteria of the *Chloroflexus*-subgroup form dense microbial mats in geothermal springs, often in close association with cyanobacteria. *Chloroflexus aurantiacus* is a thermophilic bacterium which grows optimally between 52 and 60°C and thrives in neutral to alkaline hot springs up to 70–72°C. Of all anoxygenic phototrophic bacteria isolated so far, only *Chloroflexus aurantiacus* is capable of growth up to 74°C. In contrast to the domain Archaea, no hyperthermophilic species are known from the domain Bacteria. The phylogenetically related *Heliothrix oregonensis* grows optimally between 50 and 55°C and is abundant as a flocculant surface layer in a few alkaline springs in Oregon. Hydrothermal springs of 56–66°C, which contain sulfide of geothermal origin, are dominated by a surface layer or a "unispecific" mat of *Chloroflexus* (Castenholz and Pierson, 1995). Because of the absence of cyanobacteria in some of these systems, *Chloroflexus* presumably grows autotrophically (Pierson and Castenholz, 1995). In the presence of O_2, the mats exhibit an orange color whereas they are green under anoxic conditions (Castenholz and Pierson, 1995). The orange color is the result of the enhanced carotenoid biosynthesis under oxic conditions (see Chemotrophic Growth with O_2 in this Chapter). In the absence of sulfide, *Chloroflexus* is present as a distinct orange layer beneath a surface layer of cyanobacteria and may utilize their exudates or the fermentation products generated during decomposition of cyanobacteria. Molecular oxygen represses bacteriochlorophyll synthesis in *Chloroflexus* and often is present at saturation levels in the orange layers. Since bacteriochlorop hylls a and c are still present in this layer, however, it must be assumed that bacteriochlorophylls are synthesized at anoxic conditions during nighttime (Castenholz and Pierson, 1995).

Green and purple sulfur bacteria often form conspicuous blooms in non-thermal aquatic ecosystems (Figs. 4, 5A, 5B), although moderately

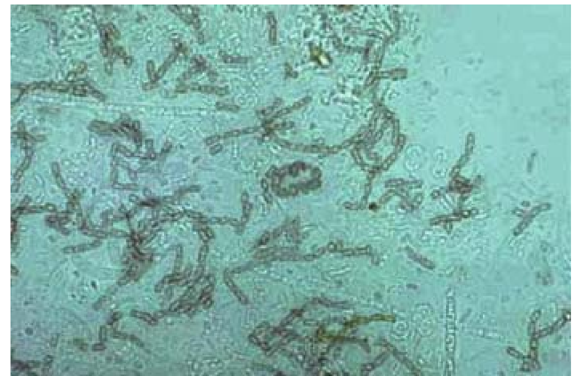


Fig. 4. Bright field photomicrograph of the bacterioplankton community thriving in the chemocline of the meromictic Buchensee (near Radolfzell, Germany) during autumn. The dominant anoxygenic phototroph at this time of the year is the green sulfur bacterium *Pelodictyon phaeoclathratiforme* (brown cells, which appear in chains or netlike colonies). In addition, phototrophic consortia ("*Pelochromatium roseum*," one consortium in the center) are found. Similar to *Pld. phaeoclathratiforme*, most of the colorless bacterial cells found in the chemocline contain gas vesicles as is evident from their highly refractile appearance in the bright field.

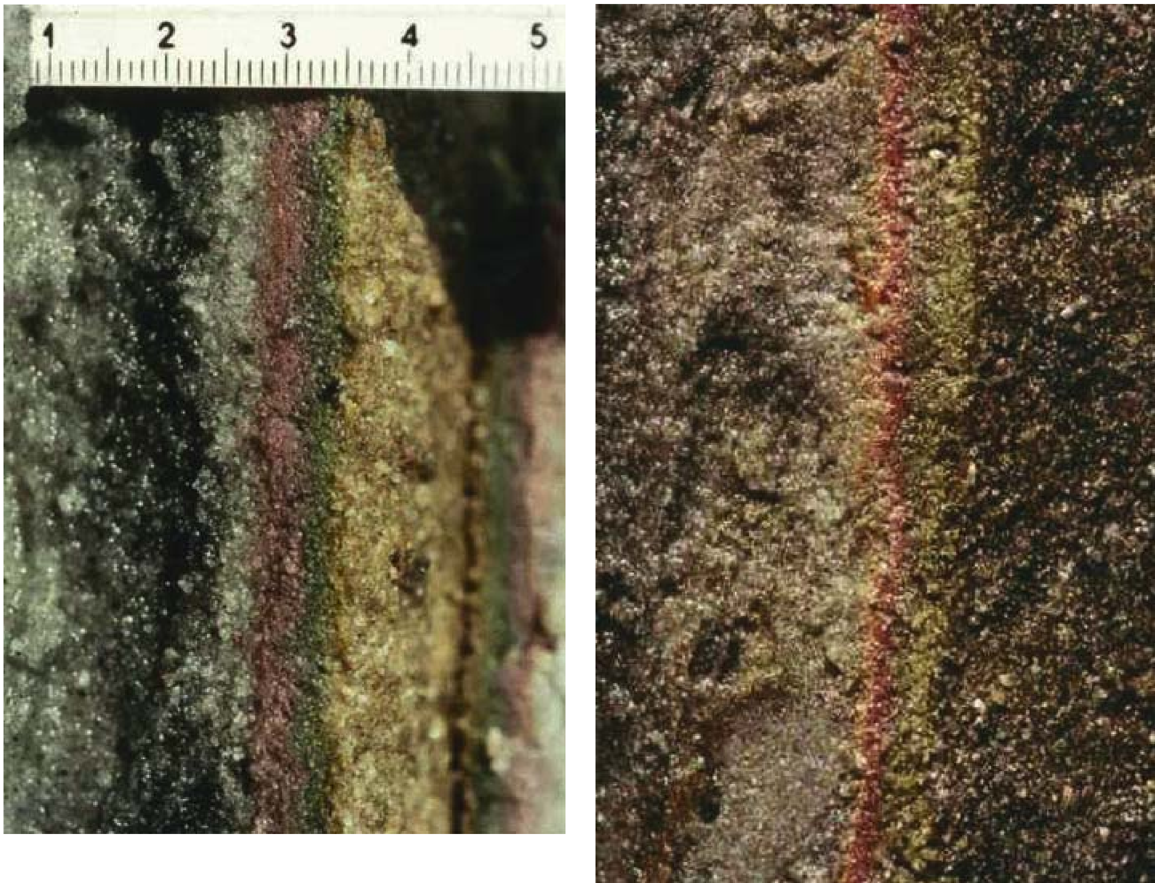


Fig. 5. Multilayered microbial mat as it is regularly found in the sandflats of Great Sippewissett Salt Marsh (Cape Cod, Massachusetts, USA). A. In most instances, the mats consist of a top green layer, an intermediate purple layer, and a grayish to blackish bottom layer. B. Fully developed microbial mats consist (from top) of an olive-green layer of diatoms and cyanobacteria, a green layer consisting mostly of cyanobacteria, a purple layer of purple sulfur bacteria, a peach-colored layer formed by BChl *b*-containing purple sulfur bacteria (morphologically similar to *Thiocapsa pfennigii*), and a greyish to blackish bottom layer.

thermophilic members of the genera *Chromatium* and *Chlorobium* have been described from hot spring mats (Castenholz et al., 1990). *Chlorobium tepidum* occurs in only a few New Zealand hot springs at pH values of 4.3 and 6.2 and temperatures up to 56°C. *Chromatium tepidum* was found in several hot springs of western North America at temperatures up to 58°C and might represent the most thermophilic proteobacterium (Castenholz and Pierson, 1995). In a recent compilation (van Gemerden and Mas, 1995), 63 different lakes and 7 sediment ecosystems harboring phototrophic sulfur bacteria were listed. Cell densities between 10^4 and $10^7 \cdot ml^{-1}$ and biomass concentrations between 10 and 1000 µg bacteriochlorophyll$\cdot l^{-1}$ are common in pelagic habitats. Of the purple sulfur bacteria, *Chromatiaceae* are typically found in freshwater and marine environments (Fig. 5A, B) whereas *Ectothiorhodospiraceae* inhabit hypersaline waters. The phototrophic sulfur bacteria grow preferentially by photolithoautotrophic oxidation of reduced sulfur compounds and are therefore limited to those environments where light reaches anoxic, sulfide-containing bottom layers. Because light and sulfide occur in opposing gradients, growth of phototrophic sulfur bacteria is confined to a narrow zone of overlap and is only possible if the chemical gradient of sulfide is stabilized against vertical mixing. In pelagic environments like lakes or lagoons, chemical gradients are stabilized by density differences between the oxic and anoxic water layers. Such density differences are either the result of thermals tratification and mostly transient (as in holomictic lakes) or are caused by high salt concentrations of the bottom water layers, in which case stratification is permanent (meromictic lakes). Pelagic layers of phototrophic sulfur bacteria extend over a vertical distance of 10 cm (van Gemerden and Mas, 1995; Overmann et al., 1991a) up to 30 m (Repeta et al., 1989) and reach biomass concentrations of 28 mg bacteriochlorophyll$\cdot l^{-1}$ (Overmann et al., 1994).

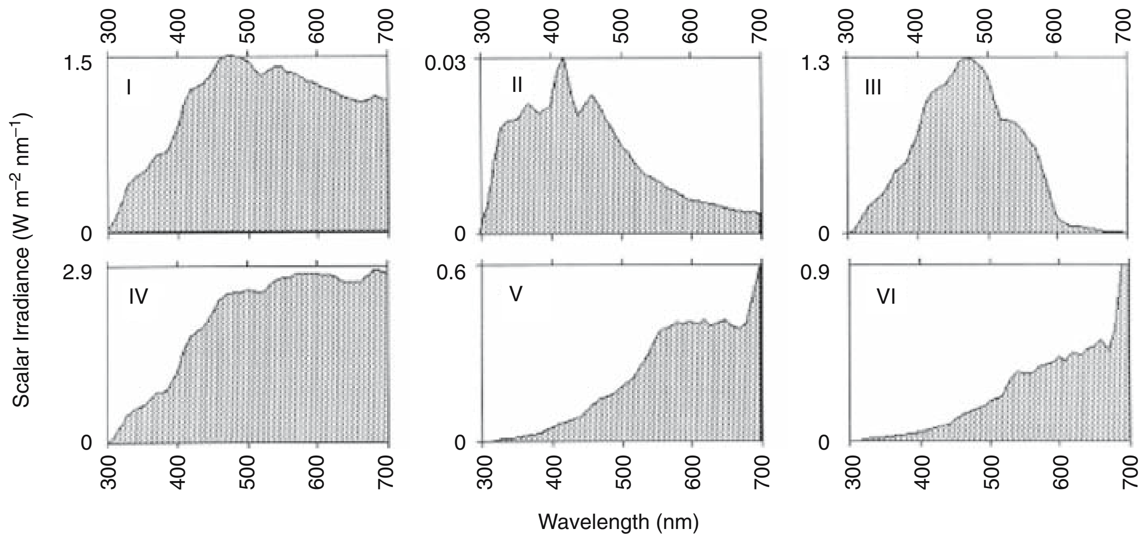


Fig. 6. Effects of the habitat on the physical exposure of cyanobacteria. The spectral scalar irradiance (sun and sky radiation) incident at ground level at noon in a clear midsummer day at 41°N is plotted in Plate I. The rest of the plates depict the in situ scalar irradiance experienced by cyanobacterial cells thriving in several habitats exposed to the incident fluxes in plate I (note different scales). Plate II: a "strong shade" habitat (North-facing surface illuminated by extremely diffuse sky radiation only), where scalar irradiance is very low but the relative importance of UV is enhanced. Plate III: a planktonic habitat (under 1 m of clear open-ocean water), where all fluxes remain fairly high and UVB and visible are more strongly attenuated than UVA. Plate IV: the surface of beach (quartz, feldspar) sand, where all UVB, UVA, and visible are higher than incident (by 120, 150, and 205%, respectively) due to light trapping effects. Plate V: 300-m deep in a wet topsoil, where UVB and U VA have been attenuated below 5% of incident but ca. 20% of the visible light remains. Plate VI: scalar irradiance within the thallus of the terrestrial cyanobacterial lichen *Collema* sp. Modified from Castenholz and Garcia-Pichel, 1999, after data from the following sources: F. Garcia-Pichel (unpublished observation); Garcia-Pichel, 1995; Büdel et al., 1997; and Smith and Baker, 1981.

Littoral sediments represent the second type of habitat of phototrophic sulfur bacteria. In these systems, turbulent mixing is largely prevented by the sediment matrix, and diffusion is the only means of mass transport. Gradients of light and sulfide are much steeper, and the fluxes of sulfide much larger compared to the pelagic environment. These conditions allow layers of phototrophic sulfur bacteria in sediments to reach much higher biomass densities (up to 900 mg bacteriochlorophyll·dm^{-3}; van Gemerden et al., 1989) than in lakes. At the same time, the layers are very narrow (1.3–5 mm; van Gemerden and Mas, 1995; Fig. 5A). This vertical distribution of anoxygenic phototrophic biomass ultimately determines the significance of microbial sulfide oxidation for the sulfur cycle in these ecosystems (see Significance of Anoxygenic Photosynthesis for the Pelagic Carbon and Sulfur Cycles in this Chapter). The spectral compos ition of light available for anoxygenic photosynthesis is considerably different between pelagic and benthic habitats (Fig. 6) and selects for different species of anoxygenic phototrophic bacteria. Whereas light of the blue to yellow-green wavelength bands dominates the depths of most lakes, infrared light is an important source of energy in benthic microbial mats (see Light Energy and the Spectral Distribution of Radiation in this Chapter).

The dominance of certain species of green sulfur bacteria (Fig. 4) or *Chromatiaceae* in pelagic environments in many cases can be explained by their specific light-harvesting capabilities (see Light Absorption and Light Energy Transfer in Prokaryotes and Competition for Light in this Chapter) and other phenotypic traits. Typically, those species that have been isolated from natural blooms in lakes are obligately photolithotrophic, lack assimilatory sulfate reduction, cannot reduce nitrate, and assimilate only few organic carbon sources (see Carbon Metabolism of Phototrophic Prokaryotes in this Chapter). This applies not only to all green sulfur bacteria but also to the dominant species of *Chromatiaceae*. Obviously, in the chemocline of lakes the metabolic versatile *Chromatiaceae* species have no selective advantage. As judged from the physiological characteristics of strains of phototrophic sulfur bacteria isolated from sediments, the pronounced diurnal variations in oxygen concentrations and salinity, together with the different light quality, select for different species composition in benthic microbial mats. The purple sulfur bacterium *Chromatium* (and the multicellular gliding colorless sulfur bacterium *Beggiatoa*) are found in many microbial mats

and exhibit diurnal vertical migrations in response to the recurrent changes in environmental conditions (Jørgensen, 1982; Jørgensen and Des Marais, 1986). Microbial mats of intertidal sediments are typically colonized by the immotile purple sulfur bacterium *Thiocapsa roseopersicina* and small motile thiobacilli (van den Ende et al., 1996).

In contrast to the phototrophic members of the γ-Proteobacteria, purple nonsulfur bacteria of the α- and β-subclasses of *Proteobacteria* do not appear to form dense accumulations under natural conditions (Biebl and Drews, 1969; Swoager and Lindstrom, 1971; Steenbergen and Korthals, 1982). However, purple nonsulfur bacteria can be readily isolated from a wide variety of marine, lacustrine and even terrestrial environments (Imhoff and Trüper, 1989; J. Overmann, unpublished observation). While comprehensive comparative quantitation of the ecological importance of purple nonsulfur bacteria is still lacking, as many as ca. 10^6 c.f.u. of purple nonsulfur bacteria could be cultivated per cm^3 of sediment in coastal eutrophic settings (Guyoneaud et al., 1996).

Generally, aerobic phototrophic bacteria thrive in eutrophic marine environments. Obligately aerobic bacteria containing bacteriochlorophyll *a* have been isolated from beach sand and seaweeds (thalli of *Enteromorpha linza* and *Sargassum horneri*; Shiba et al., 1979), and in some cases also from freshwater ponds and microbial mats. At least some of the aerobic phototrophic bacteria apparently can survive in situ temperatures of up to 54°C (Yurkov and Beatty, 1998). Aerobic phototrophic bacteria were isolated from hydrothermal plume water of a black smoker 2000 m below ocean surface (Yurkov and Beatty, 1998); acidophilic strains could be isolated from acidic mine drainage. Typically, *Methylobacterium* species are isolated from foods, soils and leaf surfaces (Shimada, 1995). Photosynthetic *Rhizobium* strains are widely distributed in nitrogen-fixing stem nodules of the tropical legume *Aeschynomene* spp. where they are present as symbiosomes. Similar strains have also been found in root and hypocotyl nodules of *Lotononis bainesii* (Fabaceae). These photosynthetic rhizobial and regular symbiosomes differ in that the former contains only one large spherical bacteroid. The photosynthesis of these endosymbionts may provide energy for nitrogen fixation and permit a more efficient growth of the host plant, since up to half of the photosynthate produced by legumes is allocated to nitrogen fixation (Fleischman et al., 1995).

Heliobacteriaceae appear to be primarily soil bacteria and have been isolated from dry paddy fields or other soils throughout the world (Madigan and Ormerod, 1995). Bacteria of this family may even represent the dominant anoxygenic phototrophic bacteria in soil (Madigan, 1992). Occasionally, strains also have been isolated from lakeshore muds and hot springs (Amesz, 1995; Madigan and Ormerod, 1995). *Heliobacterium modesticaldum* grows up to 56°C (Kimble et al., 1995). Spore formation may offer a selective advantage to *Heliobacterium modesticaldum*, *Heliophilum fasciatum*, and *Heliobacterium gestii* in their main habitat (rice field soil), which undergoes periodic drying and concomitantly becomes oxidized (Madigan, 1992). During growth of the rice plants, organic compounds excreted by their roots could provide sufficient substrates for photoheterotrophic growth of the *Heliobacteriaceae*.

Cyanobacteria as a group exhibit the widest range of habitats of all phototrophic prokaryotes due to the ubiquity of water, their preferred electron donor for the reduction of CO_2. In principle, cyanobacteria can thrive in any environment that has, at least temporarily, liquid water and sunlight. They are known from Antarctic endolithic habitats and from hot springs. More than 20 species of cyanobacteria (Castenholz and Pierson, 1995) are thermophilic. Effectively, however, no cyanobacteria are known from acidic environments (below pH 4.5) and competition with eukaryotic microalgae or higher plants may restrict their growth in other environments. Cyanobacteria are found in the plankton of coastal and open oceans and in freshwater and saline inland lakes. They thrive in the benthos of marine intertidal (Fig. 5B), lacustrine and fluvial waters and in a large variety of terrestrial habitats (soils, rocks, trees). Symbiotic associations are common.

In the marine plankton, the phycoerythrin-containing *Synechococcus* often represents a major fraction of all primary producers. The same holds true for *Prochlorococcus* (Campbell and Vaulot, 1993; Chisholm et al., 1988; Olson et al., 1990b). Compared with the high number of cyanobacterial species found in freshwater plankton, intertidal areas, and hypersaline environments, the diversity of this group is very limited in the open ocean (Carr and Mann, 1994). The predominant group invariably consists of small (<2 m) mostly nonmotile, non-nitrogen-fixing single cells assigned to the genus *Synechococcus*, which is found in the photic zone of all oceans except in the coldest areas. As a characteristic feature, the cells contain phycoerythrin as accessory photopigment which confers an orange autofluorescence on the cells. Despite their similar phenotype, marine *Synechococcus* strains are genetically heterogenous (Waterbury et al., 1986). An important component of the phytoplankton in tropical and subtropical oceans are the filamentous *Trichodesmium* spp. (Carr

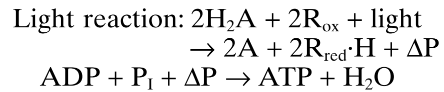
and Mann, 1994). The bundle and aggregate forming *Trichodesmium* typically develop into blooms that can extend kilometers long and are detected on the surface of oligotrophic tropical and subtropical oceans with the naked eye or with satellite imagery from space. The success of *Trichodesmium* can be mainly traced to the highly efficient nitrogen-fixing capacity of these nonheterocystous cyanobacteria. Their activities attain global magnitude for the nitrogen cycle (Capone et al., 1977). Heterocystous, nitrogen-fixing cyanob acteria of the genera *Nodularia*, *Anabaena*, and *Aphanizomenon* bloom in mesotrophic and eutrophic fresh and brackish waters. Together with the blooms of the nonheterocystous genus *Microcystis*, these cyanobacteria have become a real environmental concern, not only because of their effects of overall water quality but also because of their ability to produce toxins, which are known to have caused the deaths of humans and cattle. In the chemocline of stratified lakes, deep blooms of cyanobacteria occur frequently.

Edaphic cyanobacteria are also distributed worldwide, especially in soils of basic pH; sheathed oscillatorian forms (*Microcoleus vaginatus*, *"Schizothrix"* spp.), along with heterocystous ones (*Nostoc*, *Scytonema*) are of major ecological relevance in arid and semiarid regions where growth of higher plants is restricted. In such environments, cyanobacteria adopt a life strategy of resistance to desiccation (Potts, 1994) making use of the few occasions in which liquid water is available from rain or dew. Very intense productivity spurts occur in a matter of minutes after wetting (Garcia-Pichel and Belnap, 1996). The so-called "cyanobacterial desert crusts" contribute significantly to the biogeochemistry and to the physical stability of arid soils. Other important terrestrial habitats of cyanobacteria are the surface or subsurfa ce of rocks: extensive endolithic cyanobacterial communities, usually dominated by members of the genus *Chroococcidiopsis*, have been described from tropical, desert and polar environments (Friedmann, 1982; Wessels and Büdel, 1995).

In the course of evolution, cyanobacteria have entered into symbiotic associations with a multitude of organisms. These have reached a wide range in the degree of interdependence between partners (see Symbiosis between Phototrophic Bacteria and Eukaryotes in this Chapter).

Principles and Prerequisites of Photosynthesis

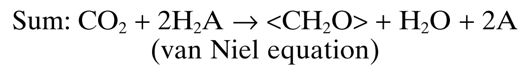
Bacterial photosynthesis can be divided into two different types of reactions 1) the light reaction, in which light energy is trapped and converted into ATP (via a proton-motive force ΔP) and a reduced redox carrier $R_{red}\cdot H^+$, and 2) the so-called dark reaction of biosynthetic carbon reduction.



$$\text{Light reaction: } 2H_2A + 2R_{ox} + \text{light} \rightarrow 2A + 2R_{red}\cdot H + \Delta P$$

$$ADP + P_I + \Delta P \rightarrow ATP + H_2O$$

$$\text{Dark reaction: } CO_2 + ATP + 2R_{red}\cdot H + H_2O \rightarrow \langle CH_2O \rangle + ADP + Pi + 2R_{ox}$$



$$\text{Sum: } CO_2 + 2H_2A \rightarrow \langle CH_2O \rangle + H_2O + 2A$$

(van Niel equation)

Microorganisms have found different ways to accomplish these two tasks.

Light Energy and the Spectral Distribution of Radiation

The present day solar irradiance at the average distance of Earth to the sun and outside the atmosphere (the so-called *solar constant*) is $1353\cdot W\ m^{-2}$ (Kirk, 1983). The spectral energy distribution of this solar radiation approximates that of a black body at 6000°K (the surface temperature of the sun). According to Wien's Law, a black body at this temperature has a maximum emission of electromagnetic energy at about 480 nm. The actual spectral energy distribution of solar radiation exhibits minima which reflect the absorption bands of hydrogen in the outer atmosphere of the sun (Fig. 7). The total light energy received by the Earth is $5.46\cdot 10^{24}\ J\cdot year^{-1}$, which would correspond to $339.4\ W\cdot m^{-2}$. The actual solar (time and space-averaged) irradiance reaching the surface of the Earth amounts only to $160\ W\cdot m^{-2}$ (Gates, 1962; Dietrich et al., 1975). This large reduction is due to Raleigh scattering by air molecules and dust particles, and of light absorption by water vapor, O_2, O_3 and CO_2 during the passage of radiation through the Earth's atmosphere. Concomitantly, the spectral distribution of solar irradiance is changed especially because water vapor absorbs infrared light (Fig. 7). At sea level, light of the wavelength regions 400–700 nm (PAR, photosynthetically available radiation) constitutes 50% of this irradiance (Kirk, 1983).

Based on estimates for global primary productivity, only 0.13% of the flux of solar energy reaching the surface of the Earth is converted into chemical energy by photosynthesis (Odum, 1983; see Introduction in this Chapter). Under natural conditions, photosynthesis of the various groups of phototrophic prokaryotes is limited by different environmental factors including light, reduced sulfur compounds, organic carbon substrates, oxygen, and temperature. The physical

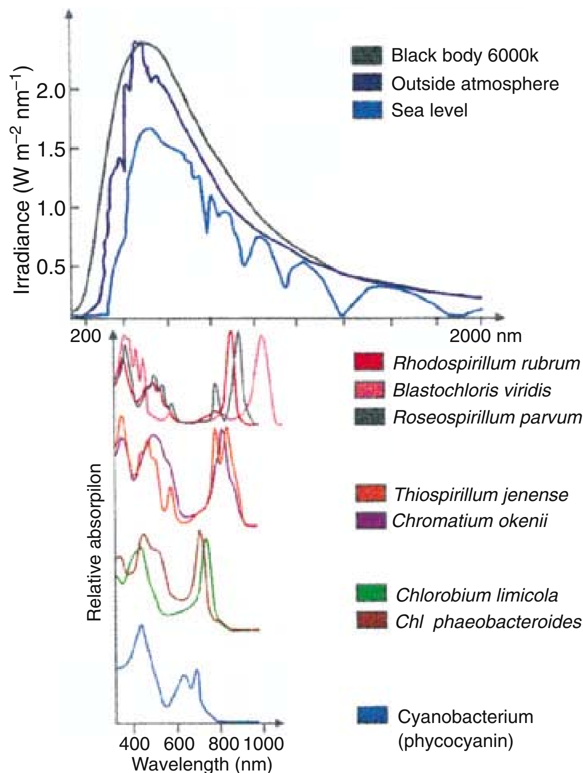


Fig. 7. Spectral energy distribution of solar radiation outside the atmosphere and at sea level as compared to the absorption spectra of various phototrophic bacteria. Absorption spectra of the purple nonsulfur bacterium *Rhodospirillum rubrum* (containing BChl *a*, spirilloxanthin), *Blastochloris viridis* (BChl *b*, 1,2-dihydroneurosporene), and *Roseospirillum parvum* (BChl *a*, spirilloxanthin, lycopenal), of the Chromatiaceae species *Thiospirillum jenense* (BChl *a*,lycopene, rhodopin) and *Chromatium okenii* (BChl *a*, okenone), of the Chlorobiaceae species *Chlorobium limicola* (BChl *c*, chlorobactene) and *Chlorobium phaeobacteroides* (BChl *e*, i sorenieratene) and of a cyanobacterium (Chl *a*, phycocyanin) are depicted.

characteristics of the medium have, through processes of absorption and scattering, a large influence on the available radiation (see Competition for Light in this Chapter). As a second major limiting factor, the availability of nutrients limits the growth of phototrophic bacteria and as a consequence, photosynthetic energy conversion.

Surface environments exposed to sky radiation (as in strong shades) may be enriched in blue and UV radiation (Fig. 6). Water is the major light-absorbing component only in very clear open ocean and inland lakes. It strongly absorbs light of the ultraviolet, red and especially infrared (wavelengths around 745 and 960 nm). As a consequence, tens of meters below the surface of clear waters the spectrum is enriched in blue wavelengths. Several meters below coastal or most lacustrine water surfaces, the spectrum is enriched towards the green wavelengths, and deep(several millimeters) in the photic zones of sediments and soils infrared wavelengths dominate. Yellow substance in lakes is mostly of terrestrial origin and particularly absorbs light of the ultraviolet and blue portion of the spectrum (Kirk, 1983). In dystrophic lakes in which high concentrations of humic compounds are the major light-absorbing components, light of the red wavelength range prevails such that green-colored species of green sulfur bacteria have a selective advantage over their brown-colored counterparts or purple sulfur bacteria (Parkin and Brock, 1980a).

In benthic and soil ecosystems, light quality differs fundamentally from that in the pelagic environment. In the visible wavelength range, radiation is strongly attenuated by mineral and biogenic particles. In sandy sediments light attenuation occurs preferentially in the wavelength range of blue light due to the reflection by sand grains (Jørgensen and Des Marais, 1986; Kühl and Jørgensen, 1992). The presence of iron minerals results in an enhanced attenuation of UV and blue wavelengths (Garcia-Pichel and Belnap, 1996). In contrast, absorption of infrared light by sediment particles is low and absorption by water is negligible due to the short optical pathlength. As a consequence of the optical properties of the sediment particulates, the red and infrared portion of the spectrum penetrate to the deepest levels. Multiple scattering causes the light fields to become rapidly diffuse, so that bacteria thriving within these environments receive light from all directions. The parameter measuring light received at a point in space from all directions is called scalar irradiance (E_0, or photon fluence rate). A third important, but counterintuitive, phenomenon is the presence of maximum irradiance values close to the surface, which are even larger than the incident scalar irradiance (Fig. 6). Below this surficial zone where the E_0 maximum occurs, E_0 attenuates exponentially (Jørgensen and Des Marais, 1986; Jørgensen and Des Marais, 1988; Kühl and Jørgensen, 1992; Lassen et al., 1992). For visible light, the measured photic depths (depths where E_0 is attenuated to 1% of the incident) varied between 3.1 mm for quartz sand and 0.45 mm for silty muds (Garcia-Pichel and Bebout, 1996b). In the ultraviolet (UV) at 310 nm, the corresponding depths were only 1.25 and 0.23 mm.

Light Absorption and Light Energy Transfer in Prokaryotes

Principle

The chlorophyll-based photosystems of bacteria convert electromagnetic energy into a redox gradient. The redox reactions are initiated by

absorption of electromagnetic energy, leading to a transition of specific molecules into an excited electronic state. An increase in the electronic energy of a molecule requires more energy than changes in vibrational or rotational states. Since the energy of light quanta is inversely related to their wavelength (Planck's Law), molecules absorb electromagnetic radiation of short wavelengths (ultraviolet and visible light) during changes in electronic energy, and longer wavelengths during changes in vibrational (near infrared radiation) and rotational energy (far infrared radiation and microwaves). Changes in the electronic state of molecules, and thus photochemically driven redox reactions by light absorption, can only occur by absorption of quanta of wavelengths <1240 nm (i.e., an energy larger than 1 eV per electron). This fact obviously limits the wavelength range that is usable for photochemical reactions. The major fraction of solar energy is present in the wavelength range between 400 and 750 nm. These wavelengths can only be harvested by organic molecules containing delocalized π-electrons in conjugated double bonds (Fig. 7).

Pigments and Light-Harvesting Complexes

To capture light for photosynthesis, phototrophic organisms employ three classes of pigment molecules: magnesium porphyrins (chlorophylls and bacteriochlorophylls, also called chlorins), open-chain tetrapyrrole bilin pigments (phycobilins), and carotenoids. However, other types of chromophores may be used in non-photosynthetic light-harvesting, as is the case of the flavins and pterines of DNA-photolyase (Tanada et al., 1997) and in specific regulatory photoreceptors (Halobacteriaceae, bacteriorhodopsin). Until recently it appeared that only the magnesium-containing chlorin molecules were employed as the major photosynthetic pigment. The aerobic photosynthetic bacterium *Acidiphilium rubrum* is the first photosynthetic organism known to employ zinc-containing bacteriochlorophyll *a* as the photochemically active pigment (Wakao et al., 1996).

Free molecules remain in the excited singlet state for as little as 10^{-8} to 10^{-9} sec and rapidly return to the ground state (fluorescence). Through the multiplicity of vibrational and rotational states associated with each electronic energy level, two different electronic energy states may overlap. In such molecules the lowermost electronic energy level (the lowest excited singlet state) is reached in a rapid series of radiationless transitions with a concomitant small decrease in free energy. The wavelengths emitted during the subsequent return of the electron to the ground state therefore is longer than those wavelengths that were absorbed (Stokes shift). Chlorophylls and bacteriochlorophylls exhibit two major absorption bands (Table 2) and, when excited in the dissolved state, a corresponding red (685 nm for chlorophyll a) or infrared (786 nm for bacteriochlorophyll *a*) fluorescence. In photosynthetically active c ells, however, only about 1% of the absorbed light energy is lost by fluorescence. It is a characteristic of the photosynthetic apparatus of living organisms, that fluorescence (hence loss of already absorbed energy) is minimized. Instead, most of the energy absorbed by the antenna pigments is channeled by vectorial and radiationless inductive dipole resonance toward the reaction centers, where it drives the photochemical redox reactions. The specific coordination of pigment molecules in photosynthetic organisms favors inductive resonance and photochemical reactions over fluorescence. Within the photosynthetic antenna, a fine modulation of the

Table 2. Major absorption maxima of chlorins in whole cells and in the dissolved state, and fluorescence maxima of whole cells of phototrophic prokaryotes.

	Absorption maxima (nm)	Fluorescence maxima (nm)	
Chlorin	Whole cells	Acetone extracts	Whole cells
Chl *a*	670–675	435, 663	680–685
Chl *b*	n.d.	455, 645	(in acetone 652)
Chl *d*	714–718	400, 697	(in acetone 745)
BChl *a*	375, 590, 805, 830–911	358, 579, 771	907–915
BChl *b*	400, 605, 835–850, 986–1035	368, 407, 582, 795	1040nm
BChl *c*	457–460, 745–755	433, 663	775
BChl *d*	450, 715–745	425, 654	763
BChl *e*	460–462, 710–725	459, 648	738
BChl *g*[a]	375, 419, 575, 788	365, 405, 566, 762	n.d.

[a]Bacteriochlorophyll *g* of the Heliobacteriaceae shows structural relationships to chlorophyll *a* because it contains a vinyl group on tetrapyrrole ring I. Like in bacteriochlorophylls *a* and *b*, pyrrole ring II is reduced, however, and the esterifying alcohol is famesol as in bacteriochlorophylls of green sulfur bacteria. As for bacteriochlorophyll *a* or *b*, the reduced state of ring II in bacteriochlorophyll *g* causes an additional though smaller absorption maximum, the Q_x band at about 567nm.
n.d., not determined.

absorption properties of the pigments occurs because of differences in their binding to the antenna proteins, so that the vectorial excitation cascade is thermodynamically favored (i.e., in a sequence involving pigments with progressively longer absorption maxima). The resulting small differences in the energy level of antenna pigments directs the transfer of excitation energy more or less to the reaction center.

A second consequence of the interactions between pigment molecules and proteins is the shift of the absorption peaks of the former towards longer wavelengths. In the case of chlorophyll *a*, the shift is comparatively small while it is larger in bacteriochlorophyll-protein complexes (up to 650 nm in bacteriochlorophyll *b*-containing phototrophic bacteria; Table 2). The shift for most carotenoids in association with proteins is as small as for chlorophyll *a*. In intact cells, carotenoids absorb mainly in the 420–550 nm wavelength region. In contrast, binding of one type of porphyrin pigment (bacteriochlorophyll *a*) by different apoproteins has led to a considerable diversification of the long-wavelength absorption maxima in purple sulfur and nonsulfur bacteria (Fig. 7). Obviously the role of proteins in pigment-protein-complexes is not confined to the proper coordination of pigment molecules but als o can represent a means to exploit wavelength regions not utilized by other phototrophic organisms. Especially in intertidal microbial mats, variations in the fine structure of the pigment-protein complexes is a means of ecological niche separation (see Competition between Phototrophic Bacteria in this Chapter). The absorption spectra of whole cells of phototrophic bacteria seem to have evolved in such a way that almost the entire electromagnetic spectrum suitable for electrochemical reactions can be exploited (Fig. 7).

The first step of porphyrin synthesis is the formation of 5-amino levulinic acid (δ-ALA). In *Chloroflexus aurantiacus*, β- and γ-Proteobacteria, *cyanobacteria*, *Heliobacteriaceae*, and green sulfur bacteria, δ-ALA is synthesized from glutamate (C5-pathway), which therefore appears to represent the more ancestral pathway. In contrast, α-Proteobacteria as well as yeasts, fungi, and animals form δ-ALA by the ALA synthase-mediated condensation of glycine with succinyl-CoA (Beale, 1995; Oh-Hama, 1989; Oh-Hama et al., 1991).

All (bacterio)chlorophylls exhibit two major absorption bands (Table 2), leaving a considerably wide gap in the absorption spectrum. The latter is partially complemented by the absorption spectrum of carotenoids found in all phototrophic bacteria or by a range of phycobiliproteins in most cyanobacteria. Owing to the presence of up to 15 conjugated double bonds, carotenoids absorb light at the short wavelength end of the visible range.

The light-harvesting antenna complexes of green sulfur bacteria and *Chloroflexus* are extramembranous ovoid organelles, so-called chlorosomes, which are attached to the inner surface of the cytoplasmic membrane and contain bacteriochlorophylls *c*, *d*, or *e*. Chlorosomes are exceptional in that proteins do not seem to be involved as ligands for most of the antenna bacteriochlorophyll molecules. Instead, interactions between the bacteriochlorophylls themselves govern the absorptive properties of the photosynthetic antenna in green sulfur bacteria (Blankenship et al., 1995; Fig. 3A). In all other phototrophic prokaryotes studied, chlorins and carotenoid molecules occur in complexes with proteins.

Chlorins in pigment-protein complexes are noncovalently bound by histidine imidazole residues, which ligate the central magnesium atom of the porphyrin (Drews and Golecki, 1995). In some cases (e.g., heliobacterial reaction center protein; Vermaas, 1994) the histidine residues are replaced by asparagine, glutamine or arginine, which may function as ligands. Noncovalent binding of carotenoids seems to be mediated largely by hydrophobic interactions. In the purple nonsulfur bacteria, the *Chromatiaceae*, and *Ectothiorhodospiraceae*, all antenna complexes (and reaction centers) are located within intracytoplasmic membranes that are differentiated from, but contiguous to, the cytoplasmic membrane of the cell. In purple nonsulfur bacteria, *Chromatiaceae*, and *Ectothiorhodospiraceae*, intracellular membranes occur as vesicles, stacks, lamellae, or tubules (Figs. 2 and 3B). Most photosynthetic species of the α-Proteobacteria (*Rhodocyclus purpureus*, *Rhodocyclus tenuis*, *Rubrivivax gelatinosus*) do not form extensive intracellular membrane systems. The photochemical apparatus of purple nonsulfur bacteria is confined to the intracellular membrane system, whereas the enzyme complexes of the respiratory chain and transport systems are located in the cytoplasmic membrane (Bowyer et al., 1985). This functional differentiation does not seem to exist in purple sulfur bacteria (*Allochromatium vinosum*, *Ectothiorhodospira mobilis*; Drews and Golecki, 1995). With one known exception, the photosynthetic apparatus in *cyanobacteria* is located on specialized intracellular membranes (thylakoids). Thylakoids may be either single or stacked, a nd are distributed concentrically (parallel to the cytoplasmatic membrane), radially, or randomly (Fig. 2). Like in chloroplasts, lateral heterogeneity (spatial separation of photosystem I in stroma lamellae and of photosystem II in grana stacks) has been found in "Prochlorophytes."

In *Heliothrix*, the *Heliobacteriaceae*, some purple nonsulfur bacteria (e.g., *Rhodocyclus tenuis*; Wakim and Oelze, 1980) and one cyanobacterium (*Gloeobacter violaceus*), the photosynthetic apparatus is located in the cytoplasmic membrane.

The light-harvesting antenna complexes of purple nonsulfur and purple sulfur bacteria are composed of two small, membrane-spanning α- and β-polypeptides to which bacteriochlorophyll *a* or *b*, and carotenoids are noncovalently bound. The polypeptide monomers aggregate within the membrane to form ring structures of 16 (LHI) or 9 (LHII) subunits, respectively (McDermott et al., 1995; Fig. 3B). According to the current structural model, the ring of 16 LHI-subunits surrounds one reaction center. Several LHII-aggregates transfer energy to this supercomplex.

In *Cyanobacteria*, light-harvesting chlorophyll *a* is present in two different types of protein complexes. The CP43 and CP47 core-antenna complexes are tightly associated with photosystem II (Barry et al., 1994). In photosystem I, however, antenna chlorophylls are an integral part of the reaction center itself (Golbeck, 1994; Fig. 3C).

A third class of light-harvesting complexes are phycobilisomes. They occur in the division *Cyanobacteria* (and in the plastids of red algae and some other groups of eukaryotic algae), and in most species are the main light-harvesting antenna structures of these bacteria. Under the electron microscope, phycobilisomes appear as hemidiscoidal to cylindrical particles attached to the cytoplasmic side of the thylacoids. In *Gloeobacter violaceus*, the cytoplasmic membrane is underlain by a continuous subcortical layer containing the phycobilisomes. Light energy absorbed by phycobilisomes is transferred preferentially to photosystem II, with chlorophyll *a* serving as antenna for photosystem I. However, short-term or partial spillover may occur, as the phycobilisomes are quite mobile (van Thor, J.J., et al., 1998). While the blue and red wavelength range is absorbed mainly by chlorophyll; the phycobilisomes harvest the blue-green, yellow, and orange regions (450–655 nm) of the light spectrum, thereby extending the spectral range of photosynthetic light-harvesting considerably (Fig. 7). The capacity of forming phycobilisomes is of selective advantage for the colonization of low light aquatic habitats (see Competition between Phototrophic Bacteria in this Chapter). Most (80%) of the phycobilisome mass is water-soluble phycobiliproteins, which contain open-chain tetrapyrrole chromophores (the phycobilins). Four types of phycobilins are known, the blue-colored phycocyanobilin (PCB), red-colored phycoerythrobilin (PEB), yellow-colored phycourobilin (PUB), and purple-colored phycobiliviolin (PXB, also sometimes abbreviated CV). They are found in various molar ratios, and form part of four recognized types of phycobiliproteins: allophycocyanin (APC), phycocyanin (PC), phycoerythrocyanin (PEC), and phycoerythr in (PE). In contrast to (bacterio)chlorophylls, the chromophores are covalently bound by thioether linkages to cysteine residues of the apoproteins. Up to three chromophores may be bound to a single α- or β-polypeptide. The phycobiliproteins are heteromonomers forming $(\alpha\beta)_3$ trimeric disks. Together with chromophore-free linker polypeptides, these disks are assembled in aggregates, the phycobilisomes, which are attached to the cytoplasmic side of photosystem II (Fig. 3C). Peripheral rod elements consisting of phycoerythrin (which harbors PEB, and sometimes also PUB) or phycoerythrocyanin (with PCB and PXB), and phycocyanin (with PCB, and in some cases small amounts of PEB) are arranged in a hemidiscoidal fashion around a core substructure consisting largely of allophycocyanin (with PCB). The different absorption properties of the phycobilins are the result of differences in the number of conjugated double-bonds (the conjugated π-electron system is shorter for PEB and PUB), in the side chains of the tetrapyrrole prosthetic groups, including also chemically distinct chromophore-protein linkages, and in the protein environments of the chromophores (Sidler, 1994). Light energy is absorbed mainly by the peripheral rods, and transferred rapidly by radiation-less downhill energy transfer from phycoerythrin (absorption maximum 495– 575 nm) or phycoerythrocyanin (575 nm) to phycocyanin (615–640 nm). Finally, allophycocyanin (650–655 nm) transfers the energy to photosystem II.

Not all cyanobacteria possess all of these different phycobiliproteins. Those synthesizing exclusively APC and PC appear blue-green. Many heterocystous cyanobacteria also produce PEC in addition to APC and PC (Bryant et al., 1982); these strains never produce PE. Dark-colored strains of many benthic genera contain large amounts of PC and PE. Red cyanobacteria, typical for deep lacustrine and marine waters produce large amounts of PE, and only small amounts of PC. Marine open ocean cyanobacteria (*Synechoccus*, *Trichodesmium*) contain large amounts of a PUB-rich PE, with absorbance maxima around 495–500 nm.

In Chl *b*-producing cyanobacteria (the former “Prochlorophytes”), the photosynthetic antennae are intrinsic to the membrane, and in *Prochlorothrix hollandica*, they contain chlorophyll *a* and β-carotene (PSI; photosystem I), or chlorophylls a and b, and zeaxanthin (PSII; photosystem II). In contrast to the other two known

species, *Prochlorococcus marinus* contains divinyl-chlorophyll *a* and divinyl-chlorophyll *b*. The presence of chlorophyll *b* and zeaxanthin and their functional connection to the reaction center of PSII enables these bacteria to absorb light in the wavelength range of 460–500 nm, and is of selective advantage under light conditions present in the lower euphotic zone of oligotrophic oceans (see Competition for Light in this Chapter). However, chlorophyll *b* represents only a minor fraction of the photosynthetic pigments. In *Prochloron*, the ratio of chlorophyll *a*/chlorophyll *b* is between 2.6 and 12.0 (Thorne et al., 1977); this ratio is even higher in *Prochlorothrix* (10–18), in which the ratio of PSI to PSII is > 3 : 1. In *Prochlorothrix hollandica*, cells grown at low light intensities exhibit the lowest chlorophyll *a*/chlorophyll *b* ratios (Matthijs et al., 1994).

A very interesting variation is exemplified by *Acaryochloris marina*, where Chl *d* is the major antenna chlorin (2% of the dry weight, whereas Chl *a* is only 0.1%) harvesting light for both photosystems (Schiller et al., 1997). *A. marina* also contains traces of a Chl *c*-like pigment in addition to more typically cyanobacterial carotenoids (α-carotene—found also in *Prochlororococcus*—and zeaxanthine—found in many cyanobacteria) and phycobiliproteins (APC and PC; Miyachi et al., 1997).

In purple bacteria, the size of the photosynthetic antenna is in the range of 20–200 bacteriochlorophyll *a* per reaction center (Zuber and Cogdell, 1995). The specific bacteriochlorophyll *a* content of aerobic bacteriochlorophyll-containing bacteria reaches only 5–10% of that of anoxygenic phototrophic bacteria (Yurkov and Beatty, 1998). At least in one strain (*Rhizobium* BTAi1), the size of the photosynthetic unit is similar to that of anoxygenic phototrophic bacteria (Evans et al., 1990), indicating that the low pigment content is due to a low number of reaction centers. In PSII of cyanobacteria, the antenna comprises 300–800 phycobilin chromophores and 47 chlorophyll *a* molecules (Sidler, 1994; Matthijs et al., 1994), whereas the reaction center protein PsaA of PSI binds 110 chlorophyll *a* molecules (Golbeck, 1994). The photosynthetic antenna of green sulfur bacteria is significantly larger than that of other anoxygenic phototrophs and comprises about 1000 bacteriochlorophyll molecules connected to one reaction center (see The Family Chlorobiaceae, Physiology section in Volume 7). This appears to be one major reason for the competitive success of green sulfur bacteria in low-light environments (see Competition for Light in this Chapter). Antenna size is smaller in *Chloroflexus* (Olsen, 1998). About 35 molecules of bacteriochlorophyll g are associated with one reaction center in *Heliobacteriaceae* (Amesz, 1995).

Efficiency of Light Harvesting

The light absorption capabilities of photosynthetic prokaryotes can be judged best by calculating which fraction *f* of the light impinging on a single cell is actually absorbed. This fraction is considerable for purple sulfur and other bacteria. The highest bacteriochlorophyll-specific attenuation coefficient k_B has been determined for a population of *Amoebobacter purpureus* (0.050 $m^2 \cdot (mg\ BChl\ a)^{-1}$; Overmann et al., 1991a). For comparison *Prochlorococcus* has a chlorophyll-specific attenuation coefficient of 0.0147–0.0232 $m^2 \cdot$(mg Chl a^{-1} (Moore et al., 1998). For *Amoebobacter*, *f* is 0.36, or 36%, as calculated from Beer's Law and using the value of k_B, the intracellular c oncentration of light-harvesting pigments C (10.3×10^6 mg $BChl \cdot m^{-3}$, calculated from a content of 85 μg BChl·(mg protein)$^{-1}$; van Gemerden and Mas, 1995; Watson et al., 1977) and the average optical path-length d of a cell (2 μm):

$$f = 100 \times \exp(-k_B \times C \times d)$$

Of the photosynthetic pigments that absorb this high fraction of incident light, the majority (typically >97%) serves in light-harvesting and transfers excitation energy to the photochemical reaction centers. The combination of antenna complexes with one reaction center constitutes the photosynthetic unit. The efficiency of energy transfer within the photosynthetic unit and its size determine the fraction of the quantum flux that is harvested.

Large concentrations of pigments result in self-shading and thus a reduced efficiency of light absorption per mole of pigment. At the cell size and intracellular pigment concentrations typical of most prokaryotic phototrophs, this decrease in efficiency is not very important (Garcia-Pichel, 1994a), but it might be significant in some extremely low-light adapted anoxygenic phototrophs like the green sulfur bacterial strain isolated from the Black Sea chemocline (Overmann et al., 1991a).

Close proximity of photosynthetic pigments enables an efficient transfer of excitation energy but at the same time also causes a so-called "package effect" (Kirk, 1983) by which self-shading of the pigment molecules exceeds that predicted by the Lambert-Beer law. The package effect is seen clearly in a flattening of absorption peaks, commonly observed when recording absorption spectra of whole cells (see The Family Chlorobiaceae, Identification section in Volume 7). Because the energy requirement for biosynthesis of additional antenna structures is rather constant, the net energy gain for a photosynthetic cell must decrease at higher intracellular pigment concentrations, which restricts the

amount of light-harvesting structures a photosynthetic cell can synthesize. Polypeptides of the photosynthetic machinery (a significant fraction of the total cell protein) amount to 20% in purple nonsulfur bacteria and >50% in phycobiliprotein-containing cyanobacteria. Interestingly, the total protein content of cyanobacterial cells is comparable to other phototrophic bacteria. Possibly, cyanobacteria contain reduced levels of proteins involved in nonphotosynthetic processes to compensate for the high energy and nitrogen expenditure of the antenna proteins.

The biosynthesis of proteins requires a major fraction of the energy expenditure of the bacterial cell (Gottschalk, 1986). In chlorosomes, the mass ratio of protein:bacteriochlorophyll is significantly lower than in other light-harvesting complexes (Table 3). Probably this is one major reason for the larger antenna size and the lower light energy requirements of green sulfur bacteria as compared to their purple and cyanobacterial counterparts (see Competition between Phototrophic Bacteria in this Chapter), and might help explain the competitive advantage gained by *Prochlorocococcus* over their close relatives *Synechococcus* in the open oceans.

Conversion of Light into Chemical Energy

PRINCIPLE The unifying principle of bacterial and archaeal photosynthesis is the light-driven generation of a proton-motive force (PMF). The PMF is subsequently used by ATP synthase to form ATP, or for active transport and motility.

In chlorophyll-based photosynthesis, redox reactions and charge separation precede the establishment of the PMF. In addition, reducing power (NAD(P)H + H^+) is generated as a primary product of the light reaction in *Cyanobacteria*. In the photochemical reaction, only the energy of the lowest excited singlet state (see Light Absorption and Light Energy Transfer in Prokaryotes in this Chapter) of the chlorophylls is used. Consequently, all absorbed light quanta have the same effect irrespective of their original energy (wavelength). When comparing the light energy available in different habitats, or the light adaptation of different phototrophic bacteria, it is therefore more meaningful to express irradiances in units of mol quanta$\cdot m^{-2} \cdot s^{-1}$ rather than $W \cdot m^{-2}$ (see Competition for Light in this Chapter).

The standard free energy for the reduction of CO_2 depends on the redox potential of the photosynthetic electron donor employed (Table 4, Fig. 8). If this energy requirement for electron transfer is compared with the energy available after absorption of photons of different wavelengths, it becomes clear that oxygenic photosynthesis is not feasible in photosystems containing the known types of chlorin pigments, and requires the absorption of two photons per electron (Fig. 8).

The biological conversion of light into chemical energy has been found to be remarkably efficient: the number of charge separation events per absorbed photon is 1.0 (Kok, 1973; Wraight and Clayton, 1973) and the efficiency of the entire photoconversion process of a red photon to chemical energy by oxygenic photosynthetic organisms is 43% (Golbeck, 1994). Whereas the efficiency of energy transfer between antenna bacteriochlorophyll and the reaction center in most cases is close to 100% (Amesz, 1995), the transfer between antenna carotenoids and the reaction center can be significantly lower, 70% in *Heliobacteriaceae* (Amesz, 1995) and even

Table 3. Pigment:protein ratio in different photosynthetic antenna complexes.

	Protein:pigment	
Antenna complex type	Mass ratio	Per pigment molecule (in Da)
Chlorosomes	0.5–2.2	420–1,840
B806-866 complex[a]	3.9–5.8	3,550–5,290
B800-850 LHII	4.4	4,000
B820 LHI	6.7	6,100
Phycobilisomes	~22.4	~12,300

[a]*Chloroflexus aurantiacus*.

Data from Olson, 1998 or calculated from Sidler, 1994, Loach and Parkes-Loach, 1995, Zuber and Cogdell, 1995. Carotenoids have been neglected in these calculations because of their lower numbers as compared to bacteriochlorophylls (B800-850 LHII), their absence in phycobilisomes, and the controversy concerning their functional significance in light-harvesting (chlorosomes). Only antenna complexes which are separate entities from reaction centers were considered. Photosystem I does not contain a distinct antenna structure; the PsaA protein of the reaction center binds 110 chlorophyll *a* molecules.

Table 4. Standard redox potentials of different electron donors of the photosynthetic light reaction.[a]

Electron donor	E_o^{-}[mV]
$^1/_2O_2/H_2O$	+820
$Fe(OH)_3 + HCO_3^-/FeCO_3$	+200
Fumarate/Succinate	+33
HSO_3/S^{0-}	−38
SO_4^{2-}/S^0	−200
SO_4^{2-}/HS^-	−218
$Fe(OH)_3/Fe^{2-}$	−236
S^0/HS^-	−278
HCO_3^-/acetate	−350
$S_2O_3^{2-}/HS^- + HSO_3^-$	−402
$H^+/^1/_2H_2$	−414
Electron acceptor	**E_o^{-}[mV]**
CO_2/<CH_2O>	−434

[a]Taken from Brune, 1989; Widdel et al., 1993; Thauer et al., 1977; Zehnder and Stumm, 1988.

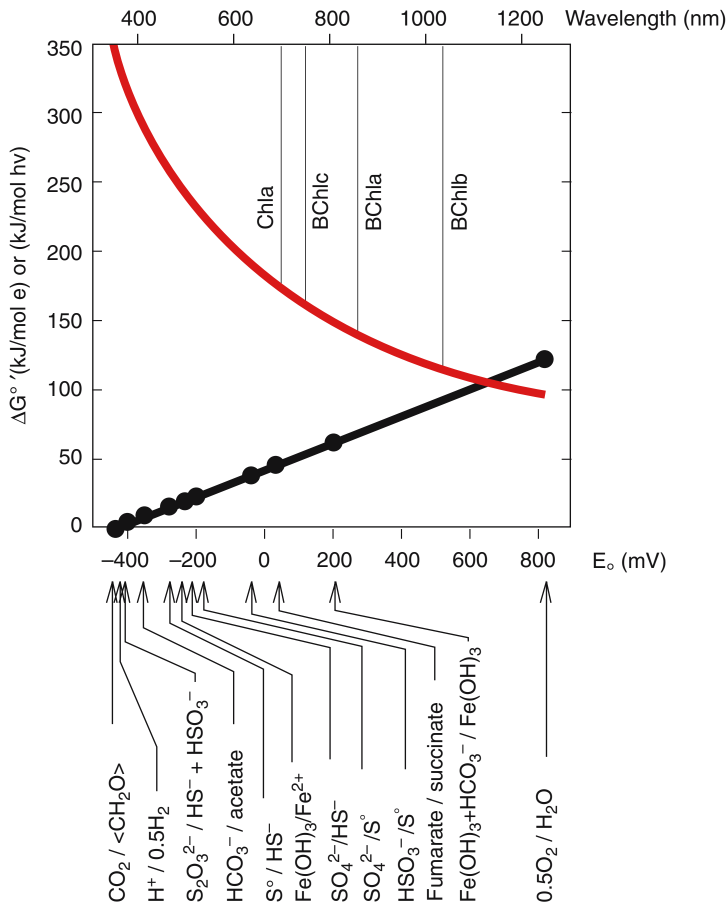


Fig. 8. Free energy of one mol quanta calculated from Planck's constant h (6.63×10^{-34} J·s), the speed of light c (2.99×10^{8} m·s^{-1}), the wavelengths of light λ, and the Avogadro constant $N_A = 6.023 \times 10^{23}$ mol^{-1} according to $\Delta G^{0\prime}_{h\nu}$; = $N_A \cdot h \cdot c \cdot \lambda^{-1}$. Free energy required for the transfer of 1 mole of electrons from an electron donor with standard redox potential $E^{0\prime}_{d}$ (see Table 4) to CO_2 calculated according to $\Delta G^{0\prime}_{el} = -F \cdot (-470 - E^{0\prime}_{d})$ using the Faraday constant F (96.5 kJ·V^{-1}·mol^{-1}). Dotted vertical lines indicate the energy that is available after absorption of light by the long wavelength Q_y absorption bands of different photosynthetic pigments.

20% in a purple nonsulfur bacterium (Angerhofer et al., 1986). When carotenoids serve as the only light-harvesting pigments, 2.5 times higher irradiances are required by *Rhodopseudomonas acidophila* to attain the same growth rates as compared to light-absorption by bacteriochlorophyll (Göbel, 1978). In aerobic phototrophic bacteria, most of the highly diverse carotenoids do not function as light-harvesting molecules but might serve in quenching of toxic oxygen radicals (Noguchi et al., 1992; Yurkov et al., 1994). The same has been proposed recently for the carotenoid isorenieratene/β-isorenieratene in brown-colored green sulfur bacteria (J. B. Arellano, J. Psencik, C. M. Borrego, R. Guyoneaud, C. A. Abella, L. J. Garcia-Gil, T. Gillbro, personal communication).

One prerequisite for the photoconversion process is the presence of a membrane that is impermeable to protons and separates two different cell compartments. Three integral membrane multisubunit protein complexes participate in the generation of ATP in all phototrophic bacteria: the photosynthetic reaction center, a cytochrome complex, and an ATP synthase. All three are highly conserved within the bacterial radiation. Reaction centers have a dimeric core and consist of two closely associated integral membrane polypeptides plus additional proteins (Fig. 3). The special protein environment of the reaction center stabilizes the excited state and prevents back reaction after charge separation by enforcing ultrafast electron transfer to other electron acceptors nearby. The transfer of excitation energy from the antenna complexes to the reaction center initiates a charge separation at a special bacteriochlorophyll dimer (special pair), which is located on the periplasmic (or lumen) side of the photosynthetic mem brane. It is this endergonic process of charge separation that is ultimately driven by light energy; all the following redox reactions are exergonic. An electric potential is established across the membrane (inside negative). In its excited state, the special pair becomes a powerful reductant and ultimately reduces a quinone (in pheophytin-type reaction centers) or ferredoxin (in FeS-type reaction centers) on the cytoplasmic side of the photosynthetic membrane. The quinol or reduced ferredoxin leaves the reaction center complex and in turn donates electrons to a membrane-bound cytochrome complex or NADH dehydrogenase. A series of redox reactions results in the establishment of a proton-motive force across the photosynthetic membrane. Finally, the PMF is converted to ATP by ATPase.

In contrast to the (bacterio)chlorophyll-based systems of bacteria, light energy conversion of Halobacteria does not involve redox reactions and is limited to a vectorial transport of protons by bacteriorhodopsin. Upon excitation by light, the prosthetic retinal undergoes a series of reversible photochemical transformations (an isomerization from the all-*trans* to the 13-*cis* form) and releases a proton into the extracellular space. The PMF thus generated is used for ATP synthesis by ATPase. Due to its low solubility, O_2 in the concentrated salt solution is present in significantly lower amount than in freshwater. Rhodopsin-mediated formation of ATP may become the sole source of energy for growth under anaerobic conditions in the light (Oesterhelt and Krippahl, 1983) and has therefore been viewed as an adaptation to the natural brine habitat of Halobacteria. Because of its distinct mechanism, archaeal "photosynthesis" is not discussed in further detail in the present section. Additional information can be found in chapters titled Introduction to the Classification of Archaea and The Family Halobacteriaceae.

Molecular Architecture of the Reaction Center

All bacteria which perform anoxygenic photosynthesis possess—or (in the case of cyanobacteria which are capable of using sulfide as electron donor) employ—only a single photosystem. The decrease in redox potential that a single photosystem can undergo upon excitation appears to be limited (Blankenship, 1992, compare Fig. 8). A combination of two different photosystems is required for the thermodynamically unfavorable utilization of water as an electron donor for photosynthesis (Fig. 3C). With the relatively simple architecture of their photosystems, all anoxygenic phototrophic bacteria depend on electron donors that exhibit standard redox potentials more negative than water (e.g., H_2S, H_2, acetate; Table 4). This molecular feature is one major reason for the narrow ecological niche of anoxygenic phototrophic bacteria in extant ecosystems (see Habitats of Phototrophic Prokaryotes in this Chapter).

Two different types of reaction centers occur in photosynthetic bacteria. Based on the chemical nature of the early electron acceptors, a pheophytin/quinone-type reaction center and a FeS-type reaction center are distinguished (Blankenship, 1992; Fig. 3A,B). The first type is found in green gliding *Chloroflexus* species, phototrophic members of the α- and β-Proteobacteria, *Chromatiaceae*, *Ectothiorhodospiraceae*, and in PSII of *Cyanobacteria*. The reaction center of *Proteobacteria* consists of three protein subunits (L, M, H) which bind four bacteriochlorophylls, two bacteriopheophytins, two quinones and one high-spin nonheme Fe^{2+} (Lancaster and Michel, 1996; Fig. 3B). Many species (e.g., *Chloroflexus aurantiacus*, *Blastochloris viridis* and *Allochromatium vinosum*) contain an additional tetraheme cytochrome c polypeptide attached to the periplasmic side of the reaction center.

Following the transfer of the electrons by ubiquinol or plastoquinol, the redox reactions at the cytochrome bc_1 (or b_6f) complex drive proton transport across the cytoplasmic membrane. Protons are translocated either into the extracellular space (anoxygenic phototrophic bacteria) or the intrathylacoidal space (cyanobacteria). The ratio of protons translocated to electrons transferred (H^+/e^- ratio) is 2. The reaction center and cytochrome bc_1 in pheophytin-type reaction centers of *Proteobacteria* and *Chloroflexus* are functionally linked by two diffusible electron carriers, ubiquinone in the hydrophobic domain of the membrane and cytochrome c_2 or auracyanin (Meyer and Donohue, 1995) in the periplasmic space. The liberated electron is transferred back to the special pair via quinone, the cytochrome bc_1 complex and soluble periplasmic soluble electron carrie r (often cytochrome c_2). Owing to this cyclic electron transport, the only primary product of photosynthesis is the proton-motive force, and the reduced pyridine nucleotide required for photosynthetic CO_2 fixation is generated by energy-dependent reverse electron flow (Fig. 3).

In oxygenic phototrophic bacteria, plastoquinone is the electron acceptor of PSII and donates electrons to the cytochrome b_6f-complex. The special pair is reduced by the manganese-containing water-splitting system located at the lumenal side of the transmembrane PSII complex (Fig. 3C).

In the pheophytin-type reaction centers of aerobic phototrophic bacteria, photoinduced charge separation occurs only in the presence of O_2 (Okamura et al., 1985). It has been proposed (Yurkov and Beatty, 1998) that oxic conditions are required for photochemical activity because the primary acceptor ubiquinone has a significantly higher midpoint redox potential than in anoxygenic photosynthetic bacteria (65 to 120 mV more positive). The primary acceptor therefore may stay in its oxidized, electron-accepting state only in the presence of O_2.

The second type of reaction center contains iron-sulfur clusters as early electron acceptors and occurs in green sulfur bacteria (Fig. 3A), *Heliobacteriaceae*, and in the photosystem I of *Cyanobacteria*. Functionally, the reaction centers of green sulfur bacteria, *Heliobacteriaceae*, and PSI of cyanobacteria are therefore similar. However, the former two are homodimeric and only

one reaction center gene has been detected, whereas the reaction center of PSI of cyanobacteria and green plants contains two nonidentical, but similar, subunits (PS I-A and PS I-B; Vermaas, 1994). In FeS-type reaction centers, the redox potential of the special pair in its reduced state (P*) is sufficiently low to permit a transfer of electrons to ferredoxin. Until recently, it has therefore been assumed that noncyclic electron flow can directly reduce $NAD(P)^+$ and does not require further energy expenditure not only in cyanobacteria but also in green sulfur bacteria. However, the sequencing of the whole genome of *Chlorobium tepidum* has not provided any indications for the presence of a ferredoxin-NADP+ oxidoreductase (D. A. Bryant, personal communication).

Electron Donors

Anoxygenic phototrophic bacteria of the α- and β-Proteobacteria use a wide variety of reduced organic carbon compounds as electron-donating substrates (see Carbon Metabolism in this Chapter; Table 4; Fig. 8). Most phototrophic sulfur bacteria are capable of using sulfide as photosynthetic electron donor. Other inorganic electron donors utilized include H_2, polysulfides, elemental sulfur, thiosulfate, sulfite, and iron (Widdel et al., 1993). Sulfide is oxidized to zero-valent sulfur, which in *Chromatiaceae* appears to be deposited as polysulfides or polythionates rather than in the form of S_8 rings (Steudel, 1989; Steudel et al., 1990). In addition, thiosulfate is formed as an oxidation product by some species (see The Family Chlorobiaceae in Volume 7; Steudel et al., 1990). The photosynthetic sulfide oxidation rates of purple sulfur bacteria are higher than required for growth and remains constant at all growth rates. As a result, storage of sulfur is at maximum at low growth rates (van Gemerden and Mas, 1995). Zero-valent sulfur is further oxidized to sulfate. In microbial mats, polysulfides and organic sulfur compounds may be significant as photosynthetic electron donor. Polysulfide oxidation has been reported for *Chlorobium limicola* f.sp. *thiosulfatophilum*, *Allochromatium vinosum*, *Thiocapsa roseopersicina*, while dimethylsulfide is utilized and oxidized to dimethylsulfoxide by the two purple sulfur bacteria *Thiocystis* sp. and *Thiocapsa roseopersicina* (van Gemerden and Mas, 1995). In addition to reduced sulfur compounds, hydrogen serves as electron donor in the majority of green sulfur bacteria, and in the metabolically more versatile species of purple sulfur bacteria (such as *Allochromatium vinosum*, *Thiocapsa roseopersicina*). In green sulfur bacteria which lack assimilatory sulfate reduction, a reduced sulfur source is required during growth with molecular hydrogen. Finally, a few species of purple nonsulfur bacteria, of *Chromatiaceae*, and of the green sulfur bacteria have been found to utilize ferrous iron as photosynthetic electron donor (Widdel et al., 1993; Heising et al., 1999).

Sulfide acts as a strong poison of PSII activity in many algae and cyanobacteria. The ability of some *Cyanobacteria* to conduct anoxygenic photosynthesis with sulfide as an electron donor to PSI (Cohen et al., 1975; Padan, 1979; Padan and Cohen, 1982), or to continue oxygenic photosynthesis in the presence of sulfide (Cohen et al., 1986), may be one of the key traits that extend the habitat of sulfide-utilizing cyanobacteria into the temporarily anoxic, sulfide-containing, layers of hot springs (Castenholz and Utkilen, 1984), marine microbial mats (De Wit and van Gemerden, 1987a; De Wit et al., 1988), and the chemoclines of meromictic lakes (Jørgensen et al., 1979; Camacho et al., 1996). Sulfide is an inhibitor of PSII and induces the synthesis of a sulfide-oxidizing enzyme system. In contrast to phototrophic sulfur bacteria, cyanobacteria oxidize sulfide to elemental sulfur or thiosulfate but do not form sulfate (De Wit and van Gemerden, 1987b). However, the use of sulfide by cyanobacteria in anoxygenic photosynthesis must be regarded as a detoxification mechanism, since their low affinity for sulfide (De Wit and van Gemerden, 1987b; Garcia-Pichel and Castenholz, 1990) renders them unable to compete with purple or green sulfur bacteria for sulfide as an electron donor.

In the natural habitat, growth of phototrophic sulfur bacteria is limited mainly by light and sulfide. Sulfide often becomes the growth-limiting factor at the top of the phototrophic sulfur bacterial layers where light intensities are highest, while sulfide has to diffuse through the remainder of the community. The affinity for sulfide during photolithotrophic growth varies between the different groups of anoxygenic phototrophs (including cyanobacteria growing with sulfide) and has been shown to be of selective value during competition experiments. Green sulfur bacteria and *Ectothiorhodospiraceae* exhibit 5 to 7 times higher affinities for sulfide than *Chromatiaceae* (van Gemerden and Mas, 1995). On the contrary, affinities for polysulfides are comparable between green sulfur bacteria and *Chromatiaceae*.

Efficiency of Growth and Maintenance Energy Requirements

For any photochemical reaction, the quantum yield is defined as the number of molecules converted per light quantum absorbed. The quantum efficiency is the ratio of energy stored in a com-

pound, to the radiant energy absorbed for its formation. The quantum requirement is the reciprocal of the quantum yield. For CO_2 fixation of purple sulfur bacteria, a quantum requirement of 8 and 10.5 mol quanta· (mol CO_2)$^{-1}$ is theoretically expected (Brune, 1989), considering that reverse electron transport is necessary. Experimentally, a quantum requirement of 12 ± 1.5 and 11.7 mol quanta·(mol CO_2^{-1} was determined, which corresponds to a quantum yield of 0.083 (Wassink et al., 1942 in Brune, 1989; Göbel, 1978).

In contrast, calculated values for the quantum requirements of green sulfur bacteria lie between 3.5 and 4.5 mol quanta·(mol CO_2)$^{-1}$, if noncyclic electron transport is assumed. However, earlier measurements had yielded much higher values (9–10; Brune, 1989). This discrepancy may be explained by the very recent finding that a gene for ferredoxin-NADP$^+$ oxidoreductase does not seem to be present in the genome of *Chlorobium tepidum* (D. A. Bryant, personal communication), which makes noncyclic electron transport rather unlikely also for green sulfur bacteria.

The quantum yield for CO_2-fixation determined for *Prochlorococcus* isolates incubated in daylight spectrum fluorescent light was between 0.086 and 0.128 mol C·(mol quanta)$^{-1}$ (Moore et al., 1998), thus reaching Emerson's theoretical maximum for O_2 evolution in oxygenic photosynthesis. In cyanobacteria, typically thriving in oxic environments where only oxidized sources of nitrogen and sulfur are available, a large proportion of the reducing power generated in the light reactions must be diverted to assimilatory nitrate or sulfate reduction, or to nitrogen fixation, so that the quantum requirement for CO_2 fixation can be substantially lower than that for oxygen evolution.

In a careful study of *Rhodobacter capsulatus* and *Rba. acidophilus* grown with lactate as electron donor in a light chemostat, a value for the maintenance light energy requirement of m_q = 0.012 mol quanta·(g dry weight·h)$^{-1}$ was determined (Göbel, 1978). The maintenance energy requirements of green sulfur bacteria are significantly lower compared to their purple conterparts (van Gemerden and Mas, 1995). This may be explained by the fact that protein turnover is highly energy demanding and that the protein content of the green sulfur bacterial antenna is much lower than in purple sulfur bacteria (Table 3).

Response to Changes in Light Intensity and Quality

Phototrophic bacteria acclimate to changes in light intensity and quality by diverse mechanisms. Anoxygenic phototrophic bacteria as well as cyanobacteria respond to a step-down in irradiance by increasing the specific pigment content and vice versa (references compiled in Sánchez et al., 1998). These changes can be accomplished either by varying the number of photosynthetic units per cell, the size of the individual photosynthetic unit, or both (see Long-term Adaptations to Changes in Light Intensity in this Chapter). Besides long-term biochemical changes in the composition and the amount of light-harvesting complexes, short-term redistribution of antenna capabilities (see State Transitions in this Chapter) occur in oxygenic phototrophs.

Many species use vertical migration, mediated by tactic responses (see Movement by Flagella in this Chapter) and formation of gas vesicles to regulate their vertical position and exposure to light. Especially in the stably stratified pelagic habitats of phototrophic sulfur bacteria, the difference in buoyant density from the surrounding water would cause a sedimentation of bacterial cells out of the photic zone and towards the lake bottom. The minimum buoyant density, which has been determined for phototrophic cells devoid of gas vesicles, was 1010 kg·m^{-3} (Overmann et al., 1991b). Actively growing cells, which contain storage carbohydrate and—in the case of *Chromatiaceae*—elemental sulfur, can easily attain much higher buoyant densities (up to 1046 kg·m^{-3}; Overmann and Pfennig, 1992). By comparison, freshwater has a considerably lower density (e.g., 996 kg·m^{-3}; Overmann et al., 1999c). As a consequence, sedimentation losses are significant for natural populations of several species of phototrophic sulfur bacteria (Mas et al., 1990). Phototrophic bacteria have developed two ways to adjust their vertical position along gradients of light intensity and spectral composition. For purple sulfur bacteria, motility in response to changes in irradiance is known to be of ecological significance in both planktonic and benthic situations. In benthic and terrestrial cyanobacteria, vertical locomotion by gliding is common. Planktonic cyanobacteria inhabiting stratified waters perform vertical migrations by changing their cellular gas vesicle content and ballast mass (intracellular carbohydrates and protein) and hence their buoyant density. Planktonic anoxygenic phototrophic bacteria do not seem to perform vertical migrations mediated by changes in gas vesicle content but rather use these cell organelles t o maintain their position within the chemocline (Overmann et al., 1991b; Overmann et al., 1994; Parkin and Brock, 1981).

Long-Term Adaptations to Changes in Light Intensity

In those photosynthetic bacteria in which the entire photosynthetic apparatus is confined to

the membrane, light absorption often is increased by formation of intracellular membrane systems (Fig. 2). In *Rhodobacter capsulatus*, the number of intracellular membrane vesicles increases by a factor of 6.3 when the cells are shifted from high to low light intensities. As a result, the area of intracellular membranes under these conditions is 2.7-fold larger than the area of the whole cytoplasmic membrane. Photosynthetic species of the β-Proteobacteria which do not form extensive intracellular membrane systems (*Rhodocyclus purpureus*, *Rhodocyclus tenuis*, *Rubrivivax gelatinosus*) increase the density of photosynthetic units in their cytoplasmic membrane (Drews and Golecki, 1995). Intracellular membranes appear to be absent in *Heliobacteriaceae* and *Heliothrix*, where pigments are confined to the cytoplasmic membrane (Fig. 2). In *Chloroflexus aurantiacus*, the increase in cellular concentrations of bacteriochlorophylls a and c is mediated by an increase in the number and volume of chlorosomes, and the percentage of cell membrane surface covered by chlorosomes (Golecki and Oelze, 1987). In a similar manner, green sulfur bacteria adapt to low light intensities by increasing the size and the cellular number of chlorosomes (see The Family Chlorobiaceae, Physiology section in Volume 7).

During induction of the photosynthesis apparatus in *Proteobacteria*, invaginations of the cytoplasmic membrane, increases in the number and size of the photosynthetic units, and bacteriochlorophyll synthesis occur simultaneously. Under anoxic conditions, the amount of pigment synthesized by anoxygenic phototrophic bacteria is inversely related to the available light intensity and varies by a factor of up to 6.6 (Göbel, 1978). After a shift to low light intensity, the ratio of light-harvesting complex I per reaction center remains constant (at about 30 bacteriochlorophylls per reaction center), whereas the relative amount of the peripheral light-harvesting complex II increases. As a result, the size of the photosynthetic unit changes by a factor of two to five. Conversely, the specific NADH dehydrogenase activity decreases as does the amount of cytochrome and ubiquinone per reaction center. In *Rba. capsulatus* and *Rba. spheroides* these changes take about 2–3 generations and the growth rate is lowered during adaptation due to energy limitation. In the purple sulfur bacterium *Allochromatium vinosum*, low-light adaptation is also accomplished by increasing the size of the photosynthetic unit (Sánchez et al., 1998). Species like *Rhodospirillum rubrum* and *Blastochloris viridis*, which harbor only one type of light-harvesting complex, increase the number of photosynthetic units (Drews and Golecki, 1995).

Similar to anoxygenic phototrophic bacteria, changes in both the number and the size of the photosynthetic unit have also been decribed for cyanobacteria. In marine *Synechococcus* strains, the cellular content of the light-harvesting phycoerythrin can be varied by a factor of 20 and decreases with increasing light intensity. In marine benthic *Microcoleus chthonoplastes*, an increase in the content of total phycobilines and a change in the ratio of PEC to PC occurs with decreasing light intensity. The latter increase the ratio of phycocyanin to chlorophyll *a* during low-light adaptation (Foy and Gibson, 1982; Post et al., 1985). Acclimation to very low light intensities usually involves an increase in the size of the photosynthetic unit, such as in metalimnetic *Oscillatoria (Leptolyngbya) redekei* and *Oscillatoria agha rdii*. Changes in both the number and the size of the photosynthetic units seem to occur in *Microcystis* (Zevenboom and Mur, 1984).

Adaptations to Low Light Intensities

The capability to adapt to low light intensities represents a competitive advantage for phototrophic organisms. An estimate of the minimum irradiance I_{min} required for survival of phototrophic cells in the environment can be calculated from a few physiological parameters, namely the pigment content of the cells, P (in mg bacteriochlorophyll·g C^{-1}); the maintenance energy requirement, m_q (in mol quanta·g $C^{-1}·s^{-1}$); the (bacterio)chlorophyll-specific attenuation coefficient, k (in m^2·mg BChl a^{-1}); the cellular dry weight content, D (in g C·m^{-3}); and the mean optical pathlength of one cell d:

$$I_{min} = m_q \cdot D \cdot d / [1 - \exp(-k \cdot D \cdot P \cdot d)]$$

Employing the appropiate values for m_q (see Efficiency of growth and maintenance energy requirements), k and P (see Light Energy and the Spectral Distribution of Radiation in this Chapter), D ($1.21·10^5$ g C·m^{-3}; Watson et al., 1977) and d (0.5 m for the smaller anoxygenic phototrophs), this yields a minimum irradiance (I_{min}) of 2 μmol quanta·$m^{-2}·s^{-1}$. In many natural habitats of anoxygenic phototrophic bacteria, irradiances of this order of magnitude or lower have been measured. *Prochlorococcus* has been found at deep water layers down to 300 m. However, these bacteria do not grow at light intensities below 3.5 mol quanta·$m^{-2}·s^{-1}$ (Moore et al., 1998) and thus appear to be less low-light adapted than the green sulfur bacterial strain MN1 isolated from the Black Sea which grows a t light intensities as low as 0.25 μmol quanta·$m^{-2}·s^{-1}$ (Overmann et al., 1991a). Lower irradiances could be used by phototrophic prokaryotes after a decrease of m_q or an increase of P or both. Both adaptations are present in strain MN1 (Overmann et al., 1991a).

Adaptations to High Light Intensities

Sessile cyanobacteria living on the surface of benthic microbial mats are typically adapted to very high light conditions and contain large amounts of sunscreen pigments. For oxygenic phototrophs, special adaptations to oxygen-dependent photoinhibition of photosynthesis are of particular relevance. The protein D1 of PSII, coded by the *psbA* gene, has been identified as the central target of photoinhibition at high light intensities. In *Synechococcus* PCC 7942, *psbA* contains actually a multigene family coding for three different forms of the protein D1, which are differentially expressed according to the light conditions. Analysis of mutants showed that the isoforms expressed under high light conditions allow for optimal performance of PSII under photoinhibitory conditions (Golden, 1994). In addition, carotenoids probably play a central role in avoiding oxygen-mediated pho tosensitized bleaching of photosynthetic pigments and photooxidation of fatty acids under high light conditions.They function as antioxidant quenchers of excited molecules (such as triplet state chlorins and singlet oxygen) in many organisms and perhaps also as inhibitors of free-radical reactions (Britton, 1995). The photoprotective xanthophyll cycle typical of green algae and higher plants is not present in cyanobacteria, but judging from its increased specific content at high light intensity, zeaxanthin seems to play an important photoprotective role in some strains (Kana et al., 1988; Masamoto and Furukawa, 1997; Millie et al., 1990). Glycosylated myxoxanthophylls seem to attain the same role in others (Nonnengießer et al., 1996; Garcia-Pichel et al., 1998; Ehling-Schulz et al., 1997). Because there is a considerable photooxidation of carotenoids themselves at high light intensities, the maintenance of high carotenoid contents requires an increased expression of their biosynthetic genes.

Chromatic Adaptation

Several species of cyanobacteria are capable of changing the amount of peripheral phycoerythrin in response to changes in the spectral composition of light. During growth in white or green light, red-pigmented PE hexamers are added to the peripheral rods whereas additional blue-pigmented PC is added under red light (Sidler, 1994). This complementary chromatic adaptation is found only in strains capable of forming PE, but not in those forming PEC. The complementary change in antenna pigment composition optimizes the light-harvesting capabilities of populations of *Oscillatoria* spp., which thrive in deeper layers of stratified lakes where light is predominantly in the blue-green to green wavelength range (Utkilen et al., 1985; Fig. 6).

Genetic Regulation in Response to Light

The synthesis of the photosystem is especially energy consuming because of the high amount of light-harvesting and reaction center protein present in phototrophically grown cells of phototrophic *Proteobacteria* (20% in purple nonsulfur bacteria). The maintenance energy requirements seem to be increased in low-light adapted cells (Sánchez et al., 1998). An effective regulation of photosynthesis gene expression therefore would prevent futile synthesis of cellular proteins. The synthesis of the photosystem in anoxygenic phototrophic bacteria is under the control of a complex regulatory network (Bauer and Bird, 1996).

The expression of light-harvesting complex I and reaction center genes is controlled 1) by the linkage of genes in superoperons, 2) at the level of transcription initiation, and 3) posttranscriptionally by the decay rate of mRNA (Bauer, 1995).

In *Rhodobacter capsulatus*, the genes coding the structural, biosynthetic and regulatory proteins for light-harvesting I and reaction center complexes are found assembled in a 46 kb-long photosynthetic gene cluster (Alberti et al., 1995). The arrangement of the genes within the cluster seems to be conserved among different phototrophic species of the α-Proteobacteria, like *Rhodobacter sphaeroides*, *Rhodocista centenaria* and *Rhodospirillum rubrum* (Bauer et al., 1993). Only the *pucBA* operon which codes for structural α- and β-polypeptides of light-harvesting complex II is found in a distant location on the bacterial chromosome (about 18 kb of the *puhA* in *Rhodobacter capsulatus*; Suwanto and Kaplan, 1989).

In anoxygenic phototrophic bacteria, transcription of the photosynthesis genes occurs only under anoxic conditions. Different photosynthesis genes exhibit varying levels of expression and degrees of regulation (Bauer and Bird, 1996). The *pufA,B,L,M* genes (coding for the α- and β-polypeptide of the light-harvesting complex I and the reaction center L and M structural polypeptides) as well as *puhA* (coding for the structural polypeptide subunit H) are tightly coregulated, transcribed at a high rate under anoxic conditions and strongly regulated (15- to 30-fold). An inverted repeat sequence located between *pufA* and *pufL* affects the longevity of the respective mRNA primary transcript. A reduction of light leads to an activation of *puf* and *puh* gene expression by the *hvrA* gene product, which probably directly interacts with the two promoter regions. Light of 450 nm exhibits

the most severe repressing effect, indicating that a flavin-binding protein (possibly HvrA itself) is the photoreceptor. Notably in aerobic phototrophic bacteria, a blue light sensitive system seems to regulate biosynthesis of bacteriochlorophyll *a* (Shimada, 1995).

The intracellular bacteriochlorophyll concentrations appear to affect *puf* and *puc* gene expression not only at the transcriptional but also the posttranscriptional level in *Rhodobacter capsulatus* (Rödig et al., 1999). The polycistronic organization allows the coordinate expression of the structural polypeptides of light-harvesting complex I and the two integral membrane-proteins of the reaction center. Since, however, many light-harvesting I complexes are required per reaction center in *Proteobacteria*, additional regulatory mechanisms must exist. Differential degradation of various portions of the polycistronic mRNA are one means to regulate the stoichiometry of different components of the photosynthetic apparatus. The synthesis of different amounts of gene products is achieved by posttranscriptional regulation (Rödig J. et al., 1999). Because of a h ighly stable secondary terminator structure at its 3´-end and the absence of specific recognition sites for endonucleolytic cleavage, the mRNA coding the two light-harvesting polypeptides has much higher stability than that of the entire *puf* gene transcript. The degradation of the downstream *pufLM* section of the mRNA is mediated by an endonuclease. A similar regulation mechanism may exist for the polycistronic mRNA of bacteriochlorophyll synthesis genes (*bchFNBHLM-F1696*) and the *puhA*, and operate in regulation of light-harvesting complex II expression.

A shift to low light intensities results in an increase especially of light-harvesting complex II. The corresponding *pucBA* operon is highly expressed but only moderately regulated (4-fold). In the purple nonsulfur bacterium *Rhodobacter capsulatus*, four-fold less *puc* mRNA but at the same time four times as many light-harvesting II complexes were detected after a shift from high to low-light conditions (Zucconi and Beatty, 1988). Therefore regulation by light most likely involves posttranscriptional regulation. A posttranscriptional regulation appears to occur (Bauer, 1995).

Bacteriochlorophyll and carotenoid biosynthesis genes are only weakly expressed and moderately (2 to 4-fold) regulated. Light intensity may control the rate of bacteriochlorophyll degradation (by oxidative degradation of bacteriochlorophyll; Biel, 1986) rather than the rate of synthesis (Biel, 1995). This is another distinct difference from the regulation by oxygen, where inhibition of δ-aminolevulinate synthase by molecular oxygen appears to occur (see Chemotrophic Growth with O_2 in this Chapter). Bacteriochlorophyll may be stabilized by insertion in pigment-protein complexes, however. The promotor of the bacteriochlorophyll synthesis gene *bchC* is of the sigma-70 type and leads to one large superoperon (Yurkov and Beatty, 1998). In contrast, an alternative sigma factor appears to recognize the strongly regulated structural *puf* and *puh* genes (Bauer, 1995). These differences explain the independent and different levels of regulation observed for the two classes of genes.

Recently the promoter for the carotenoid biosynthesis genes *crtB* and *crtP* were identified in *Synechocystis* PCC 6803, and shown to be light regulated (Fernández-González et al., 1998).

State Transitions

In cyanobacteria, state transitions involve redirecting the pathways of excitation energy transfer from light-harvesting complexes to both photosystems, and can be recognized by fluorescence analysis. Cyanobacteria can reach two energetically different states, in which one of the photosystems is preferentially excited. This is achieved with fast changes in the coupling between the light-harvesting complexes and the reaction center (van Thor et al., 1999). Evidence is accumulating that at least in the chlorophyll *b*-containing phototrophic bacteria ("Prochlorophytes"), the short-term regulation occurs by a mechanism similar to that in green chloroplasts (Matthijs et al., 1994). In the latter, polypeptides of the PSII antenna (LHCII) are rapidly phosphorylated during overexcitation of this photosystem, and as a consequence detach from PSII and migrate to the stromal thylakoids. This mechanisms ensures a bala nced energy distribution between PSII and PSI. The net result of state transitions is the balanced function of both photosystems and an optimization of the quantum yield for photosynthesis during short-term changes, such as those that planktonic cells might experience during vertical transport by water currents.

Movement by Flagella

Phototrophic *Proteobacteria* swim by means of flagella, whereas one species of the green sulfur bacteria (*Chloroherpeton thalassium*), members of *Chloroflexus* subgroup and cyanobacteria move by gliding. Of the α-Proteobacteria, most phototrophic species are motile. Peritrichous or lateral flagella are only found in *Rhodomicrobium vannielii* and the swarming phase of *Rhodocista centenaria*. About two thirds of the known *Chromatiaceae* species are motile. Larger forms (*Chromatium okenii*, *Chr. weissei*, *Chr. warmingii*, *Chr. buderi*, *Thiospirillum jenense*)

are motile by means of bipolar multitrichous tufts of flagella. *Thiospirillum jenense* is bipolarly flagellated. Forms with smaller cells are monotrichously flagel lated (small *Chromatium* species, *Lamprocystis*, *Thiocystis*, *Thiorhodococcus*, *Thiorhodovibrio*). All *Ectothiorhodospiraceae* are flagellated. A new mode of motility has been described for a unicellular cyanobacterium which moves in a similar fashion to flagellated bacteria but apparently lacks a flagellum (Waterbury et al., 1985).

True phototaxis is the ability to move towards or away from the direction of light. Cyanobacteria are the only prokaryotes displaying true phototaxis (Garcia-Pichel and Castenholz, 1999). Phototaxis may not be of competitive value for microorganisms adapted to live at low light intensities in the subsurface of sediments, soils and mats because the light fields may be close to diffuse deep below the surface. However, directed movements can still be of much use in microorganisms dwelling at or close to the sediment surface, where the light fields contain a significant downward directionality. Photophobic responses are changes in the direction of movement in reaction to abrupt changes in light intensity (Castenholz, 1982; Häder, 1987). In the step-up photophobic response, organisms will reverse direction when sensing an increase in light intensit y, which results in a net accumulation of organisms at lower light intensities. In a step-down photophobic (or scotophobic) response, the organisms will tend to accumulate in the region of higher light intensity. Photophobic responses are the basis of photomovement in all flagellated bacteria (Armitage, 1997), and in most gliding cyanobacteria (Castenholz, 1982).

In swimming cells of phototrophic *Proteobacteria*, a decrease in light intensity triggers a reversal of flagellar rotation (*Rhodospirillum rubrum*, *Chromatium* spp.) or an increase in stopping frequency (*Rhodobacter sphaeroides*). Owing to a memory effect, cells of the latter species retain a higher stopping frequency for up to 2 min, which prevents the cells from being trapped in the dark but instead permits reorientation of the cells and a return to higher light intensities (Armitage et al., 1995). As a result of this scotophobic response, the cells accumulate in the light and at wavelengths corresponding to the absorption maxima of photosynthetic pigments. A change in light intensity of as little as 2% can be sensed (Armitage et al., 1995). Active electron transport is required for the scotophobic response.

The formation of flagella in *Chromatium* species is induced by low sulfide concentrations and low light intensities. These two environmental variables are mutually dependent: the lower the light intensity, the higher the sulfide concentration at which a given strain can persist in its motile stage (Pfennig and Trüper, 1989). In the natural environment of purple sulfur bacteria, gradients of light and sulfide are opposed to each other. The control of motility by the two interdependent environmental variables (instead of only one) enables *Chromatium* cells to return either from low sulfide/high light environment above the chemocline or from the high sulfide/low light environment below the chemocline back to their habitat.

In its pelagic habitat, *Chromatium okenii* may display diurnal migrations with a vertical amplitude of about 2 m (Sorokin, 1970). In other lakes, vertical migrations of *Chromatium minus* extended over a distance of 30–35 cm (Lindholm et al., 1985; Pedrós-Alió and Sala, 1990). Vertical migration of nonthermophilic *Chromatium*, and of *Chromatium tepidum* also has been observed in ponds and in intertidal or hot spring microbial mats (Castenholz and Pierson, 1995; Jørgensen, 1982; Pfennig, 1978). In the latter environments, *Chromatium* cells migrate upwards to the surface of the mat and enter the overlaying water as a result of positive aerotaxis during the night. The cells contain high amounts of intracellular sulfur globules, which are formed during incomplete sulfide oxidation by anoxygenic photosynthesis during daytime. It is assumed that migration into microoxic layers enables the cells to grow chemoautotrophically by oxidation of sulfide or intracellular sulfur with molecular oxygen (Jørgensen, 1982; Castenholz and Pierson, 1995).

If phototrophic sulfur bacteria would solely follow the light gradient, their scotophobic response would ultimately lead them into oxic water layers. Both the scotophobic behavior and aerotaxis respond to the rate of intracellular electron flow (presumably sensed as changes in the redox state of an intermediate). Because the two tactic reponses interact through a common signal, a combination of light and molecular oxygen elicits a differential response. *Rhodobacter sphaeroides* exhibits pronounced aerotaxis when precultivated aerobically, but negative aerotaxis when grown anaerobically in the light. Conversely, cells only swim towards higher light intensities in anoxic medium. A pulse of oxygen in the light causes a transient fall in the membrane potential which probably represents the primary tactic signal. As a result, the bacteria move towards environments where electron transport rate is increased (Armitage et al., 1995).

Rhodocista centenaria exhibits a characteristic swarming behavior. In liquid media, cells move with a single polar flagellum. Upon contact with solid agar media, formation of a large number of lateral flagella is induced. Lateral flagella allow whole colonies to swarm towards or away from the light (Ragatz et al., 1994). The supposedly

true phototaxis of these swarming colonies (Ragatz et al., 1995) has later been proven to actually be aerotaxis following microgradients within the colonies (Sackett et al., 1997). The light sensing system in this species appears to be more complex, since infrared light leads to positive, and visible light to negative phototaxis. In microbial mats, infrared light penetrates to much greater depths than light of the visible wavelength range (see Competition for Light in this Chapter). It has been suggested that the ratio of visible to in frared light may be used to maintain an optimum position in such environments (Armitage et al., 1995; Ragatz et al., 1995).

Cyanobacteria are the only prokaryotes displaying true phototaxis (Garcia-Pichel and Castenholz, 1999). Surface dwelling cyanobacteria such as *Lyngbya* spp. from hot springs mats and intertidal sediments and the motile phases (hormogonia) of terrestrial *Nostoc* spp. from desert soils exhibit this type of movement. The bundle-forming *Microcoleus chthonoplastes* also is able to display a "populational phototaxis" in that bundles of trichomes of this cyanobacterium are able to steer in the direction of the incoming light, whereas single trichomes are apparently not able to do so (Prufert-Bebout and Garcia-Pichel, 1994). True phototaxis is a mechanism for the orientation of cells at or close to the sediment surface, where the light field contains a significant downward directionality. In contrast, phototaxis does not provide a selective advantage for bacteria thriving in the subsurface of sediments, soils and mats because of the diffuse light field. In natural microbial mats photophobic responses to changes in light intensity are probably involved in the migrations of gliding bacteria (Nelson and Castenholz, 1982; Pentecost, 1984). In microbial mats, some strains of cyanobacteria are able to migrate vertically following their optimal light intensity over the diel cycle (Garcia-Pichel et al., 1996). The upward migrations of cyanobacteria in mats is preferentially prevented by short wavelengths, especially by UV radiation (Garcia-Pichel and Castenholz, 1994b; Bebout and Garcia-Pichel, 1995; Kruschel and Castenholz, 1988) and not by red nor green light.

Phototrophic consortia are structural associations between a colorless central bacterium and several surrounding cells of pigmented epibionts (see Interactions between Phototrophic Bacteria and Chemotrophic Bacteria in this Chapter; The Family Chlorobiaceae in Volume 7; Fig. 5). Intact consortia of the type "Chlorochromatium aggregatum" exhibit a scotophobic response and accumulate in a spot of white light. In phototrophic consortia, only the central colorless bacterium carries a flagellum (J. Glaeser and J. Overmann, unpublished observation). The action spectrum of scotophobic accumulation corresponds to the absorption spectrum of the green sulfur bacterial epibionts, however. It has to be concluded that a rapid signal transfer exists between the light-sensing but immotile epibionts and the colorless motile rod (Fröstl and Overmann, 1998).

Gas Vesicles

Buoyancy-conferring gas vesicles are common in green sulfur bacteria, *Chromatiaceae*, and cyanobacteria. Gas vesicles are cylindrical structures with conical ends; their length and width are variable and species-specific. The sheath of gas vesicles are composed of proteins (Walsby, 1994). The gas mixture within the gas vesicles is the same as in the surrounding medium and is at the same partial pressures. Gas vesicles occur in a third of the species of *Chromatiaceae* (belonging to the genera *Amoebobacter*, *Lamprobacter*, *Lamprocystis*, *Thiodictyon*, *Thiopedia*, *Thiolamprovum*) and some green sulfur bacteria (genera *Ancalochloris*, *Pelodictyon*, *Chloroherpeton*). Of the *Ectothiorhodospiraceae*, only *Ectothiorhodospira vacuolata* forms gas vesicles during stationary phase. This reflects the distribution of both families of purple sulfur bacteria in nature, where *Chromatiaceae* typically colonize low-light stratified aquatic environments, whereas *Ectothiorhodospiraceae* typically inhabit more shallow saline ponds and sediments. Gas vesicles also are present in *Prochlorothrix hollandica*. In planktonic habitats, cells of cyanobacteria and phototrophic sulfur bacteria often contain gas vesicles, which indicates a selective advantage of this cellular property.

Gas vesicle formation in the green sulfur bacterium *Pelodictyon phaeoclathratiforme* is induced exclusively at light intensities <5 mol quanta·m^{-2}·s^{-1} (Overmann et al., 1991b). This appears to be the reason for the rare observation of gas vesicles in pure cultures of green sulfur bacteria which routinely are incubated at much higher light intensities. A transfer of *Amoebobacter purpureus* strain ML1 to the dark results in an increase of the gas vesicle volume by a factor of nine (Overmann and Pfennig, 1992). Ambient temperature controls gas vesicle formation in *Thiocapsa pendens* (Eichler and Pfennig, 1986).

The buoyancy of many species of *Cyanobacteria* is regulated by the formation of gas vesicles. Highly buoyant cells may float towards the surface of stagnant water bodies. When the turgor pressure within the surrounding cytoplasm rises, such as by accumulation of low molecular weight photosynthates during periods of intense photosynthesis, the critical pressure may be exceeded and the gas vesicles collapse. New vesicles are formed by de novo synthesis rather than by re-inflation of collapsed vesicles. Short-term regula-

tion of cell buoyant density occurs in cyanobacterial species thriving in stratified lakes, like *Aphanizomenon flos-aquae*, *Anabaena flos-aquae*, and green-colored *Oscillatoria* spp. (Konopka et al., 1978; Oliver and Walsby, 1984; Utkilen et al., 1985). In these species, the proteinaceous gas v esicle sheaths are weak enough to permit a collapse at high intracellular turgor pressures as they are reached during periods of intense photosynthesis. By this mechanism, cells lose buoyancy within 30 minutes and thus can sink out of surface layers of stratified lakes. After de novo synthesis of gas vesicles in lower water layers, utilization of photosynthates, and a decrease of turgor pressure, cells rise back to the surface during the night. Rapid, turgor-mediated reduction of buoyancy together with gas vesicle formation thus represents an adaptation to the pronounced diurnal variations in light intensity and the limitation of growth by inorganic nutrients as they occur during summer stratification in the surface layer of eutrophic lakes. In some instances (e.g., *Microcystis aeruginosa*) diurnal migrations are mediated by an increase of carbohydrate ballast alone and gas vesicles do not collapse even at maximum turgor pressure (Kromkamp and Mur, 1984; Thomas and Walsby, 1985).

In contrast, gas vesicles of red-colored *Oscillatoria aghardii* and of phototrophic sulfur bacteria are mechanically stronger and do not collapse even at maximum cell turgor pressure. A decrease in the cellular gas vesicle content is therefore the result of their dilution during growth and division of the cells, and thus proceeds rather slowly (Overmann et al., 1991b; Overmann and Pfennig, 1992). Bacteria of this category mostly colonize the low-light environments shortly above or within the chemocline of stratified lakes where photosynthetic rates typically are strongly limited by light and hydrostatic pressure is high. Gas vesicles in green sulfur bacteria are rigid enough to persist at hydrostatic pressures down to depths of 38 m (Overmann et al., 1991b). The cyanobacterium *Trichodesmium* contains extremely stable gas vesicles (mean crit ical collapse pressures up to 3.7 MPa, corresponding to a depth of 370 m; Walsby, 1978). The differences in strength of gas vesicles formed by different species is related to their shape (especially the diameter) and the primary structure of the GvpC protein of their sheath (Walsby, 1994).

In addition to the formation of gas vesicles, a new type of buoyant density regulation was detected in *Pelodictyon phaeoclathratiforme*. Cells of this species form large extracellular slime layers during the stationary phase which leads to an increase of the cellular volume by a factor of three (Overmann et al., 1991b).

Advantages of the Vertical Movement by Flagella and by Gas Vesicles

Theoretically, motility based on flagellar movement and vertical migration by means of gas vesicle formation have different advantages under natural conditions. Movement by flagella requires a permanent, (albeit sometimes low) fraction of metabolic energy (proton-motive force), whereas gas vesicle synthesis represents an initial one-time investment of a higher amount of metabolic energy. Once formed, gas vesicles keep bacterial cells in their habitat without any further demand for energy. The purple sulfur bacterium *Lamprobacter modestohalophilus* is capable of both flagella and gas vesicle formation. Motile cells are usually devoid of gas vacuoles and initially dominate during growth in fresh media. Later, cells become immotile and form gas vesicles and slime capsules (Gorlenko et al., 1979). In a very similar manner, cells of *Ectothiorhodospira vacuolata* are flagellated at low sulfide concentrations and light inte nsities, and become immotile and form gas vesicles in stationary phase (Imhoff et al., 1981). This supports the view that flagellar movement of purple sulfur and purple nonsulfur bacteria is favored under conditions of continuous energy supply, while gas vesicle formation represents an adaptation to conditions of starvation. Within one lake ecosystem, vertical migration of a flagellated species (*Chromatium minus*) was observed while the gas-vacuolated *Amoebobacter* did not change its vertical position (Pedrós-Alió and Sala, 1990).

A minimum quantum requirement of flagellar motility can be estimated from data in the literature. A vertical migration over a distance of 2 m (the maximum amplitude of vertical migration observed in nature) during 6 hours corresponds to a swimming speed of 93 $\mu m \cdot s^{-1}$. At a similar speed of 100 $\mu m \cdot s^{-1}$ the frequency of flagellar rotation is >100 s^{-1} in *Rhodobacter sphaeroides* and requires between 200 and 1000 H^+ per rotation (Armitage et al., 1995). This yields a proton translocation rate of $\sim 6 \times 10^4$ $H^+ \cdot s^{-1}$ at a swimming velocity of 100 $m \cdot s^{-1}$. Based on an absorbing cross sectional area of the cell of 1 m^2, an absorption of 36% of the incident light (see Efficiency of Light Harvesting in this Chapter), a ratio of protons translocated to electrons transferred (H^+/e^- ratio) of 2 (see Conversion of Light into Chemical Energy in this Chapter), and assuming that each photon absorbed leads to transport of an electron, the proton translocation rate of 6×10^4 $H^+ \cdot s^{-1}$ would be reached at an underwater irradiance of 0.2 mol quanta$\cdot m^{-2} \cdot s^{-1}$. However, all available quanta would be required just for motility at this irradiance and no vertical migration would be possible during the night. Therefore motility by flagella will be of compet-

itive advantage only at significantly higher irradiances. In many lakes, underwater irradiances in layers of phototrophic sulfur bacteria are ≤1 mol quanta$\cdot m^{-2} \cdot s^{-1}$ (Overmann and Tilzer, 1989a; Overmann et al., 1999a). Under these conditions, purple sulfur bacteria harboring gas vesicles dominate over flagellated forms in the chemocline community (Fig. 4). At least in some lakes, gas vesicles appear to be of selective advantage also at higher underwater irradiances (Overmann et al., 1991b; Overmann and Pfennig, 1992).

Interestingly, the extremely low-light adapted *Chlorobium phaeobacteroides* strain MN1 isolated from the chemocline of the Black Sea was not capable of gas vesicle formation. The green sulfur bacterial layer is located at an 80-m depth and with respect to light intensity represents the lower limit for growth of a phototrophic organism (see The Family Chlorobiaceae in Volume 7). The isolated strain exhibits an extremely low maintenance energy requirement. It therefore appears that gas vesicle formation is too energy demanding at the very low light intensities available at an 80-m depth in the Black Sea.

Carbon Metabolism of Phototrophic Prokaryotes

In the natural environment, the principal carbon source of phototrophic bacteria in many instances is CO_2 (Madigan et al., 1989; Sinninghe Damsté et al., 1993; Takahashi et al., 1990). In *Cyanobacteria*, *Chromatiaceae*, *Ectothiorhodospiraceae* and purple nonsulfur bacteria, CO_2 is assimilated by the reductive pentose phosphate or Calvin cycle. Employing this cycle, the formation of one molecule of glyceraldehyde-3-phosphate requires 6 $NAD(P)H+H^+$ and 9 ATP. By comparison, the reductive tricarboxylic acid cycle used for CO_2-assimilation by green sulfur bacteria requires 4 $NADH+H^+$, 2 reduced ferredoxins, and only 5 ATP. As two of the reactions of the reductive tricarboxylic acid cycle (the α-oxoglutarate synthase and pyruvate synthase rea ctions) require reduced ferredoxin as electron donor, this pathway of CO_2 fixation can only proceed under strongly reducing conditions. Furthermore, reduced ferredoxin is a primary product of the light reaction only in FeS-type reaction centers. Ultimately, the lower demand for ATP is possible because of the adapatation of green sulfur bacteria to the strongly reducing conditions of their natural environment. CO_2-fixation by the hydroxypropionate cycle in *Chloroflexus aurantiacus* requires 8 ATP per glyceraldehyde-3-phosphate and therefore is energetically less favorable than in green sulfur bacteria.

Organic carbon as it is present in canonical microbial biomass ($<C_4H_8O_2N>$; Harder and van Dijken, 1976) is considerably more reduced than CO_2. Given the high energy demand of autotrophic growth, the capability for assimilation of organic carbon compounds is of selective advantage especially if natural populations are limited by light or by low concentrations of electron-donating substrates, as is typically the case for phototrophic sulfur bacteria. At limiting concentrations of sulfide or thiosulfate, the cell yield of green sulfur bacteria is increased three times if acetate is available as an additional carbon source (Overmann and Pfennig, 1989b). Acetate represents one of the most important intermediates of anaerobic degradation of organic matter (Wu et al., 1997). That almost all anoxygenic phototrophic bacteria (with the exception of *Rhodopila globiformis*; Imhoff and Trüper, 1989) are capable of acetate assimilation is therefore not surprising. In most phototrophic *Proteobacteria*, acetate is assimilated by acetyl-CoA synthetase and the enzymes of the glyoxylate cycle. In green sulfur bacteria, the ferredoxin-dependent pyruvate synthetase, PEP synthetase, and reactions of the reductive tricarboxylic acid cycle serve this purpose. The capacity for organotrophic growth seems to correlate with the presence of α-oxoglutarate dehydrogenase. The latter is a key enzyme for the complete oxidation of the carbon substrates in the tricarboxylic acid cycle (Kondratieva, 1979), whereas a complete cycle is not needed for the photoassimilation during the presence of inorganic electron donors. The range of carbon substrates utilized and the capacity for photoorganotrophy or chemoorganotrophy varies considerably among the different groups of phototrophic pr okaryotes (Pfennig and Trüper, 1989).

Organic carbon compounds not only are assimilated but also can serve as photosynthetic electron donors in purple nonsulfur bacteria, some *Chromatiaceae* and *Ectothiorhodospiraceae*, all *Heliobacteriaceae*, and members of the *Chloroflexus* subdivision.

Green sulfur bacteria are the least versatile of all phototrophic prokaryotes. All known species are obligately photolithotrophic and assimilate only very few simple organic carbon compounds (acetate, propionate, pyruvate). Few strains have been shown to assimilate fructose or glutamate. Whereas green sulfur bacteria have a higher growth affinity for sulfide than purple sulfur bacteria, acetate seems to be used by purple sulfur bacteria at an affinity 30 times higher than in green sulfur bacteria (Veldhuis and van Gemerden, 1986). In addition, uptake of acetate in *Chlorobium phaeobacteroides* is inhibited by light (Hofman et al., 1985).

Based on their metabolic flexibility, two groups can be distinguished among the *Chromatiaceae*. Several species (*Chromatium okenii*, *Chr. weissii*, *Chr. warmingii*, *Chr. buderi*, *Chr. tepidum*, *Thiospirillum jenense*, *Lamprocystis roseopersicina*, *Thiodictyon elegans*, *Thiodictyon bacillosum*, *Thiocapsa pfennigii*, *Thiopedia rosea*) are obligately phototrophic, strictly anaerobic and photoassimilate acetate and pyruvate only in the presence of CO_2 and sulfide. Assimilatory sulfate reduction is absent in these species (Pfennig and Trüper, 1989). However, particularly those species with limited metabolic flexibility form dense blooms under natural conditions (see Coexistence of Phototrophic Sulfur Bacteria in this Chapter). The second physiological group within the *Chromatiaceae* comprises the small *Chromatium* species (*Chr. gracile*, *Chr. minus*, *Chr. minutissimum*), *Allochromatium vinosum*, *Lamprobacter modestohalophilus*, as well as *Thiocystis* spp., *Thiocapsa*. Most of these species use thiosulfate as electron donor and a wide range of organic carbon compounds including glucose, fructose, glycerol, fumarate, malate, succinate, formate, propionate, and butyrate for photoassimilation, and often are capable of assimilatory sulfate reduction. In some species (especially *Allochromatium vinosum*), these organic carbon substrates also serve as electron-donor for phototrophic or chemotrophic growth.

Most *Ectothiorhodospiraceae* species are capable of photoorganotrophic growth, with *Ectothiorhodospira halophila* and *Ectothiorhodospira halochloris* being the exceptions. The spectrum of electron-donating carbon substrates for photoorganotrophic growth resembles that found in the versatile *Chromatium* species (Pfennig and Trüper, 1989). Assimilation of acetate and propionate proceeds by carboxylation and therefore depends on the presence of CO_2.

Chloroflexus aurantiacus grows preferably by photoorganoheterotrophy (Pierson and Castenholz, 1995). The carbon substrates utilized comprise acetate, pyruvate, lactate, butyrate, C_4-dicarboxylic acids, some alcohols, sugars and amino acids (glutamate, aspartate). This versatility has been seen as the major cause for the profuse growth of *Chloroflexus* in microbial mats where accompanying microorganisms, especially cyanobacteria, may provide the required carbon substrates (Sirevåg, 1995). However, high rates of formation of low-molecular-weight organic carbon substrates by the anaerobic food chain have also been observed in other stratified systems, where the dominating anoxygenic phototrophs could utilize only a narrow range of carbon substrates (Overmann, 1997; Overmann et al., 1996). The refore, the presence of low-molecular-weight organic carbon substrates is not necessarily the most selective factor in the natural environment.

Slow photolithoautotrophic growth with H_2S or H_2 as electron-donating substrates has been shown in laboratory cultures of *Chloroflexus aurantiacus* and in hot spring populations (Pierson and Castenholz, 1995). Carbon fixation proceeds by carboxylation of acetyl-CoA and via hydroxypropionyl-CoA as an intermediate and yields glyoxylate as the net product (hydroxypropionate cycle; Holo, 1989; Strauß and Fuchs, 1993; Eisenreich et al., 1993). So far this cycle has not been found in any other member of the Bacteria. Glyoxylate is further assimilated into cell material with tartronate semialdehyde and 3-phosphoglycerate as intermediates (Menendez et al., 1999).

The highest metabolic versatility is found in phototrophic α- and β-Proteobacteria (purple nonsulfur bacteria). All representatives grow photoorganoheterotrophically and (with the exception of *Blastochloris viridis*) photolithoautotrophically with H_2 in the light. In addition to the substrates used by versatile purple sulfur bacteria, the spectrum of substrates that can serve as electron donors comprise long-chain fatty acids (like pelargonate), amino acids (aspartate, arginine, glutamate), sugar alcohols (sorbitol, mannitol), or aromatic compounds (benzoate; Imhoff and Trüper, 1989). With the exception of *Rubrivivax gelatinosus*, none of the purple nonsulfur bacteria is capable of degradation of polymers and therefore depends on the anaerobic food chain for the supply of electron-donating substrates required for growth. This dependence and the competition with chemotrophs for the carbon substr ates might be the major reason why dense blooms of purple nonsulfur bacteria do not occur under natural conditions (see Habitats of Phototrophic Prokaryotes in this Chapter). Some species are capable of also using reduced sulfur compounds as electron donors. However, most species oxidize sulfide to elemental sulfur only (Hansen and van Gemerden, 1972).

In *Heliobacteriaceae*, only a limited number of carbon substrates can serve as photosynthetic electron donor including pyruvate, ethanol, lactate, acetate, and butyrate. High levels of sulfide are inhibitory (Madigan, 1992; Madigan and Ormerod, 1995).

Cyanobacteria are obligate autotrophs par excellence; however, small molecular weight organic compounds such as acetate, sugars and amino acids are assimilated. In the case of amino acids, the presence of various efficient uptake systems has been interpreted as a means of recovery of leaked organic nitrogen, rather than a true chemotrophic capability (Montesinos et al., 1997). Certain strains of cyanobacteria can grow facultatively as chemoheterotrophs in the

dark (Rippka et al., 1979), but even under these conditions all of the photosynthetic machinery is synthesized. This lack of regulation implies that chemotrophy has played no significant evolutionary role in these organisms.

Chemotrophic Growth with O_2

Ecophysiology of Chemotrophic Growth

In lakes, purple sulfur and green sulfur bacteria are confined to environments where light reaches sulfide-containing water layers. The physiological properties restrict the distribution of these bacteria in the pelagic habitat (Pfennig, 1978). Dense accumulations of anoxygenic phototrophic bacteria, which apparently are growing chemotrophically, are only known for *Chloroflexus* (see Habitats of Phototrophic Prokaryotes in this Chapter). Although populations of other anoxygenic phototrophic bacteria do not seem to grow permanently by chemotrophy, the ability of many strains to shift to an aerobic chemotrophic mode of growth is of selective advantage in environments like intertidal sediments.

Green sulfur bacteria and *Heliobacteriaceae* are obligate anaerobes. Under oxic conditions, the reaction of reduced ferredoxin of the type I reaction center with molecular oxygen would create superoxide and other activated oxygen species. *Heliobacteriaceae* are rapidly damaged by exposure to molecular oxygen. This has been attributed not only to the formation of toxic oxygen radicals but also the destruction of the unsaturated fatty acids present in the cell membrane by activated oxygen species (Madigan and Ormerod, 1995). In green sulfur bacteria, it has been observed that the energy transfer from light-harvesting bacteriochlorophylls c/d/e to bacteriochlorophyll *a* drops by a factor of 10 after an increase in redox potential due to the quenching by chlorobium quinone. This mechanism may protect the cells during brief anoxic/oxic transitions. (see The Family Chlorobiaceae, Physiology section in Volume 7).

All other groups of phototrophic prokaryotes comprise species that not only generate metabolic energy by photosynthesis but are also capable of chemosynthesis with O_2.

Chloroflexus aurantiacus is capable of growth as an aerobic heterotroph. During phototrophic growth, β-carotene, γ-carotene, and hydroxy-γ-carotene-glucoside are the major carotenoids, whereas echinenone and myxobactone predominate in aerobically grown cells (Pierson and Castenholz, 1995). Unlike in purple nonsulfur or purple sulfur bacteria, synthesis of some carotenoids by *C. aurantiacus* is greatly enhanced under aerobic conditions (Pierson and Castenholz, 1974). The expression of the chlorosome CsmA protein is transcriptionally or posttranscriptionally regulated by oxygen (Theroux et al., 1990).

Almost all known species of phototrophic α- and β-Proteobacteria (purple nonsulfur bacteria) are capable of microaerophilic or aerobic chemoorganoheterotrophic growth with oxygen as terminal electron acceptor. Of the purple sulfur bacteria, *Ectothiorhodospira* species, and eight small-celled species of the *Chromatiaceae* (*Thiocapsa rosea*; *Chromatium gracile*; *Chr. minus*; *Allochromatium vinosum*; *Thiocystis violascens*; *Thiocapsa roseopersicina*; *Thiocystis violacea*; *Thiorhodovibrio winogradskyi*) can grow by chemolithotrophy, oxidizing sulfide or thiosulfate with molecular oxygen (De Wit and van Gemerden, 1987b; Kämpf and Pfennig, 1980; Overmann and Pfennig, 1992). Only few species grow also chemoorganotrophically with organic carbon substrates as electron donor of respiration. The group of facultatively chemotrophic *Chromatiaceae* includes typical inhabitants of benthic microbial mats like *Thiocapsa roseopersicina* and *Thiorhodovibrio winogradskyi*. This is not surprising considering the pronounced oxic/anoxic fluctuations in this type of habitat. The cells of purple sulfur bacteria in benthic systems are often immotile and form aggregates together with sand grains, apparently as an adaptation to the hydrodynamic instability of the habitat (van den Ende et al., 1996). At the same time, however, immotile cells are exposed to strong diurnal variations in oxygen concentrations. The growth affinities for sulfide are lower for chemotrophically growing *Thiocapsa roseopersicina* than for colorless sulfur bacteria, which may explain that no natural populations of purple sulfur bacteria are known that grow permanently by chemotrophy (see Interactions between Phototrophic Sulfur Bacteria and Chemotrophic Bacteria in this Chapter).

When grown anaerobically in the light, facultatively chemotrophic species of the purple nonsulfur and purple sulfur bacteria contain a potentially active repiratory system and exhibit ≥50% of the respiratory activity of chemotrophically growing cells (De Wit and van Gemerden, 1987a; Kämpf and Pfennig, 1980; Overmann and Pfennig, 1992; Pfennig, 1978). In cells that still contain bacteriochlorophyll, respiration is inhibited by light. This indicates that respiration and photosynthesis are coupled (e.g., by the membrane potential or common redox carriers; Richaud et al., 1986). An example is the soluble cytochrome c_2 which has a dual function in *Rhodobacter sphaeroides* where it is needed for electron transfer from the cytochrome bc_1 complex to the reaction center during ph otosynthesis, and to the cytochrome c oxidase during

respiration with molecular oxygen. During photosynthetic growth, expression of cytochrome c_2 is increased. At limiting concentrations of electron donating substrate, photosynthesis is preferred over respiration as long as the intracellular bacteriochlorophyll content is maintained at a sufficiently high level (4–7 g bacteriochlorophyll a·mg protein$_{-1}$ in *Thiocapsa roseopersicina* at light saturation; De Wit and van Gemerden, 1990a).

Growth continues after a shift to microoxic or aerobic conditions. Under oxic conditions the synthesis of pigments and of pigment-binding proteins of the photosynthetic apparatus ceases. The number of intracellular membrane vesicles is reduced dramatically and the composition of membrane lipids is altered. The pigment content in purple sulfur bacteria is inversely related to the ambient oxygen concentration (Kämpf and Pfennig, 1986). At 25% air saturation (52 M) of oxygen, pigment synthesis in *Thiocapsa roseopersicina* is completely repressed and cells become colorless (De Wit and van Gemerden, 1987b). In continuous cultures of purple sulfur bacteria, active degradation has not been observed and intracellular bacteriochlorophyll concentrations follow the washout curve. Thus bacteriochlorophyll does not seem to be actively degraded but is diluted out by cell division (De Wit and van Gemerden, 1987b). Concomitantly, the activities of respiratory enzymes (NADH dehydrogenase, cytochrome c oxidases) are increased in chemotrophically grown cells. When the cells of *Thiocapsa roseopersicina* become colorless, they use only one third of the electron donor for reduction of CO_2. The remaining two thirds are used for energy generation and respired. Correspondingly, the protein yield reaches one third of that of phototrophically grown cells (De Wit and van Gemerden, 1987b; De Wit and van Gemerden, 1990b).

In aerobic phototrophic bacteria, aerobic growth is stimulated by light that is absorbed by bacteriochlorophyll *a*. This stimulation is only transient, however, since bacteriochlorophyll synthesis is repressed even by low light intensities (Yurkov and van Gemerden, 1993) thus leading to a loss of the photosynthetic apparatus under continuous illumination.

Respiration in *cyanobacteria* involves a full respiratory chain including a cytochrome aa_3 terminal oxidase. Monomeric sugars are degraded using the oxidative pentose phosphate cycle. A complete tricarboxylic acid cycle has never been shown for any cyanobacterium. The NADPH formed in sugar catabolism is fed to the membrane-bound electron transport chain at the level of plastoquinone. This is in contrast to green chloroplasts, in which plastoquinol is autoxidized (Peltier and Schmidt, 1991). The respiratory electron transport chain of cyanobacteria is located in both the plasma and the thylakoidal membrane, and it shares many functional components with photosynthetic electron transport. The role of exogenous respiration of organic substrates is probably minor under natural conditions. Under anoxia, the known electron acceptor alternatives to oxygen for cyanobacterial chemoorganotrophy are some organic compounds and elemental sulfur. Fermentation seems to be a relatively widespread ability in benthic and bloom-forming cyanobacteria, but it is not universal (Moezelaar and Stal, 1994).

Genetic Regulation by O_2

A shift from anoxic to oxic growth conditions requires the expression of new proteins and cofactors. On the genetic level the formation of the photosynthetic apparatus and the intracytoplasmic membrane system is regulated by two main environmental variables, light intensity (see Response to Changes in Light Intensity and Quality in this Chapter) and molecular oxygen. The two factors act independently of one another and are involved in different mechanism of regulation of bacteriochlorophyll synthesis (Arnheim and Oelze, 1983). Compared to light, molecular oxygen acts as a stronger repressor, however. Although oxygen is a major factor controlling the formation of the photosynthetic apparatus in most of the facultatively phototrophic *Proteobacteria*, *Rhodovulum sulfidophilum* and *Rhodocista centenaria* are exceptional in that these species form the photosynthetic apparatus under both aerobic and anaerobic conditions (Hansen and Veldkamp, 1973; Nickens et al., 1996). Photopigment synthesis is not repressed by O_2 in *Rhodocista centenaria*.

The regulation of bacteriochlorophyll synthesis in purple nonsulfur bacteria is complex. The cells synthesize very little bacteriochlorophyll, probably because of the inhibition of bacteriochlorophyll biosynthesis enzymes (the δ–aminolevulinic acid synthesis and enzymes for the conversion of coproporphyrin; Oelze, 1992) by O_2. Oxygen does not seem to exert an effective transcriptional control. Under oxic conditions the transcription of bacteriochlorophyll synthesis genes decreases 2-fold, while that of light-harvesting I and reaction-center genes decreases by a factor of 30–100 (Bauer, 1995). The tetrapyrrole synthesis pathway has four different branches (leading to heme, bacteriochlorophyll, siroheme and vitamin B_{12}). While the bacteriochlorophyll content is drastically reduced in the presence of oxygen (Arnheim and Oelze, 1983), heme synthesis remains unaffect ed (Lascelles, 1978). The intracellular activity of δ-aminolevulinic acid synthase, the key enzyme of tetrapy-

rrol synthesis in α-Proteobacteria, is reduced in the presence of oxygen. Regulation by oxygen may occur also during some later steps of tetrapyrrole synthesis. It appears that oxygen inhibits magnesium chelatase, thereby increasing the protoporphyrin IX pool, which in turn leads to increased formation of heme. Feedback inhibition of δ-aminolevulinate synthase by heme would then slow down the synthesis of intermediates but still guarantee the amount needed for heme biosynthesis (Beale, 1995; Biel, 1995; Rebeiz and Lascelles, 1982).

After return to anoxic conditions the synthesis of the photosynthetic apparatus and intracellular membranes occurs in a light-independent manner. Anoxygenic photosynthetic bacteria contain a distinct light-independent protochlorophyllide reductase, composed of probably three subunits (BchN, BchB, and BchL). In angiosperms, the reduction of the fourth ring of the Mg-tetrapyrrole intermediate by NADPH-protochlorophyllide oxidoreductase is a light-dependent step in the chlorophyll biosynthetic pathway. This protein represents one of the only two enzymatic transformations known to require light (Suzuki and Bauer, 1995). Cyanobacteria, green algae and gymnosperms contain both, the light-dependent and light-independent protochlorophyllide reductase. The capacity to synthesize (bacterio)chlorophyll in the dark is of significance for the competitive success of *Chromatiaceae* in intertidal microbial mats. During anoxic conditions in the dark, *Thiocapsa roseopersicina* can synthesize bacteriochlorophyll *a* at maximum rate. Under the fluctuating conditions as they are observed in benthic microbial mats (oxic light, anoxic dark phase), purple sulfur bacteria therefore can maintain a photosynthetic mode of growth as long as bacteriochlorophyll synthesis during the night compensates for the wash out of pigments during the day (De Wit and van Gemerden, 1990b).

A multicomponent regulatory cascade controls the coordinate expression of the light-harvesting and reaction center *puf*, *puh*, and *puc* genes and involve various transcription factors (Bauer, 1995; Bauer and Bird, 1996). In *Rhodobacter capsulatus*, a redox-sensitive repressor (CrtJ) binds under oxic conditions to a conserved palindrome sequence in promotors of bacteriochlorophyll, carotenoid, and light-harvesting complex II genes. A second system for the regulation of the *puf*, *puh*, and *puc* operons probably consists of three components, a membrane-spanning sensor kinase (RegB), a soluble response regulator (RegA), and a hypothetical activator of the nonspecific alternative sigma factor σ^P (RegX). A decrease in oxygen tension causes autophos phorylation of the membrane-spanning sensor kinase RegB, which then phosphorylates the cytoplasmic response regulator RegA. The latter acts as intermediate and probably transfers its phosphate to a putative third DNA-binding component that activates gene expression. The RegA-RegB system also is involved in regulation of the expression of cytochrome c_2 and the Calvin cycle CO_2 fixation genes and therefore is of general significance for the regulation of cellular metabolism.

The transcripts of the photosynthetic gene cluster exceed 10 kb and extend from pigment biosynthesis genes across promoter regions and into the genes for light-harvesting complex I and reaction center proteins. In *Rhodobacter capsulatus*, transcription of the genes coding structural polypeptides of the reaction center and light-harvesting complex I are not the only peptides initiated at their respective promotors. The transcripts of the bacteriochlorophyll biosynthesis *bchCA* operon extends through the promoter and coding sequences of the downstream *puf BALM* operon, and the transcript of the carotenoid biosynthesis *crtEF* operon extends through both (Wellington et al., 1992). Similarly, the *bchFBKHLM-F1696* and *puhA* operons are transcriptionally linked. The linkage of operons of different components of the phot osynthetic apparatus in such superoperons also has been detected in other species of purple nonsulfur bacteria and may play a significant role in the adaptation of cells to changes in environmental oxygen tension. According to a model (Wellington et al., 1992), the presence of superoperons ensures a rapid physiological response to a decrease in oxygen tension. In the presence of oxygen, a basal level of light-harvesting I and reaction center polypeptides is constantly formed and incorporated into the membrane, but these polypeptides disappear again in the absence of bacteriochlorophyll (Dierstein, 1984; Drews and Golecki, 1995) due to degradation. After a shift from oxic to anoxic conditions, the presence of a basal level of structural polypeptides considerably shortens the lag time for the change from aerobic respiratory to anaerobic photosynthetic growth. During this lag ph ase, the cellular amount of structural polypeptides of the photosynthetic apparatus is further increased by increasing the transcription rate of the *puf* and *puh* genes.

Oxygen does not only regulate the transcription of photosynthesis genes but also later steps in gene expression. Posttranscriptional regulation involves mRNA processing (mRNA degradation) and possibly some later steps (Rödig J. et al., 1999).

In most bacteria, the formation of multiple sigma factors is a prerequisite for the coordination of the regulation of a large number of genes in response to changes in environmental condi-

tions. Sigma factors are dissociable subunits that confer promoter specificity on eubacterial core RNA polymerase and are required for transcription initiation. In phototrophic bacteria, the diversity of sigma factors of the σ^{70} family as they are present in the different phylogenetic groups appears to be correlated with their metabolic flexibility. In the unicellular cyanobacteria *Synechococcus* sp. and *Synechocystis* sp., nine different sigma factors (one member of group 1, four members of group 2, and four members of group 3) have been found, whereas one group 1 and three group 2 sigma factors have been found in *Chloroflexus* spp. In contrast to most other bacteria, the green sulfur bacterium *Chlorobium tepidum* contains only one group 1, but no alternative group 2 sigma factor (Gruber and Bryant, 1998). In *Chloroflexus*, one group 2 σ^{70} factor (SigB) is transcribed at fourfold higher levels during aerobic growth and therefore appears to be involved in the shift in metabolism. It has been proposed that SigB is involved in regulation of pigment synthesis (Gruber and Bryant, 1998).

Significance of Anoxygenic Photosynthesis for the Pelagic Carbon and Sulfur Cycles

The carbon fixation of phototrophic sulfur bacteria has been determined in a wide range of habitats, mostly inland lakes (Overmann, 1997; van Gemerden and Mas, 1995). The theoretical maximum of primary production by phototrophic sulfur bacteria has been estimated to be 10,000 mg $C{\cdot}m^{-2}{\cdot}d_{-1}$. Purple and green sulfur bacteria can contribute up to 83% of total primary productivity in these environments. This high number notwithstanding, anoxygenic primary production only represents a net input of organic carbon to the food web if 1) the anaerobic food chain is fueled by additional allochthonous carbon from outside and 2) aerobic grazers have access to the biomass of phototrophic sulfur bacteria. Based on recent experimental evidence, these conditions are met at least in some aquatic ecosystems (Overmann, 1997).

With the exception of geothermal springs, the sulfide required by phototrophic sulfur bacteria for CO_2-assimilation originates from sulfate or sulfur reduction during the terminal degradation of organic matter. This organic matter cannot be provided solely by anoxygenic phototrophic bacteria, since growth (hence accumulation of reduced carbon) constantly diverts electrons from their cycling between anoxygenic phototrophic bacteria and sulfate-reducing bacteria. At least part of the sulfide formation is therefore fueled by carbon that has already been fixed by oxygenic photosynthetic organisms within or outside the ecosystem. Consequently anoxygenic photosynthesis represents not new, but secondary primary production. A complete degradation of the carbon fixed by phototrophic sulfur bacteria in the anaerobic food chain (and thus an efficient recycling of electrons) in an anoxygenic primary production has been estimated to exceed oxygenic photosynthesis by as much as ten times (Overmann, 1997). In reality, anoxygenic photosynthesis surpasses that of phytoplankton mostly in oligotrophic lakes. In many oligotrophic lakes, the input of allochthonous carbon derived from terrestrial sources in the watershed is significant (Rau, 1980; Sorokin, 1970). In an oligotrophic saline meromictic lake (Mahoney Lake, B.C., Canada), purple sulfur bacteria together with the anaerobic food chain efficiently converted allochthonous organic carbon into easily degradable bacterial biomass (Overmann, 1997). It appears likely that phototrophic sulfur bacteria have this ecological function also in other aquatic ecosystems.

The presence of hydrogen sulfide in layers of phototrophic sulfur bacteria may prevent their biomass from entering the grazing food chain. This has been substantiated by stable carbon and sulfur isotope data, which indicated that phototrophic sulfur bacteria are not consumed to a significant extent by higher organisms (Fry, 1986). In addition, a quantitative analysis of loss processes conducted in a few lakes indicates that predation must be of minor significance (Mas et al., 1990; van Gemerden and Mas, 1995). In contrast, recent investigations have revealed that at least in one lake ecosystem, a major fraction of purple sulfur bacterial biomass enters the aerobic food chain via rotifers and calanoid copepods (Overmann et al., 1999b; Overmann et al., 1999c). The key environmental factors that caused this efficient link between anoxic and oxic water layers were the autumnal upwelling of phototrophic bacteria into oxic water layers by mixing currents, and the formation of gas vesicles and large cell aggregates by the dominant species, *Amoebobacter purpureus*.

Sulfide formation by sulfate- and sulfur-reducing bacteria and sulfide oxidation back to sulfur and sulfate occur at comparable rates in several lakes (Overmann et al., 1996; Parkin and Brock, 1981). This leads to a closed sulfur cycle and a detoxification of sulfide without concomitant depletion of oxygen (Pfennig, 1978).

The significance of phototrophic sulfur bacteria for the oxidation of sulfide in stratified environments is critically dependent on their cell density rather than the absolute biomass per surface area of the ecosystem (Jørgensen, 1982). Dense populations in laminated microbial mats can account for 100% of the total sulfide oxidation in those systems, whereas some dilute pelagic populations oxidize only very small amounts (e.g., 4% in the Black Sea) of the sulfide

diffusing from below into the chemocline (Overmann et al., 1991a; Overmann et al., 1996).

No information on the ecological significance of aerobic phototrophic bacteria is available to date.

Interactions with Other Microorganisms

Competition for Light Blue light prevails in very clear open oceans (Fig. 6) where marine *Synechococcus* cells thrive under conditions of low photon flux (~10 mol quanta·m^{-2}·s^{-1}; Carr and Mann, 1994). Two ecotypes of the marine *Synechococcus* exist which differ in the intracellular ratio of phycourobilin to phycoerythrobilin (Waterbury et al., 1986). Two subpopulations are distinguished according to the predominant chromophore associated with the phycoerythrin. Phycourobilin-rich strains are characteristic of the open oceans whereas strains with a lower PUB content predominate in shelf waters (Olson et al., 1990a). Compared to PEB-containing antennae (absorption maximum, ~550 nm), incorporation of PUB (absorption maximum, ~495 nm) increases the efficiency of light absorption significantly in deeper water lay ers of oligotrophic oceans.

Similarly, coexisting and phylogenetically closely related but genetically distinct populations of *Prochlorococcus* are adapted for growth at different light intensities, which results in their broad depth distribution (Moore et al., 1998). The low-light-adapted ecotype has a higher intracellular content of chlorophylls a and b, a higher chlorophyll b/a ratio, and exhibits a higher maximum quantum yield reaching the theoretical maximum of 0.125 mol C·(mol quanta)$^{-1}$. Its properties enable this ecotype to colonize very low water layers. It has been suggested that the distribution of different ecotypes in the same water column would result in greater integrated production than could be achieved by a single ecotype (Moore et al., 1998).

Based on the specific physiological properties of oxygenic and anoxygenic phototrophic bacteria, multilayered microbial communities frequently develop in stratified pelagic and in benthic (Fig. 5A,B) habitats. Cyanobacteria, eukaryotic algae and even plants (Lemna) form the topmost layers overlying populations of *Chromatiaceae* and green sulfur bacteria (Dubinina and Gorlenko, 1975; Caldwell and Tiedje, 1975; Pfennig, 1978; Camacho et al., 1996; Pierson et al., 1990; Pierson et al., 1990).

Phototrophic sulfur bacteria require the simultaneous presence of light and sulfide, which usually restricts their occurrence to layers well below the surface of lakes and sediments. As a consequence of the absorption of light in the overlying water, the light energy available to phototrophic sulfur bacteria in most pelagic environments is rather low (0.02–10% of surface light intensity; van Gemerden and Mas, 1995; Parkin and Brock, 1980b; Camacho et al., 1996). Similar values have been determined for purple layers in benthic microbial mats (Kühl and Jørgensen, 1992; Pierson et al., 1990; Garcia-Pichel et al., 1994c). A tight correlation between anoxygenic photosynthesis and the amount of light reaching phototrophic sulfur bacteria strongly suggests that light i s the main environmental variable controlling the anoxygenic photosynthesis (van Gemerden and Mas, 1995). Therefore, a selective pressure for efficient light harvesting and maximum quantum yield exists in anoxygenic phototrophs. The same holds true for a few niche-specialized, deep-dwelling cyanobacteria.

The ecological niches of green sulfur bacteria and *Chromatiaceae* show considerable overlap because both groups grow preferably or exclusively by photolithotrophic metabolism, using ambient sulfide as electron-donating substrate. Different species of the same group should be even more competitive. Besides differences in maintenance energy demand, in adaptation to low light intensities and metabolic flexibility, another important factor determining the species composition of phototrophic sulfur bacteria in their natural habitats is the spectral composition of underwater light. In the overlying layers, light is absorbed by water itself, dissolved yellow substance (gilvin), phytoplankton and inanimate particulates. The limited wavelength range available at great depth selects for species of anoxygenic phototrophic bacteria with complementary absorption spectra. In many lacustrine habitats, light absorption by phytoplankton exceeds that of gilvin or water itself (Kirk, 1983), and light of the blue green to green wavelength range reaches layers of phototrophic sulfur bacteria. Those *Chromatiaceae* which contain the carotenoid okenone (Fig. 7) dominated in 63% of the natural communities studied (van Gemerden and Mas, 1995). It was proposed that energy transfer from carotenoid antenna pigments to the reaction center is more efficient in okenone-forming strains than in other purple sulfur bacteria (Guerrero et al., 1986). In addition, the capability of gas vesicle formation, and the different kinetics of sulfide oxidation (see Coexistence of Phototrophic Sulfur Bacteria in this Chapter) appear to be of selective value for the colonization of pelagic habitats. Below accumulations of purple sulfur bacteria, the green-colored forms of the green sulfur bacteria dominate because of their superior capability to harvest the light reaching them, which has its spectrum shifted to a maximum intensity at 420–450 nm (Table 2) (Montesinos et al., 1997). In contrast, the brown-colored forms of the green sulfur bacteria dominate in lakes

where the chemocline is located at depths greater than 9 m and in eutrophic lakes with a pronounced light absorption in the oxic zone.

A similar niche separation occurs in the phototrophic consortia (see The Family Chlorobiaceae in Volume 7), which encompass green-colored or brown-colored epibionts (Overmann et al., 1999b). The ecological niche of the brown-colored green sulfur bacteria may be attributed to their use of significantly lower light intensities than purple sulfur bacteria for phototrophic growth and to their lower maintenance energy requirements (see Light Absorption and Light Energy Transfer in Prokaryotes in this Chapter; The Family Chlorobiaceae in Volume 7). An extremely low-light adapted strain of the green sulfur bacterium *Chlorobium phaeobacteroides* has been isolated from the chemocline of the Black Sea located at an 80-m depth (Overmann et al., 1991a). This isolate (strain MN1) could grow at light intensities as low as 0.25 μmol quanta·m^{-2}·s^{-1}.

In sedimentary environments with their particular optical properties (Fig. 6), the irradiance reaching anoxygenic phototrophic bacteria may be reduced to ←1% of the surface value for light in the visible region, while >10% of the near infrared light is still available (Kühl and Jørgensen, 1992; see Light energy and the spectral distribution of radiation). As a consequence, the long wavelength Q_y bands of bacteriochlorophylls are significant for light-harvesting in sediments, whereas light absorption of anoxygenic phototrophic bacteria in lakes is mediated by carotenoids and the Soret bands of bacteriochlorophylls. In microbial mats, the spectral quality of the scalar irradiance is strongly modified as it penetrates. The presence of populations of phototrophic microorganisms impose strong absorption signatures on the spectrum of the scalar irradiance (Jørgensen and Des Marais, 1988; Pierson et al., 1987). As a result of vertical niche separation, benthic microbial mats can consist of up to five distinctly colored layers that are formed (from the top) by diatoms and cyanobacteria, cyanobacteria alone, purple sulfur bacteria with bacteriochlorophyll a, purple sulfur bacteria with bacteriochlorophyll b, and green sulfur bacteria (Nicholson et al., 1987). In this vertical sequence different wavelength bands of red and infrared light (compare Table 2, Fig. 7) are successively absorbed by the different microbial layers (Pierson et al., 1990). Distinct blooms of bacteriochlorophyll *b*-containing anoxygenic phototrophic bacteria have been observed only in benthic habitats. Employing this pigment, the phototrophic Proteobacteria *Blastochloris viridis*, *Blastochloris sulfoviridis*, *Thiocapsa pfennigii*, *Halorhodospira halochloris*, *Halorhodospira abdelmalekii* harvest light of a wavelength range (1020–1035 nm), which cannot be exploited by any other photosynthetic organism.

Until recently, no strain of anoxygenic photosynthetic bacteria was known that could absorb light in the wavelength range between 900 and 1020 nm. Because of the prevalence of infrared radiation in the anoxic layers of microbial mats and the strong competition for this wavelength region, bacteria containing other types of photosynthetic antenna complexes would have a high selective advantage. Recently, the α-Proteobacterium *Rhodospira trueperi* was isolated, which contains bacteriochlorophyll *b* in a light-harvesting complex with a maximum absorption at 986 nm (Pfennig et al., 1997). Employing a selective enrichment strategy, the α-Proteobacterium *Roseospirillum parvum* could be isolated which harbors another new type of photosynthetic antenna complex. Here, bacteriochlorophyll *a* is the light-harvesting pigment and in vivo exhibits an absorption maximum at 911 nm (Glaeser and Overmann, 1999, Fig. 7). Both isolates originate from benthic microbial mats, indicating that the diversity of pigment-protein complexes in *Proteobacteria* is higher than previously assumed. The variation in the in vivo absorption spectra of the same pigment must be the result of differences in binding to light-harvesting proteins. In contrast, changes in the absorption spectra of the light-harvesting complex of green sulfur bacteria are the result of chemical alterations (e.g., methylation) of the pigment molecules (Bobe et al., 1990) because pigment-pigment interactions dominate in the chlorosomes (see Light Absorption and Light Energy Transfer in Prokaryotes in this Chapter).

Because methanogenesis is the predominant pathway of terminal degradation in rice fields, *Heliobacteriaceae* probably compete with the photoheterotrophic purple nonsulfur bacteria in their natural environment (Madigan and Ormerod, 1995). Owing to the presence of bacteriochlorophyll g, *Heliobacteriaceae* take advantage of a wavelength region of the electromagnetic spectrum, which is not absorbed by other phototrophic bacteria. As a result of the small and fixed size of the photosynthetic antenna (see Light Absorption and Light Energy Transfer in Prokaryotes in this Chapter), these bacteria are adapted to higher light intensities than other anoxygenic phototrophic bacteria (≈1,000 mol quanta·m^{-2}·s^{-1}).

In addition to the capacity of absorbing light in the long wavelength range, metabolic flexibility is of highly selective value for the colonization of benthic habitats with their high fluctuations in oxygen and sulfide concentrations (see Chemotrophic growth with O_2).

However, the composition of communities of phototrophic sulfur bacteria is not solely deter-

mined by competition. The simultanous presence of green sulfur bacteria and *Chromatiaceae* possibly is also based on syntrophic interactions (see Coexistence of Phototrophic Sulfur Bacteria in this Chapter).

Coexistence of Phototrophic Bacteria Within the *Chromatiacea* the small-celled genus *Chromatium* species exhibit a considerably greater metabolic flexibility than the large-celled species (see Carbon Metabolism and Chemotrophic Growth with O_2 in this Chapter). In addition, small-celled species like *Allochromatium vinosum* have a higher growth affinity for sulfide. Based on these pure culture data, it is therefore unexpected that large-celled forms in fact dominate in natural ecosystems. The large-celled *Chromatium weissei* oxidizes sulfide twice as fast as the small-celled *Allochromatium vinosum*. Whereas the former preferentially oxidizes sulfide to zero-valent sulfur, the latter oxidizes a larger fraction directly to sulfate. Under fluctuating conditions as they occur in the chemocline of lakes, *Chromatium weissei* is capable of rapidly oxidizing sulfide at the onset of illumination, thereby accumulating zero-valent sulfur. During the remaining light period and because of its higher affinity for sulfide, *Allochromatium vinosum* utilizes most of the sulfide. Continuous cocultures of both species have thus been established by illumination in light-dark cycles (van Gemerden, 1974).

Furthermore, stable coexistence of two organisms is feasible in the presence of two substrates for which the two competitors have complementary affinities. Stable syntrophic interactions can be established in laboratory cocultures of purple sulfur (*Allochromatium vinosum*) and green sulfur bacteria (*Chlorobium limicola* f.sp. thiosulfatophilum; van Gemerden and Mas, 1995). Because of its higher affinity, the green sulfur bacterium oxidizes sulfide to zero-valent sulfur. The extracellular sulfur is mobilized as polysulfide, which can be used instantaneously as electron donor of the purple sulfur bacterium. The presence of sulfide inhibits the green sulfur bacterium from using polysulfide (see The Family Chlorobiaceae in Volume 7). Sulfide and polysulfide thus are the mutual substrates for the two different phototrophic sulfur bacteria.

Purple and green sulfur bacteria also have complementary affinities for sulfide and acetate (see Carbon metabolism). Accordingly, stable continuous cocultures of *Chlorobium phaeobacteroides* and *Thiocapsa roseopersicina* can be established (Veldhuis and van Gemerden, 1986).

Interactions Between Phototrophic Sulfur Bacteria and Chemotrophic Bacteria A considerable number of strains of *Chromatiacae* is capable of switching to a chemolithotrophic growth mode after prolonged incubation in the presence of molecular oxygen (see Chemotrophic Growth with O_2 in this Chapter). Under these conditions, purple sulfur bacteria compete with colorless sulfur bacteria like *Thiobacillus* spp. Compared to thiobacilli, the purple sulfur bacterium *Thiocapsa roseopersicina* attains a higher growth yield under chemolithotrophic conditions (De Wit and van Gemerden, 1987a). However, the growth affinity for sulfide of the colorless sulfur bacteria is up to 47 times higher than that of *Chromatiacae* (De Wit and van Gemerden, 1987b; van Gemerden and Mas, 1995). Therefore *Chromatiacae* growing exclusively by chemolithotrophy would be rapidly outcompeted by colorless sulfur bacteria.

Culture experiments indicate that *Thiocapsa roseopersicina*, a typical inhabitant of laminated microbial mats in temperate environments, can replenish its photosynthetic pigments during anoxic periods in the dark, thereby maintaining a phototrophic growth mode also during the subsequent oxic light period (De Wit and van Gemerden, 1990b). Based on microelectrode measurements, purple sulfur bacteria in marine microbial mats of the North Sea barrier islands are exposed to oxygen during most of the day, whereas anoxic conditions prevail during the night (De Wit et al., 1989). Thus, the anoxygenic phototrophs cannot grow during the night and face competition for sulfide by colorless sulfur bacteria during the day. Because of their higher affinity for sulfide, the latter would be expected to outcompete phototrophically growing purple sulfur bacteria. In cocultures of *Thiocapsa roseopersicina* and *Thiobacillus thioparus*, sulfide is indeed entirely used by the colorless sulfur bacterium in the presence of oxygen. If oxygen concentrations are limiting, however, sulfide is oxidized incompletely by the chemolithotroph and soluble zero-valent sulfur formed (either as polysulfide or polythionates) that in turn is used by the purple sulfur bacterium for phototrophic growth (van den Ende et al., 1996). Both diurnal fluctuations between oxic light and anoxic dark periods and syntrophism based on sulfur compounds may permit a stable coexistence of these groups and explain their simultaneous presence in natural microbial mats.

Stable associations can be established between green sulfur bacteria and sulfur- or sulfate-reducing bacteria (see The Family Chlorobiaceae in Volume 7; Interactions with Chemotrophic Bacteria in this Chapter). These associations are based on a cycling of sulfur compounds but not carbon (see Significance of Anoxygenic Photosynthesis for the Pelagic Carbon and Sulfur Cycles in this Chapter). The simultaneous growth of both types of bacteria is fueled by the

oxidation of organic carbon substrates and light. In a similar manner, cocultures of *Chromatiaceae* with sulfate-reducing bacteria have been established in the laboratory (van Gemerden, 1967).

The most spectacular type of association involving phototrophic bacteria is represented by the phototrophic consortia. These consortia consist of green sulfur bacterial epibionts that are arranged in a regular fashion around a central chemotrophic bacterium. A rapid signal transfer exists between the two partners and permits phototrophic consortia to scotophobotactically accumulate at preferred light intensities and wavelengths. In this association, the immotile green sulfur bacteria attain motility like purple sulfur bacteria. The high numbers of phototrophic consortia found in many lakes indicate that this strategy must be of high competitive value under certain environmental conditions.

A commensal relationship may exist between coccoid epibiotic bacteria and the purple sulfur bacterium *Chromatium weissei* (Clarke et al., 1993). This unidentified epibionts attaches to healthy cells but does not form lytic plaques on lawns of host cells like the morphologically similar parasite *Vampirococcus* (see Significance of Bacteriophages and Parasitic Bacteria in this Chapter). Possibly, the epibiont grows chemotrophically on carbon compounds excreted by the purple sulfur bacterium.

A syntrophic interaction between cyanobacteria and sulfate-reducing bacteria appears to exist in microbial mats where both types of microorganisms occur in close spatial proximity, if not intermixed with each other. In these ecosystems, the excretion of organic carbon substrates by cyanobacteria may provide the electron-donating substrates for sulfate-reducing bacteria (Jørgensen and Cohen, 1977; Skyring and Bauld, 1990; Fründ and Cohen, 1992). The glycolate produced by photorespiration (Fründ and Cohen, 1992), as well as the formate, acetate and ethanol produced by glycogen fermentation (Moezelaar and Stal, 1994) most likely are the substrates excreted by cyanobacteria.

Despite a pronounced limitation of sulfate reduction by carbon substrates (Overmann et al., 1996; Overmann, 1997), no close syntrophic relationship was found between purple sulfur and sulfate-reducing bacteria in a meromictic lake. In this specific environment degradation of biomass by the entire anaerobic food chain rather than excretion of small carbon molecules and their direct utilization by sulfate-reducing bacteria provides the electron-donating substrates for sulfate-reducing and sulfur-reducing bacteria.

Symbioses Between Phototrophic Bacteria and Eukaryotes Only one example is known for an intracellular symbiosis of anoxygenic phototrophic bacteria with an eukaryotic organism. The ciliate *Strombidium purpureum* inhabits the photic zone of sulfide-containing marine sands and harbors 200–700 purple endosymbionts. Symbionts are arranged along the periphery of the host cell and contain intracellular tubular or vesicular membranes, bacteriochlorophyll *a* and spirilloxanthin (Fenchel and Bernard, 1993a; Fenchel and Bernard, 1993b). The ciliate shows a photosensory behavior, accumulating at wavelength that corresponds to the absorption maxima of the endosymbionts. It has been suggested that the intracytoplasmic purple bacteria increase the efficiency of the fermentative host by using its end products for anoxygenic photosynthesis. Furthermore, respiration of the bacteria may protect the host against oxygen toxicity.

In the course of evolution, *Cyanobacteria* have entered into symbiotic associations with a multitude of organisms (Schenk, 1992). Besides all eukaryotic phototrophs, from microalgae to *Sequoia sempervirens*, which have intracellular cyanobacterial symbioses, the most common extracellular symbioses of nonheterocystous cyanobacteria are in the form of cyanolichens and involve the unicellular genera *Chroococcidiopsis*, *Gloeocapsa*, "*Chroococcus*," and *Gloeothece*, as well as members of the genera *Nostoc*, *Calothrix*, *Scytonema*, *Stigonema*, and *Fischerella* as photobionts. Heterocystous cyanobacteria in the genus *Nostoc* form extracellular symbioses with liverworts and hig her plants (Cycads, duckweed). *Anabaena* enters in symbiosis with water ferns of the genus *Azolla*. *Prochloron* strains, large-celled *Synechocystis* and small-celled *Acaryochloris marina* are known from extracellular symbioses with ascidians in tropical or subtropical marine waters; *Prochloron* is found as ectosymbiont on the marine didemnid ascidian *Lissoclinum patella* (Lewin and Withers, 1975). Extracellular symbioses of the *Pseudanabaena*-like "*Konvophoron*" occur in Mediterranean invertebrates. Finally, intracellular symbioses of nonheterocystous cyanobacteria are known with tropical sponges ("*Aphanocapsa*", *Oscillatoria*, *Synechocystis*, *Proc hloron*), with green algae (*Phormidium*) and dinoflagellates (unidentified). Heterocystous cyanobacteria occur intracellularly in oceanic diatoms of the genera *Hemiaulus* and *Rhizosolenia* (and the cyanobacterium *Richelia intracellularis*). The cyanobacterial symbiont consists of a short cell filament with a terminal heterocyst (Mague et al., 1977). The numbers of filaments varies with host species. *Nostoc* thrives intracellularly in *Trifolium* (clover) and also in the terrestrial non-lichenic fungus *Geosiphon pyriforme*. With the notable exception of lichenic photobionts, many symbiotic cyanobacteria have resisted cultivation in spite of continued efforts.

Significance of Bacteriophages and Parasitic Bacteria In addition to grazing, light and nutrient limitation, cyanophage infection of cyanobacteria may be a significant factor limiting primary productivity in the marine environment. However, because of inactivation by solar radiation and resistence of the host cells, the role of cyanophages has remained unclear (Bergh et al., 1989; Proctor and Fuhrman, 1990; Suttle et al., 1990; Suttle et al., 1993; Waterbury and Valois, 1993).

Several bacteria have been discovered that attack phototrophic bacteria (Guerrero et al., 1986; Nogales et al., 1997). *Vampirococcus* attaches to the cell surface of *Chromatium* spp. where it divides, forming chains of up to three cells. Concomitantly, the cytoplasm of the host cell appears to be degraded. *Daptobacter* penetrates the cell envelope and divides intracellularly by binary fission. In contrast to *Vampirococcus*, *Daptobacter* has been cultivated in the absence of the host and grows by fermentative metabolism. *Bdellovibrio* has a broad host range, and under laboratory conditions attacks also purple sulfur bacteria. *Bdellovibrio* forms daughter cells by multiple division in the periplasmic space of the host cell. The Gram-negative chemotrophic bacterium *Stenotrophomonas maltophilia* is a non-obligatory parasite of green sulfur bacteria, which causes cell lysis and ghost formation (Nogales et al., 1997). Its host range is not limited to green sulfur bacteria. The presence of parasitic bacteria in water samples becomes evident by the formation of lytic plaques on lawns of host bacteria (Esteve et al., 1992; Nogales et al., 1997). Up to 94% of the cells of phototrophic sulfur bacteria may be infected by parasitic bacteria in natural samples. Since infection is largely limited to nongrowing cells, the impact of parasitism on populations of phototrophic sulfur bacteria appears to be limited (van Gemerden and Mas, 1995).

Evolutionary Considerations

Porphyrins are found in all organisms from archaebacteria through plants to animals, and are indispensable as prosthetic groups for energy conservation. In contrast, the partially reduced derivates of porphyrins, the (bacterio)chlorophylls, are synthesized by members of only a few bacterial divisions (Fig. 1). This indicates that the capability for synthesis of porphyrins is a very ancient trait, whereas only a few prokaryotes acquired the capability to form photosynthetic pigments. Photosynthesis requires the presence of various complex protein structures and cofactors, and thus the expression of a large number of different genes (see Photosynthetic Gene Cluster in this Chapter). Previously, it had therefore appeared justified to consider all phototrophic prokaryotes as a monophyletic group only distantly related to nonphototrophic bacteria (Pfennig and Trüper, 1974; Trüper and Pfennig, 1978). Two lines of evidence have been used to recon struct the evolution of photosynthesis.

Fossil Evidence The oldest fossils of microorganisms have been dated back to the early Archaean (3.8 billion years ago) and may represent remains of cyanobacteria (Awramik, 1992). They consist of chemical fossils and stromatolites that have been detected especially in sedimentary rocks of the Pilbara region, Western Australia, and the Barberton Mountain Land, South Africa. Stromatolites are laminated convex domes and columns of cm to dm size and have been found in 3.5 to 0.8 billion year old rocks. Although scarce in biosynthetic molecular skeletons, the insoluble, high-molecular-weight organic matter (kerogen) contains isotopic evidence for autotrophic carbon fixation. The ratio of stable carbon isotopes ($\delta^{13}C$ values) are in the range of –35.4 to –30.8°/oo, which is typical for CO_2-carbon fixed by the ribulose-1,5-bisphosphate cycle (Hayes et al., 1983). In addition, the se ancient sediments contain laminated domes and columns of cm to dm size, which in analogy to extant stromatolites have been interpreted as organosedimentary structures produced by the trapping, binding, and precipitation activity of filamentous microorganisms, most likely cyanobacteria.

Alternatively, it has been proposed that anoxygenic photosynthetic bacteria and not the oxygenic cyanobacteria formed the oldest stromatolites. Based on the phylogenetic analysis of the 16S rRNA gene sequence (Oyaizu et al., 1987) and the ecophysiology (Ward et al., 1989) of the filamentous green photosynthetic bacterium *Chloroflexus aurantiacus*, similar anoxygenic phototrophic bacteria may be the more likely candidate microorganisms that built the most ancient stromatolites. However, according to analyses of the nucleotide sequences of its reaction center polypeptides and primary sigma factor (see Molecular Evidence in this Chapter), *Chloroflexus aurantiacus* does not represent a deep branch of bacterial evolution. Gypsum layers within the supposed stromatolites have been interpreted as indicators of sulfide oxidation by either anoxygenic phototrophs or colorless sulfur-oxidizing bacteria (Awramik, 1992). However, similar structures have been discovered in lacustrine, and thus sulfur-depleted, settings with little input of allochthonous organic carbon (Buick, 1992). Therefore, at least some 2.7 billion year-old stromatolites are more likely to have harbored oxygenic cyanobacteria. Taken together with the fossil evidence, this would indi-

cate that diversification of the major groups of phototrophic microorganisms did occur during the early Archaean (Awramik, 1992).

Because of the indefinite character of the fossil evidence, 16S rRNA sequences and components of the photosynthetic apparatus of the different photosynthetic prokaryotes have been used to gain additional insight into the evolution of photosynthesis.

Molecular Evidence Chlorophyll-based photosystems are only found in the Bacteria and chloroplasts, suggesting that this type of energy conversion originated in the bacterial lineage after the divergence of Archaea and Eukarya. So far, photosynthetic species have not been discovered in the very early lineages of the bacterial radiation (e.g., the thermophilic oxygen reducers and *Thermotogales*; Fig. 1). Because most species of these lineages are chemolithotrophic, it has been proposed that chemolithoautotrophy preceded phototrophy during the evolution of the Bacteria (Pace, 1997). This conclusion is supported by the fact that in phylogenetic trees based on protein sequences of elongation factor EF-Tu and the β-subunit of ATP synthase, only the *Aquificales* and *Thermotogales* branch deeper than the majority of the bacterial divisions, while the *Chloroflexus* subdivision does not (Stackebrandt et al., 1996), thus indicating that *Chloroflexus* does not represent the descendant of a more ancient ancestor than other phototrophic bacteria.

At present, five of the known bacterial lineages comprise phototrophic species (Fig. 1, see Taxonomy of Phototrophy among Prokaryotes in this Chapter). Based on 16S rRNA sequences, extant phototrophic species of different lineages are only very distantly related to each other. Furthermore, one lineage, the *Chloroflexus* subgroup, represents an early branch in the evolution of the Bacteria. Given the complexity of the photosynthetic apparatus, it is unlikely that photosynthesis has evolved more than once during the evolution of the domain Bacteria (Woese, 1987). The phylogenetic analysis indicates that either an early ancestor of most known bacteria had acquired the capacity for photosynthetic growth (Stackebrandt et al., 1988) or, alternatively, that the genes coding the photosynthetic apparatus were transferred laterally between phylogenetically distant bacteria. The evidence for the various scenarios of the evolution of bacterial photosynthesis is discussed in the present section.

Originally, it had been proposed (Oparin, 1938; Gest and Schopf, 1983a) that anaerobic, heterotrophic prokaryotes capable of fermenting hexose sugars were among the earliest life forms and that electron transport and photosynthesis evolved as a response to the depletion of organic nutrients from the primordial soup. Based on one hypothesis (the Granick hypothesis; Granick, 1965), the biosynthetic pathway of photosynthesis pigment molecules may be taken as a recapitulation of evolution such that compounds with shorter biosynthetic pathways reflect the more ancestral state. The synthesis of bacteriochlorophyll requires one additional enzymatic reduction than that of chlorophyll. Because chlorophyll precedes bacteriochlorophyll in the biosynthetic pathway, the former should have existed earlier in nature. It has been proposed (Pierson and Olson, 1989) that a non-oxygenic photosynthetic ancestor containing chlorophyll *a* and the two types of reaction centers evolved prior to the major radiation event of the Bacteria. During the subsequent radiation, oxygen evolution appeared in one line of descent whereas either the quinone or the FeS-type photosystem was lost in other lineages, concomitant with the emergence of the different bacteriochlorophylls. Besides avoiding an a priori lateral gene transfer of the complete photosynthetic gene cluster, this Pierson-Olson hypothesis takes into account the ecological conditions of the early biosphere in which the absence of oxygen and ozone caused a predominance of radiation in the blue and UV wavelength range, which in turn would render the red-shifted absorption maxima of bacteriochlorophylls of little selective advantage (Boxer, 1992).

As an argument against the Granick and Pierson-Olson hypotheses, several types of phototrophic bacteria that would be expected are apparently missing in nature. As an example, anoxygenic chlorophyll-containing forms have never been found, although it has been argued that the 8-hydroxychlorophyll-containing *Heliobacteriaceae* represents this type inasmuch as bacteriochlorophyll g is easily converted to chlorophyll *a* by oxidation. Bacteriochlorophylls occur in both types of reaction centers, the pheophytin-type (*Proteobacteria*, *Chloroflexus*) and the FeS-type. This could indicate that the presence of bacteriochlorophyll represents a primitive trait. The chlorophyll-first hypothesis postulates that bacteriochlorophyll has replaced chlorophyll independently in at least three different bacterial lineages. Chlorophyll, however, is presently only found in oxygen-evolving organisms of the phylum *Cyanobacteria* whic h, based on 16S rRNA sequence comparison, represents the most recently evolved group of phototrophic bacteria (Woese, 1987, Fig. 1). *Cyanobacteria* contain two different photosystems and thus have the most complex photosynthetic apparatus. In addition, the much higher complexity of the oxygen-evolving PSII of oxygenic phototrophic organisms may imply that it

appeared later than the other photosystems during evolution.

As another argument against the Pierson-Olson hypothesis, chlorophyll itself should have been of little selective advantage in Earth's early biosphere and it has been proposed that quinone-iron complexes represented the first photosynthetic unit (Boxer, 1992). In contrast to the complex porphyrin pigments, quinones can form spontaneously from acetyl thioesters (Hartmann, 1992). Furthermore, the discrepancy between the presence of chlorophyll exclusively in the most highly evolved bacteria and its shorter biosynthetic pathway may be explained by the finding that the chlorin reductase, which catalyzes the additional step of the biosynthetic pathway for bacteriochlorophyll, is phylogenetically older than the enzyme (protochlorophyllide reductase) that catalyzes the preceeding step. This enzyme is present in both the chlorophyll- and bacteriochlorophyll-containing bacteria (Burke et al., 1993). An ancien t reductase may have been able to perform both, the reduction of protochlorophyllide and of chlorin, such that bacteriochlorophyll was the photochemically active pigment in the last common ancestor of all extant phototrophic bacteria.

An analysis of the distribution of the different types of reaction centers among the different bacterial phyla and the amino acid sequences of reaction center proteins (Blankenship, 1992) provides an alternative hypothesis for the evolution of photosynthesis, namely the possibility of lateral transfer of photosynthesis genes. Both the pheophytin/quinone and the FeS-type reaction centers are found in phylogenetically distant groups (e.g., a pheophytin/quinone reaction center in *Chloroflexus* and phototrophic members of the α-Proteobacteria). Even more significantly, a phylogenetic analysis of the amino acid sequences of pheophytin-type reaction center polypeptides from the three different bacterial lineages *Chloroflexaceae*, *cyanobacteria* and α-Proteobacteria indicated that the reaction center of *Chloroflexus aurantiacus* is more closely related to that of phototrophic members of the α-Proteobacteria than to the PSII reaction center of cyanobacteria (Blankenship, 1992). Thus the reaction center of *Chloroflexus* must have evolved after (and not prior to) the divergence of the D1/D2 branch from the L/M line of descent. Another essential component of the photosynthetic apparatus of *Chloroflexus* and green sulfur bacteria are the light-harvesting chlorosomes. Based on amino acid sequence comparison of protein constituents, chlorosomes of both groups have a common evolutionary origin (Wagner-Huber et al., 1988). Similarly, a comparison of the amino acid sequences of the group 1 σ^{70} primary sigma factor also has demonstrated a close relationship to the green sulfur bacteria with respect to this component of the central housekeeping function (Gruber and Bryant, 1998). Other features of *Chloroflexus aurantiacus* appear to be unique (like the lipid and carotenoid composition), or ancient (like the hydroxypropionate pathway of CO_2-fixation). Recently, the activity of the key enzymes of this pathway have been reported for some archaea (Menendez et al., 1999) such that *Chloroflexus aurantiacus* seems to represent a "chimeric" organism.

Based on the most parsimonious assumption that homodimeric reaction centers are ancestral to homodimeric ones, the reaction centers of green sulfur bacteria and *Heliobacteriaceae* would resemble most the reaction center of the ancestor of all extant bacteria. It has been hypothesized (Gruber and Bryant, 1998) that the reaction center of *Chloroflexus aurantiacus* was acquired by a recent lateral gene transfer event that may have replaced a type I reaction center with a type II (FeS) reaction center, whereas other features like primary sigma factor or chlorosomes still reflect the common descent of *Chloroflexus* and the green sulfur bacteria. Alternatively, it has been suggested that transfer of the genetic information of the relatively simple chlorosomes occurred after the evolution of the two classes of reaction centers and that the green sulfur bacteria represent a relatively modern evolutionary invention (Stackebrandt et al., 1996).

The presence of two homologous polypeptides in all known reaction centers would suggest a single gene duplication event in an early ancestor of all phototrophic bacteria. As an additional result of the phylogenetic analysis of the amino acid sequences of pheophytin-type reaction center polypeptides from the three different bacterial lineages (*Chloroflexaceae*, *cyanobacteria* and α-Proteobacteria; Blankenship, 1992), the most likely occurrence of two independent gene duplications is suggested—one leading to the reaction center of PSII in cyanobacteria and green plants (polypeptides D1 and D2) and another to the reaction center of *Chloroflexus* and purple nonsulfur bacteria (polypeptides L and M). Another, third, independent gene duplication has to be assumed during the evolution of the FeS-type reaction center. The reason for the paraphyletic development of the three lineages may be a functional advan tage of dimeric reaction centers over monomeric ones.

Yet another evolutionary scenario for photosynthetic reaction centers (Vermaas, 1994) has been based on the finding that the sixth membrane-spanning region of the heliobacterial (FeS- or PSI-type) reaction center shows a great similarity to the sixth membrane-spanning

region of the CP47 antenna polypeptide of (the quinone-type) PSII, and the preceeding N-terminal five hydrophobic regions still show significantly greater similarity to CP47 (and to another PSII antenna protein, CP43) than to the respective portion of PSI. According to this model, an ancestral homodimeric antenna/reaction center complex comprised 11 putative transmembrane regions and contained two quinones and an F_x-type Fe_4S_4 iron-sulfur center. Relatively few modifications may have led to the homodimeric complex of green sulfur bacteria and *Heliobacteriaceae*, whereas a gene duplication event and divergent evolution led to the heterodimeric PSI. As a para llel line of descent, splitting of the ancestral reaction center complex into a reaction center and a separate antenna protein may have occurred. Operon duplication, loss of the FeS, and divergent evolution are assumed to have resulted in two separate lineages. By association with an additional water-splitting enzyme system, PSII was formed. In contrast, the separate antenna polypeptide was lost and replaced by a modified antenna complex (light-harvesting I) during evolution of the reaction center of Proteobacteria and *Chloroflexus*. Significantly, however, this theory does not explain the occurrence of the quinone-type reaction center in these latter two groups, which are phylogenetically very distant. In addition, the combination of a reaction center typical for *Proteobacteria* with an antenna structure characteristic for green sulfur bacteria would still need to be explained by lateral gene transfer of either of the two components.

Based on the obvious discrepancy between the phylogeny of ribosomal RNA and reaction center proteins, the hypothesis of lateral transfer of photosynthesis genes between distantly related groups of bacteria has been put forward. Lateral gene transfer as yet seems to provide the simplest explanation for the distribution pattern of photosynthesis genes within the bacterial radiation (Blankenship, 1992; Nagashima et al., 1993; Nagashima et al., 1997). Such a lateral gene transfer would encompass reaction center structural genes, genes coding for other electron transfer proteins, and genes needed for the biosynthesis of pigments and cofactors. In purple nonsulfur bacteria the majority of these genes indeed form a single cluster of 46 kb (which does not encompass the genes for the light-harvesting II complex, however; Bauer and Bird, 1996; Wellington et al., 1992; Yildiz et al., 1992). The genetic organization may be taken as evidence for lateral gene transfer as the cluster represents only ~1.3% of the total genome size. It should be mentioned, however, that clustering of most photosynthesis genes may also be due to structural or regulatory constraints. Supporting the latter argument (Yildiz et al., 1992), photosynthesis genes in α-Proteobacteria are transcriptionally coupled in superoperons involving overlapping transcripts. The particular genetic organization is the prerequisite for adaptation of the cells to changing light intensity (see Genetic Regulation in Response to Light in this Chapter) and oxygen tension (see Genetic Regulation by O_2 in this Chapter). Therefore a selective pressure may exist to retain the linkage order and would make the genetic organization of the photosynthesis genes less suitable for phylogenetic inference. Furthe rmore, the high correlation between the phylogenetic trees for 16S rRNA and cytochrome c in phototrophic members of the α-Proteobacteria has been taken as evidence that a lateral transfer of photosynthesis genes did not occur at least within this phylogenetic group (Woese et al., 1980). Thus, the presence of reaction centers in aerobic bacteriochlorophyll-containing α-Proteobacteria may represent an atavistic trait, and the genes coding the reaction center might have been lost frequently during the evolution of aerobic representatives in this group (Stackebrandt et al., 1996).

Because the pigment composition of the oxygenic photosynthetic "Prochlorophytes" is very similar to that of green plant chloroplasts, and like the latter "Prochlorophytes" have appressed thylakoid membranes, it has been proposed that the chloroplasts of green plants evolved from an endosymbiotic "prochlorophyte" (van Valen and Maiorana, 1980; Lewin, 1981). In contrast to the other oxygenic phototrophs, *Prochlorococcus* contains divinyl isomers of chlorophylls a and b, and α- instead of β-carotene (Chrisholm et al., 1992; Goericke and Repeta, 1992). However, based on sequence comparison of 16S rRNA (Urbach et al., 1992) and the *rpoC1* (Palenik and Haselkorn, 1992) genes, the three known prochlorophyte lineages (*Prochloron*, *Prochlorothrix*, and *Prochlorococcus*) are no direct ancestors of chloroplasts. In addition, these analyses revealed that "Prochlorophytes" most likely are of polyphyletic origin and that the use of chlorophyll *b* as additional light-harvesting pigments must have developed at least four times during evolution. In this case, too, a horizontal transfer of the respective biosynthesis genes could be invoked to explain the distribution pattern of chlorophyll *b* among the different members of the cyanobacterial division (Palenik and Haselkorn, 1992). Immunological studies and differences in the chlorophyll a/chlorophyll *b* ratio of the antennae isolated from different "Prochlorophytes" indicate that the capacity to bind chlorophyll *b* arose several times and independently from the cyanobacterial ancestors, and thus confirm the results of s equence comparisons of the *16S rRNA* and *rpoC1* genes.

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Prokaryotes (2006) 2:86–101
DOI: 10.1007/0-387-30742-7_4

CHAPTER 1.4

The Anaerobic Way of Life

RUTH A. SCHMITZ, ROLF DANIEL, UWE DEPPENMEIER AND GERHARD GOTTSCHALK

Introduction

Molecular oxygen in appreciable amounts is found only in those areas on earth that are in direct contact with air or are inhabited by organisms carrying out oxygenic photosynthesis. The solubility of oxygen in water is low. In equilibrium with air at 1.013 bar and at 20°C, pure water will contain approximately 9 mg/liter of dissolved oxygen. In aqueous systems, aerobic organisms rapidly consume dissolved oxygen, so that deeper layers of many waters and soils (especially if they are rich in organic compounds), as well as mud and sludge, are practically anaerobic. Nevertheless, these areas are inhabited by numerous organisms that fulfill the important ecological role of converting insoluble organic material to soluble compounds and gases that can circulate back into aerobic regions. Other important anaerobic habitats are the rumen, the intestinal tract, and man-made anaerobic digestors of sewage treatment plants.

Anaerobic prokaryotes that can live in the above-mentioned environments are either phototrophs, which, of course, can only flourish if light is available, or chemotrophs. With respect to their relationship to aerobic metabolism, three groups of organisms capable of growth in an anaerobic environment can be identified:

1. Organisms that are aerobes but can use alternate electron acceptors such as nitrate or nitrite when exposed to an anaerobic environment. The electron transport from NADH to these acceptors is coupled to the phosphorylation of ADP, as is the electron transport to oxygen.
2. Organisms that are facultative aerobes. The enterobacteria are the most prominent representatives of this group. These organisms grow as typical aerobes in the presence of oxygen; in its absence, they carry out fermentations.
3. Obligately anaerobic bacteria that are characterized by the inability to synthesize a respiratory chain with oxygen as terminal electron acceptor. They are restricted to life without oxygen.

The diversity of microorganisms able to thrive under anaerobic conditions is overwhelming. Up to now more than 200 genera of obligate anaerobic microorganisms have been described. Obligate anaerobes are found in all three domains. The eukaryotes are represented by anaerobic fungi, ciliates and flagellates, the archaea by the methanogens, which comprise 23 genera, and by the most hyperthermophilic genera *Pyrolobus*, *Pyrodictium* and *Pyrococcus*. Most genera of the obligate anaerobes belong to the bacteria. Especially prominent are the 32 genera characterized by their ability of dissimilatory reduction of sulfate, sulfite or sulfur. Spore formers are well represented, e.g., by the genera *Clostridium*, *Sporomusa*, *Desulfotomaculum*, *Moorella* and *Thermoanaerobacterium*. There are halophiles such as the genera *Haloanaerobacter* and *Sporohalobacter* and alkaliphiles like *Anaerobranca*. A few genera comprise more than a dozen species: *Bacteroides*, *Bifidobacterium* and *Clostridium* (the genus which by far contains the most species), *Desulfotomaculum*, *Desulfovibrio*, *Eubacterium* and *Thermococcus*. Quite a few genera are represented just by one species, e.g., *Acetitomaculum*, *Acetonema*, *Chrysiogenes*, *Desulfobacula*, *Hippea*, *Stetteria* and *Succinispira*. Autotrophic CO_2-fixation is widespread among the acetogenic anaerobes such as *Acetobacterium woodii*, *Clostridium aceticum* and *Moorella thermoautotrophica* and especially among the methanogens of which only a few representatives are unable to grow with CO_2 plus H_2, e.g., *Methanosaeta concilii*, *Methanosarcina acetivorans* and the *Methanosphaera* species. A few sulfate-reducing bacteria utilize CO_2, such as *Desulfobacterium autotrophicum* and *Desulfosarcina variabilis*. The ability to fix molecular nitrogen is probably more common among anaerobes than known at the moment. Several clostridia are able to do so, with *Clostridium pasteurianum* being the first species demonstrated to have nitrogenase activity. Methanogens express active nitrogenase under nitrogen-limited growth conditions as has been demonstrated for *Methanosarcina barkeri*, *Methanosarcina mazei* and *Methanococ-*

Table 1. Reactions yielding ATP by substrate-level phosphorylation in anaerobes.

Reaction	Enzyme	ΔG_{abs}^0 (kJ/mole)
1,3-Bisphosphoglycerate + ADP ⇔ 3-phosphoglycerate + ATP	Phosphoglycerate kinase	–24.1
Phosphoenolpyruvate + ADP ⇔ pyruvate + ATP	Pyruvate kinase	–23.7
Acetyl phosphate + ADP ⇔ acetate + ATP	Acetate kinase	–12.9
Butyryl phosphate + ADP ⇔ butyrate + ATP	Butyrate kinase	–12.9
Carbamoyl phosphate + ADP ⇔ carbamate + ATP	Carbamate kinase	–7.5
N^{10}-Formyl FH_4[a] + ADP + P_i ⇔ formate + FH_4 + ATP	Formyl-FH_4 synthetase	+8.32
Glycine + 2H + ADP + P_i ⇔ acetate + NH_3 + ATP	Glycine reductase	about –46.0

[a]FH_4, tetrahydrofolic acid.

cus maripaludis. Many more anaerobes can be expected to do so.

So obligate anaerobes are known now for all-important anaerobic habitats on earth. Because of their inability to utilize oxygen, they had to develop their strategies to conserve energy in the form of ATP, to metabolize substrates and to cope with some of their own products such as ethanol, lactate, butyrate or acetate. Some of the characteristic features of the anaerobes will be outlined.

Novel Ion Translocation Reactions Involved in Energy Conservation

It is a fact that several anaerobic microorganisms produce ATP only by substrate-level phosphorylation. Growth on sugars or on amino acids coupled to the formation of ethanol, lactate, butyrate or acetate very often indicates that substrate-level phosphorylation is involved (Thauer et al., 1977). This holds true for lactic acid bacteria and also for many clostridia. Some of the reactions employed for ATP synthesis by these bacteria and by other anaerobes are listed in Table 1. It can be seen that the reactions 1 to 4 listed in Table 1 are part of the glycolytic pathway of acetate and butyrate formation. Carbamoyl phosphate is formed in the conversion of arginine to ornithine, and thereby becomes available for ATP synthesis. The conversion of N^{10}-formyl FH_4 (N^{10}-formyl tetrahydrofolic acid; an intermediate of methyl group oxidation) to formate, and FH_4 gives rise to ATP synthesis. Glycine reductase is involved in the reductive part of the Stickland reaction, the pairwise fermentation of amino acids. This interesting reaction will be discussed in detail below.

There are fermentations in which at first sight reactions giving rise to ATP synthesis cannot be identified. Such processes are for instance hydrogen-dependent fermentations; some are summarized in Table 2. Here it has been assumed for quite some time that electron transport processes might be coupled to ion translocation and that the ion-motive force generated might support ATP synthesis. Experimental proof for this assumption has been provided in recent years.

Wolinella succinogenes grows on fumarate and H_2 according to the equation given in Table 2. Clearly, this organism must gain ATP by electron transport phosphorylation. The electron transport chain that catalyzes this reaction (Fig. 1B) consists of hydrogenase, menaquinone and fumarate reductase (Lancaster and Kröger, 2000). Using vesicles and reconstituted liposomal systems the generation of a proton-motive force could be demonstrated in the course of H_2-dependent menaquione reduction as catalyzed by the hydrogenase (Gross et al., 1998). A number of other bacteria also can take advantage of ion-translocating electron transport system using fumarate as a terminal electron acceptor (Kröger et al., 1992). Formate, NADH or H_2 are typical electron donors, and succinate or propionate are formed as catabolic end products.

The pathway (as employed by the methanogens) for CO_2-reduction to methane by H_2 is depicted in Fig. 2. It has been demonstrated in recent years that one reaction, the methyl group transfer from methyltetrahydromethanopterin to coenzyme M, is coupled to the translocation of sodium ions (Deppenmeier et al., 1996). This

Table 2. H_2-dependent fermentations.

Reaction			Change of free energy
Fumarate + H_2	→	succinate	$\Delta G^{o\prime} = -86$ kJ/mol
$CO_2 + 4\ H_2$	→	$CH_4 + 2\ H_2O$	$\Delta G^{o\prime} = -131$ kJ/mol
$2\ CO_2 + 4\ H_2$	→	$CH_3{\cdot}COO^- + H^+ + 2\ H_2O$	$\Delta G^{o\prime} = -95$ kJ/mol
$SO_4^{2-} + 4\ H_2 + H^+$	→	$HS^- + 4\ H_2O$	$\Delta G^{o\prime} = -152$ kJ/mol
2 FeOOH + H_2 + 4 H^+	→	2 Fe^{2+} + 4 H_2O	$\Delta G^{o\prime} = -110$ kJ/mol

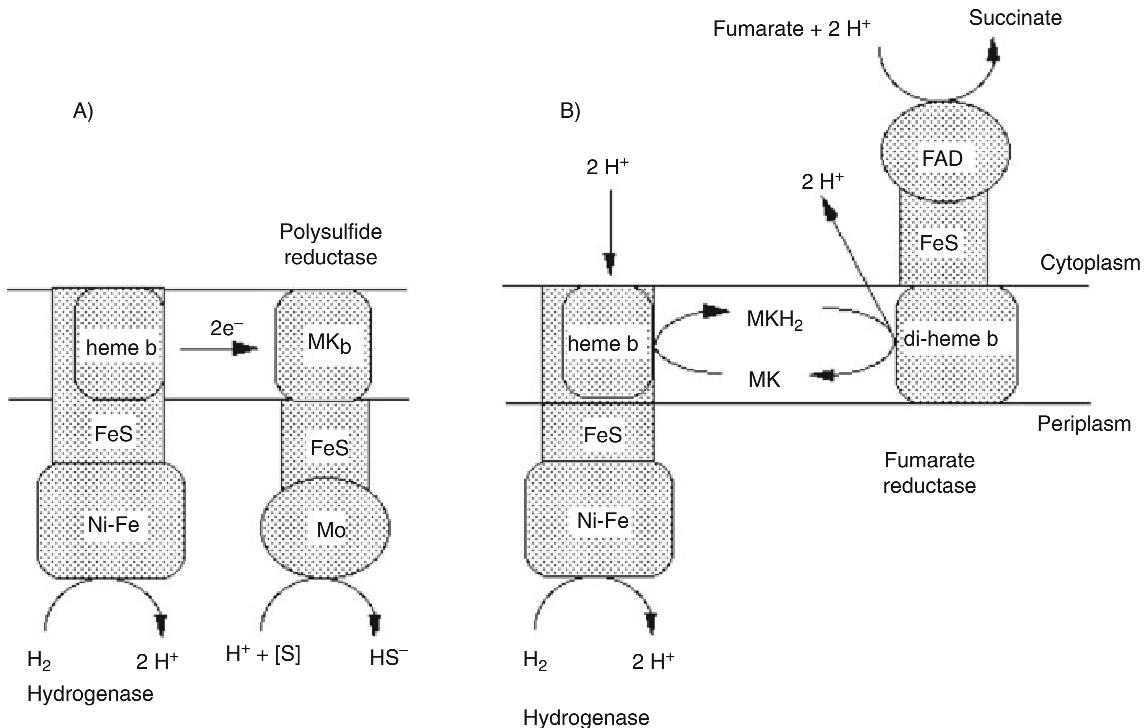
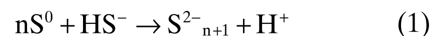


Fig. 1. Anaerobic respiration in *Wolinella succinogenes*. A) Polysulfide reduction: The membrane-bound hydrogenase is composed of three subunits (HydABC) and contains heme *b*, iron-sulfur clusters (FeS) and the nickel/iron center (Ni-Fe) for hydrogen oxidation. The gene products PsrA, B and C form the polysulfide reductase which contains a molybdopterin guanine dinucleotide (Mo), iron sulfur clusters (FeS). A menaquinone (Mkb) is tightly bound to the protein. Electron transfer is probably mediated by diffusion and collision of the enzymes. B) Fumarate reduction: The hydrogenase is identical to the one shown in Fig. 1A. The fumarate reductase consists of three subunits (*frdCAB*). A diheme cytochrome *b* anchors the enzyme in the membrane (di-heme *b*). The catalytic subunit carries a covalently bound FAD. These subunits are connected by an iron-sulfur protein (FeS). Electron transfer from the hydrogenase to the fumarate reductase is mediated by menaquinone.

system represents a novel type of sodium ion pump, which will be discussed below in connection with other sodium ion pumps. Some methanogens (e.g., *Methanosarcina* spp.) employ two novel membrane-bound electron transport systems generating an electrochemical proton gradient. The systems are composed of the heterodisulfide reductase and either a membrane-bound hydrogenase or an $F_{420}H_2$ dehydrogenase (Bäumer et al., 2000), which is functionally homologous to the proton-translocating NADH dehydrogenase (complex I of the respiratory chain). It has been shown that all of these enzymes are involved in proton translocation. Interestingly, the electron transport systems of these organisms contain electron carriers (such as cytochromes and the novel redox carrier methanophenazine), not found in methanogens utilizing only H_2 + CO_2 (Deppenmeier et al., 1999).

A number of archaea as well as of bacteria reduce elemental sulfur with H_2 to H_2S (Hedderich et al., 1999). Examples are *Pyrodictium occultum*, *Stetteria hydrogenophila* and *Desulfurobacterium thermolithotrophum*, but also the already mentioned *Wolinella succinogenes* in which a H_2:polysulfide reductase was characterized consisting of a nickel-iron hydrogenase, menaquinone and a molybdenum iron sulfide-containing polysulfide reductase (Fig. 1A). Because the solubility of elemental sulfur in water is extremely low, it is believed that polysulfide is the actual electron acceptor (Hedderich et al., 1999). It is formed in an H_2S environment according to:



$$nS^0 + HS^- \rightarrow S^{2-}{}_{n+1} + H^+ \qquad (1)$$

Proton gradients are also established in the process of dissimilatory sulfate reduction. Here, the electron transfer from H_2 to sulfite is coupled to ATP synthesis via a chemiosmotic mechanism (Badziong and Thauer, 1980). Shewanella putrefaciens (not an obligate anaerobe) can grow with Fe^{3+} and H_2. The mode of energy conservation is not known as yet.

Diffusion gradients may also be exploited for the generation of a proton-motive force. As long as the intercellular lactate concentration is high

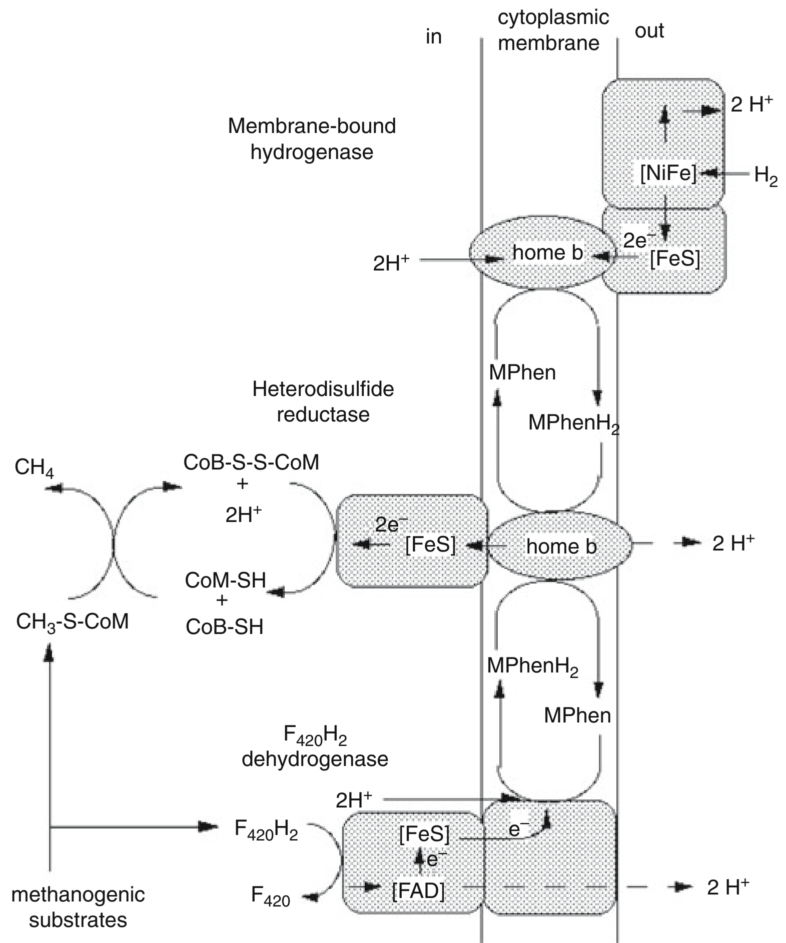
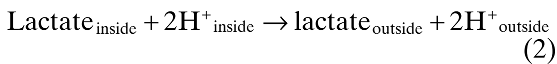


Fig. 2. Membrane-bound electron transport chain in *Methanosarcina mazei*. In the course of methanogenesis, methyl-coenzyme M (CH3-S-CoM) is formed and is reductively cleaved by the methyl-CoM reductase which uses coenzyme B (HS-CoB) as electron donor. The reaction results in the formation of methane and a heterodisulfide (CoB-S-S-CoM) from HS-CoM and HS-CoB. The disulfide functions as electron acceptor of the anaerobic respiratory chain. Molecular hydrogen or reduced coenzyme F_{420} ($F_{420}H_2$) serves as electron donors. The $F_{420}H_2$ dehydrogenase contains FAD and FeS clusters and is responsible for the oxidation of $F_{420}H_2$. Electrons are transferred to methanophenazine (MPhen). The reduced form of this novel cofactor is the electron donor of the heterodisulfide reductase. This enzyme contains heme b and iron-sulfur clusters. It catalyzes the reduction of CoM-S-S-CoB. The H_2-dependent electron transport system is composed of a membrane-bound hydrogenase which is very similar to the corresponding enzyme from *Wolinella* (Fig. 1). Methanophenazine functions as mediator of electron transport to the heterodisulfide reductase.

as compared to the extracellular one, it can be exported accompanied by two protons:



$$\text{Lactate}_{\text{inside}} + 2\text{H}^+{}_{\text{inside}} \rightarrow \text{lactate}_{\text{outside}} + 2\text{H}^+{}_{\text{outside}} \quad (2)$$

Thus, the proton/product symport helps lactate acid bacteria to increase their ATP yield (Konings et al., 1997).

Sodium Ion Pumps

Cells have the tendency to expel sodium ions from the interior. Usually expulsion is catalyzed by sodium-proton antiporters, but a number of obligately anaerobic microorganisms have primary sodium ion pumps at their disposal. In these organisms certain exergonic reactions are coupled with Na^+-translocation across the cytoplasmic membrane. One example was given already: the methyltetramethanopterin:coenzyme M methyltransferase reaction which is present in all methanogens and which is responsible for the Na^+-dependence of growth and methane formation of this group of archaea. This enzyme system is an extremely complex one consisting of eight different subunits and containing B_{12} as cofactor (Gottschalk and Thauer, 2001). A related enzyme system may occur in *Acetobacterium woodii* and related organisms that are Na^+-dependent and generate a sodium ion-motive force during acetogenesis (Heise et al., 1989). This, however, is not true for all acetogens. Organisms such as *Clostridium aceticum* and *Moorella thermoautotrophica* are not Na^+ dependent; they contain cytochromes and apparently generate a proton gradient instead of a sodium ion gradient (Hugenholtz and Ljungdahl, 1990).

Certain decarboxylases have been found to function as primary Na^+ pumps. They are membrane bound and they contain biotin. These enzymes occur in organisms such as *Propionigenium modestum*, *Acidaminococcus fermentans* or *Klebsiella pneumoniae*, and the acids are decarboxylated with Na^+ extrusion are oxaloacetate, methylmalonyl-CoA, glutaconyl-CoA or malonyl-acyl carrier protein (malonyl-ACP; Dimroth, 1997; Dimroth and Schink, 1998). A scheme is depicted in Fig. 3.

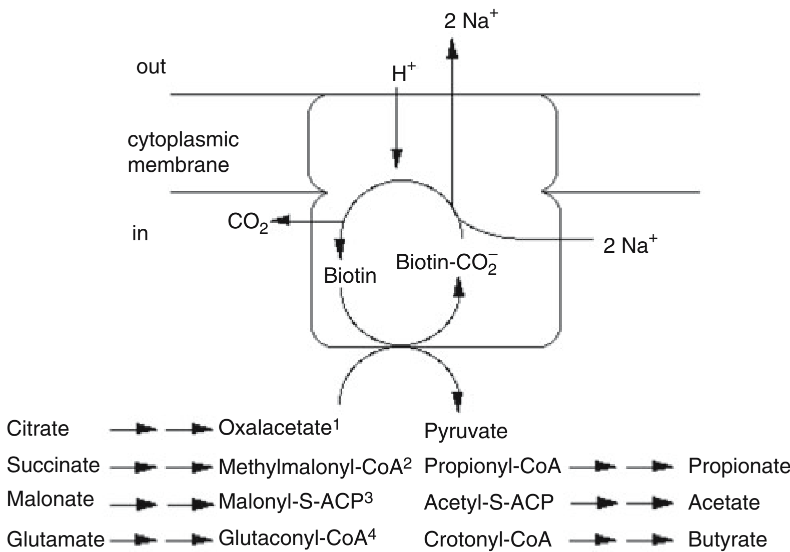


Fig. 3. Decarboxylation reactions coupled to sodium ion translocation: 1) Oxaloacetate decarboxylase (e.g., is used by *Klebsiella pneumoniae* to ferment citrate); 2) Methylmalonyl-coenzyme A (CoA) decarboxylase (e.g., is used by *Propionigenium modestum* for succinate metabolism); 3) Malonyl-S-acyl carrier protein (ACP) decarboxylase (e.g., is used by *Malonomonas rubra* growing on malonate); and 4) Glutaconyl-CoA decarboxylase (e.g., is used by *Acidaminococcus fermentans* to ferment glutamate).

Degradative Pathways

With respect to the degradation of substrates, the anaerobes have disadvantages and advantages. One difficulty is that in the absence of an external electron acceptor anaerobes must balance their oxidation and reduction reactions. The electron donors and acceptors are derived from organic molecules of medium redox states such as sugars, organic acids, heterocyclic compounds and amino acids. Often more reduced (e.g., ethanol) and more oxidized (e.g., CO_2) products are formed. In a few fermentations, the redox state of the substrate and the product is the same, e.g., the fermentation of hexoses to two lactates or three acetates. Highly oxidized or reduced compounds such as carbon dioxide or hydrocarbons, respectively, are only suitable for fermentation together with inorganic electron donors or acceptors.

Another disadvantage of anaerobes is, of course, that oxygen cannot be employed for the initial attack of certain substrates such as hydrocarbons. On the other hand, there are a number of advantages. Oxygen-sensitive systems can be taken advantage of radical reactions or even of radical enzymes. So under the dictate of balanced redox reactions and with the involvement of unique enzymes and reactions, a fascinating array of unusual fermentations has evolved; some will be discussed now.

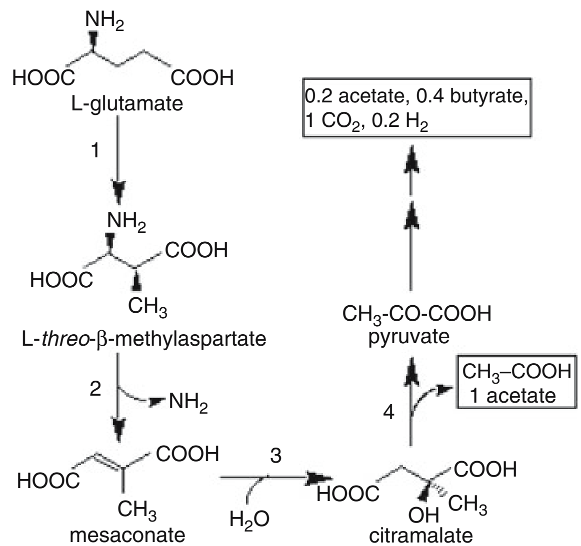


Fig. 4. Pathway of L-glutamate fermentation by *Clostridium tetanomorphum*: 1) Glutamate mutase (coenzyme B_{12}-dependent); 2) β-Methylaspartase; 3) Citramalate dehydratase; and 4) Citramalate lyase.

Coenzyme B_{12}-Dependent Pathways

When *Clostridium tetanomorphum* or *Clostridium cochlearium* grows on L-glutamate, the substrate is prepared for a cleavage into a two-carbon and a three-carbon compound in an interesting way. Under the catalysis of glutamate mutase (a B_{12}-containing enzyme), L-glutamate is converted to L-*threo*-β-methylaspartate (Buckel and Golding, 1996b). This carbon-skeleton rearrangement facilitates the elimination of ammonia and formation of mesaconate by β-methylaspartase. Subsequently, mesaconate is hydrated to citramalate, which then is cleaved into acetate and pyruvate (Buckel, 1980; Fig. 4). Oxidative decarboxylation of pyruvate results in

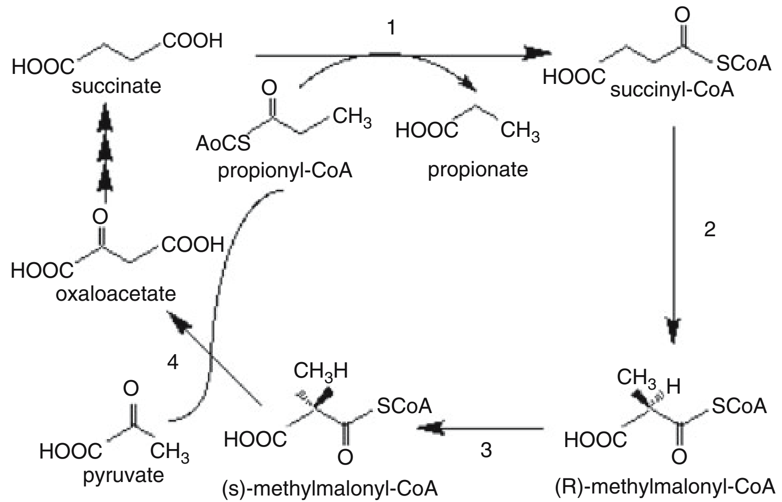


Fig. 5. Interconversion of succinate and propionate by methylmalonyl-CoA mutase: 1) Propionate CoA-transferase; 2) Methylmalonyl-CoA mutase (coenzyme B_{12}-dependent); 3) Methylmalonyl-CoA epimerase; and 4) Transcarboxylase (biotin-containing).

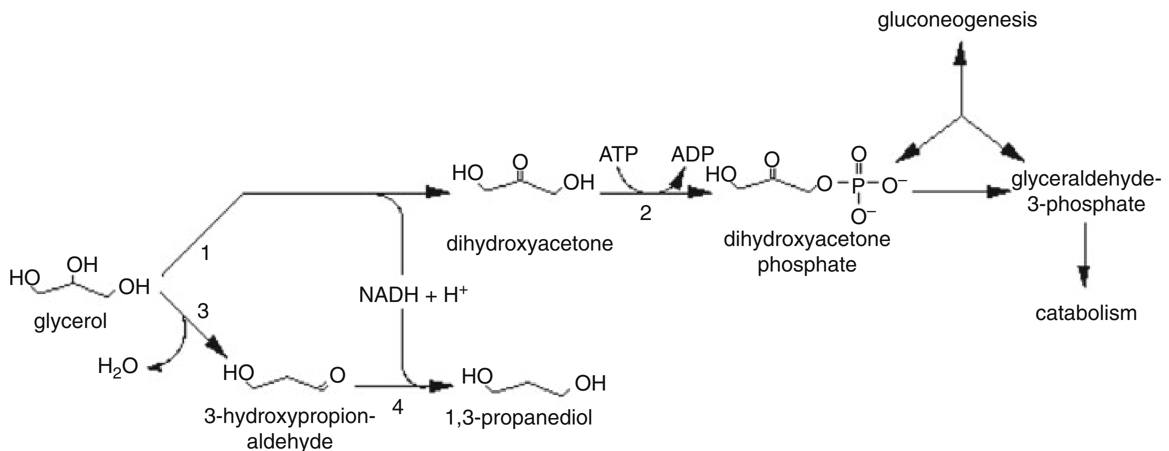


Fig. 6. Pathway of glycerol fermentation by *Citrobacter freundii*: 1) Glycerol dehydrogenase; 2) Dihydroxyacetone kinase; 3) Glycerol dehydratase (coenzyme B_{12}-dependent); and 4) 1,3-Propanediol dehydrogenase.

the formation of acetyl-CoA and reduced ferredoxin, which is reoxidized during the synthesis of butyryl-CoA from two moles of acetyl-CoA. Then, ATP is synthesized in the acetate and butyrate kinase reactions (Barker, 1981). By this pathway, a degradation of glutamate via the tricarboxylic acid cycle is circumvented; the latter would not be feasible because of an unbalanced generation of reducing equivalents in the form of NADH and $FADH_2$.

Coenzyme $B1_2$-dependent rearrangements like the glutamate mutase reaction proceed via radical intermediates; they are per se oxygen sensitive although another reaction of this type, the methylmalonyl-CoA mutase reaction, proceeds in higher eukaryotes such as man. This reaction is also of key importance in propionic acid bacteria and many other anaerobes because it allows the interconversion of succinate and propionate (Fig. 5).

A fermentation that involves a coenzyme B_{12}-dependent reaction and proceeds only under anaerobic conditions is the glycerol conversion to 1,3-propanediol. This fermentation was discovered in enteric bacteria such as *Citrobacter freundii* and *Klebsiella pneumoniae*; it proceeds as depicted in Fig. 6. Glycerol is oxidized to dihydroxyacetone, which is converted further to dihydroxyacetone phosphate. To balance the fermentation, a portion of glycerol is dehydrated to 3-hydroxypropionaldehyde in a coenzyme B_{12}-dependent reaction. Subsequently, the aldehyde is reduced to the major fermentation product 1,3-propanediol, which is of great biotechnological interest. The bottleneck of the pathway is the coenzyme B_{12}-dependent glycerol dehydratase

that is rapidly inactivated during glycerol dehydration (Daniel et al., 1998).

Degradation of Amino Acids and α-Hydroxy Carboxylic Acids

Novel reactions occur in a number of anaerobes for the utilization of α-amino acids and α-hydroxy carboxylic acids. If redox balance allows, these acids can be oxidized, of course, to the corresponding α-keto acids and then very easily metabolized further. So lactate or alanine can be oxidized to pyruvate and further to acetyl-CoA. This often is not possible because an acceptor for the electrons generated is not available. A commonly used pathway involves the reduction of the α-keto acids generated by deamination of amino acids to the corresponding hydroxy carboxylic acids, followed by activation to the CoA ester and dehydration to an enoyl-CoA (Fig. 7). A simple dehydration of α-hydroxy carboxylic acids is not feasible because it would have to proceed against the rule of Markovnikov (Jones, 1961). A well-studied example is the dehydration of α-hydroxyglutaryl-CoA to glutaconyl-CoA carried out by *Acidaminococcus fermentans*. The enzyme, α-hydroxyglutaryl-CoA dehydratase, is extremely oxygen sensitive and contains [Fe-S] clusters, reduced riboflavin, and $FMNH_2$. The activation of the dehydratase is catalyzed by an activator protein and requires a reducing agent and catalytic amounts of ATP and Mg^{2+}. A novel mechanism involving thiol ester-derived radical anions (ketyls) has been postulated for these dehydrations (Buckel, 1996a).

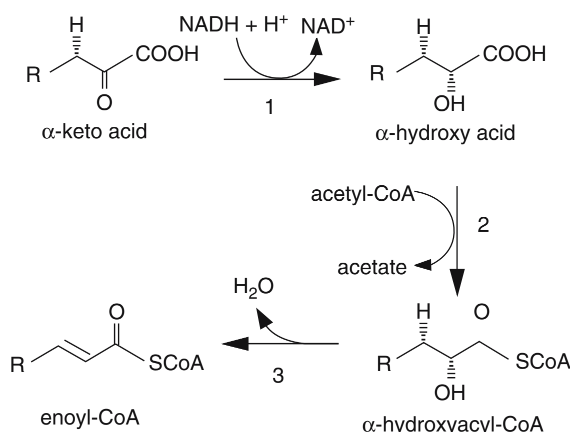
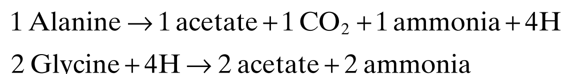


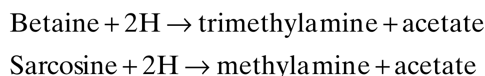
Fig. 7. α-Hydroxy acid pathway: 1) α-Hydroxy acid dehydrogenase; 2) CoA transferase; and 3) α-Hydroxyacyl-CoA dehydratase.

Another way to deal with certain α-amino acids is reductive deamination. Such deaminations are part of the Stickland reaction in which amino acids are fermented pairwise. Alanine, for instance, is oxidized and the reducing equivalents generated are transferred to glycine:



$$1\ \text{Alanine} \rightarrow 1\ \text{acetate} + 1\ CO_2 + 1\ \text{ammonia} + 4H$$
$$2\ \text{Glycine} + 4H \rightarrow 2\ \text{acetate} + 2\ \text{ammonia}$$

The structurally related compounds betaine and sarcosine can also serve as hydrogen acceptors (Naumann et al., 1983; Hormann and Andreesen, 1989), methylamines being formed instead of ammonia:



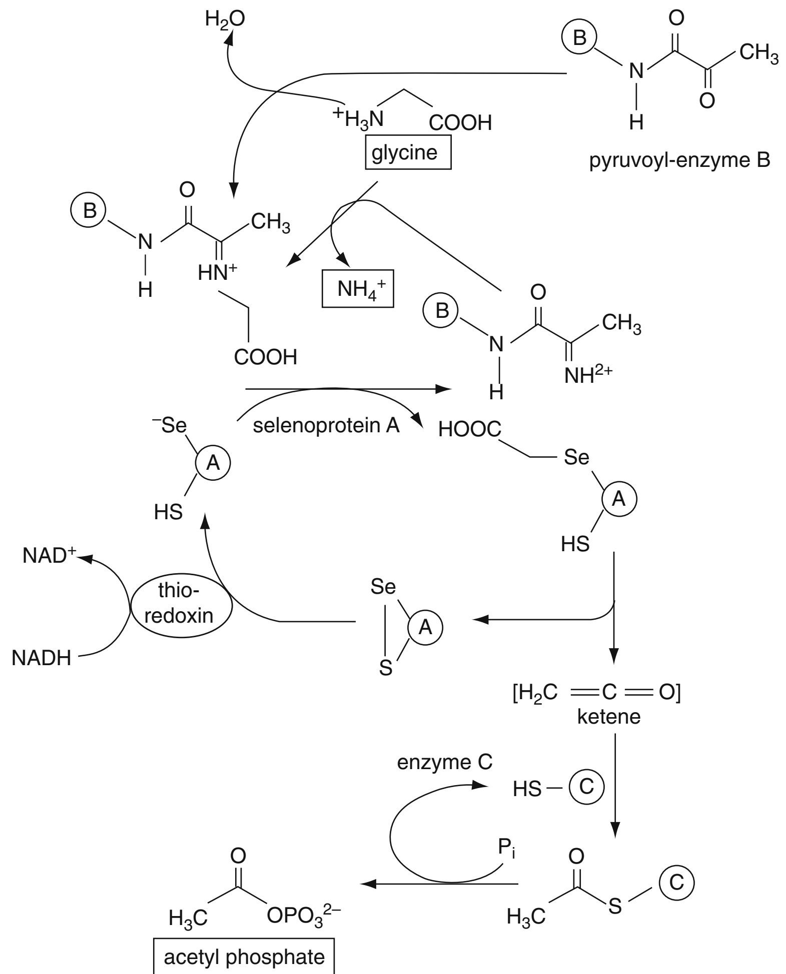
$$\text{Betaine} + 2H \rightarrow \text{trimethylamine} + \text{acetate}$$
$$\text{Sarcosine} + 2H \rightarrow \text{methylamine} + \text{acetate}$$

Acetate formation from glycine proceeds via acetyl phosphate, and the last step of acetate formation is catalyzed by acetate kinase giving rise to ATP synthesis by substrate-level phosphorylation. The key enzyme of glycine fermentation (glycine reductase) was well studied in *Eubacterium acidaminophilum* (Andreesen, 1994). The enzyme consists of four proteins including one selenoprotein (enzyme A), a pyruvoyl-protein (enzyme B), enzyme C, and thioredoxin. The reaction mechanism is depicted in Fig. 8. The pyruvoyl residue of enzyme B forms a Schiff-base with glycine, which then reacts with the Se^--anion of protein A to yield a carboxymethylselenocysteine residue linked to protein A and the iminopyruvoyl protein. Subsequently, ammonia is released by hydrolysis or in the next turnover. Elimination of ketene yields the oxidized protein A-Se-S intermediate, which is reduced by thioredoxin. Reduction of thioredoxin is catalyzed by thioredoxin reductase with NADH or another electron donor. The hypothetical ketene intermediate adds to the cysteine residue of protein C. An acetylcysteine is formed, which is cleaved by phosphate (P_i) to form acetyl phosphate. Again, this is a complex reaction, which only can be visualized to occur in anaerobes.

Degradation of Aromatic Compounds and Hydrocarbons

Most of the aromatic compounds studied to date are first transformed to benzoyl-CoA, the central intermediate of the best-studied pathway for anaerobic degradation of aromatic compounds (Harwood et al., 1999). Benzoyl-CoA then

Fig. 8. Mechanism of glycine reductase.



undergoes a reductive attack (Schink et al., 2000). The key enzyme for this attack is the benzoyl-CoA reductase, which was purified from the denitrifying bacterium *Thauera aromatica* and characterized as a FAD- and iron-sulfur cluster-containing enzyme complex (Boll and Fuchs, 1995). Under hydrolysis of ATP, one electron is added to the thiol ester carbonyl of benzoyl-CoA and the resulting radical intermediate is reduced further to cyclohexa-1,5-dienecarboxyl-CoA (Buckel and Golding, 1999; Fig. 9). This reaction may be of general importance for the anaerobic degradation of aromatic compounds. Recently, it was shown that the reductive strategy for destabilization of the ring is not the only one used in anaerobic degradation of aromatic compounds. Anaerobic degradation of 3,5-dihydroxybenzoate by *Thauera aromatica* (Philipp and Schink, 2000) and 1,3-dihydroxybenzene by *Azoarcus anaerobius* (Philipp and Schink, 1998) proceeds by a novel mechanism. Phenolic compounds with their hydroxyl groups in *meta* position to each other are hydroxylated by membrane-bound enzymes yielding hydroxyhydroquinone, which is later dehydrogenated to the nonaromatic compound hydroxybenzoquinone. Thus, oxidation rather than reduction is used to overcome the stability of the aromatic ring.

Radical Enzymes

Glycyl radical enzymes are involved in a number of anaerobic reactions. Well-studied examples are the pyruvate formate lyase (Knappe et al., 1984), the anaerobic ribonucleotide reductase (Licht et al., 1996), and the benzyl succinate synthase (Leuthner et al., 1998). The latter initiates the breakdown of toluene under anaerobic conditions. These glycyl radical enzymes are formed from their precursor enzyme in a reaction, which requires S-adenosyl methionine. The pyruvate formate lyase of *Escherichia coli* is synthesized

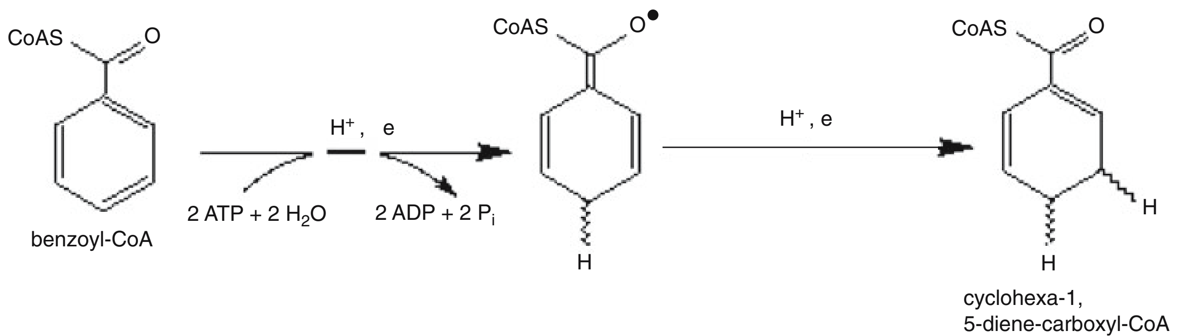
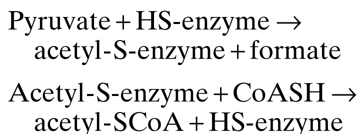


Fig. 9. Mechanism of benzoyl-CoA reductase.

as an inactive and coenzyme-free protein. The enzyme is posttranslationally modified by S-adenosyl methionine and a reduced flavodoxin in a reaction catalyzed by an activase. A hydrogen atom is abstracted from a specific glycine residue, yielding methionine and 5′-deoxyadenosine from S-adenosyl methionine. The formed free radical (HS-enzyme) is involved in a two-step reaction:



$$\text{Pyruvate} + \text{HS-enzyme} \rightarrow \text{acetyl-S-enzyme} + \text{formate}$$

$$\text{Acetyl-S-enzyme} + \text{CoASH} \rightarrow \text{acetyl-SCoA} + \text{HS-enzyme}$$

Pyruvate formate lyase, like the other glycyl radical enzymes, is rapidly inactivated by oxygen.

Anaerobic alkane-degrading bacteria have also been isolated recently. Alkanes are used as substrates by several species of sulfate-reducing microorganisms (Aeckersberg et al., 1998). Another group of anaerobic hydrocarbon-degrading bacteria is dependent on syntrophic associations with methanogens. The biochemistry of the process is still poorly understood but it can be speculated that again radicals are generated to initiate this breakdown (Zengler et al., 1999).

A number of potentially hazardous compounds in our environment are halogenated (e.g., pentachlorophenol or perchloroethene). These compounds can be partially or completely degraded under anaerobic conditions. This degradation occurs by reductive dehalogenations. Organisms such as *Desfulfitobacterium dehalogenans*, *Dehalobacter restrictus* or *Dehalospirillum multivorans* contain corrinoid-proteins, which exhibit dehalogenase activities (Holliger et al., 1999). There is evidence that these H_2-dependent fermentations are also coupled with the generation of a proton-motive force.

Anaerobic Food Chains

The anaerobic degradation of complex organic matter depends on the cooperation of various trophic groups of anaerobic bacteria and archaea. Two possible schemes for anaerobic food chains, as they occur in nature in the absence or in the presence of sulfate, are presented in Fig. 10. Polymers such as polysaccharides, proteins and nucleic acids are initially converted to oligomers and monomers and subsequently fermented by the "classical" primary fermentative bacteria. In the absence of sulfate, the products acetate, methanol, methylamines, CO_2 and H_2 can be used directly by methanogenic bacteria to convert them to methane and carbon dioxide. Alcohols longer than one carbon atom, fatty acids longer than two carbon atoms and branched or aromatic fatty acids are degraded by the secondary fermenters to acetate, C1-compounds and H_2, which are subsequently used by the methanogens. Because the reactions catalyzed by the secondary fermentative bacteria are mostly endergonic under standard conditions, they depend on a very efficient cooperation with the subsequent partners. Such cooperations are called syntrophic relationships, in which the pool size of shuffling intermediate has to be kept small to allow efficient degradation. In sulfate-rich anaerobic habitats, such as marine sediments, sulfate-reducing bacteria further degrade the primary fermentation products. As many sulfate reducers are metabolically more versatile than methanogenic bacteria, they can use and oxidize all classical fermentation products to carbon dioxide, simultaneously reducing sulfate to sulfide (Hansen, 1994; Jansen and Hansen, 1998; Zengler et al., 1999; Fig. 10B). In addition to the primary fermentations that have already been mentioned, three important points should be briefly discussed here: the fate of acetate under anaerobic conditions, production of H_2, and the syntrophic relationships.

Acetate is the end product of a number of fermentations starting from substrates with two (e.g., ethanol) or more carbon atoms (e.g., glucose), but it is also produced by acetogenic organisms from one-carbon compounds (e.g.,

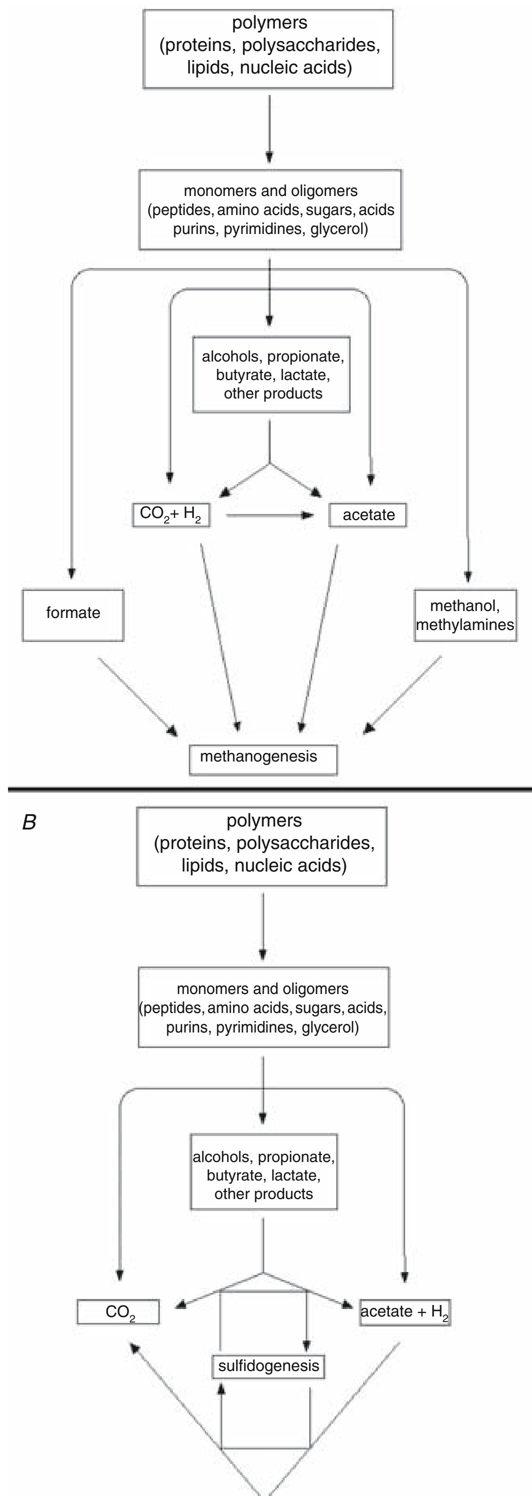
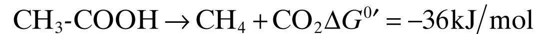


Fig. 10. Anaerobic food chains. (A) Methanogenesis. As a terminal process, all organic material is metabolized to methane via a few methanogenic substrates: CO_2 + H_2, acetate, formate, methanol and methylamines. (B) Sulfidogenesis. As a terminal process, incomplete oxidizers convert various products to CO_2 and acetate, and the complete oxidizers couple sulfate reduction with acetate oxidation to CO_2. In addition, H_2 can be used for sulfate reduction.

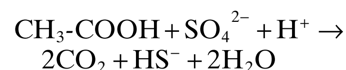
methanol) and from $H_2 + CO_2$. Because so many pathways lead to the formation of acetate under anaerobic conditions, the further degradation of acetate is of great importance for carbon flow under anaerobic conditions. Among the methanogenic archaea, only species of the genera *Methanosarcina*, *Methanosaeta* and *Methanothrix* are able to utilize and degrade acetate to methane and carbon dioxide (e.g., *Methanosarcina barkeri*, *Methanothrix thermophila* and *Methanosaeta concilii*). The degradation occurs according to the following equation (Thauer et al., 1989):



$$CH_3\text{-}COOH \rightarrow CH_4 + CO_2 \Delta G^{0\prime} = -36\,kJ/mol$$

Initially acetate is activated to acetyl-CoA by acetate kinase and phosphotransacetylase or directly by acetyl-CoA synthetase (*Methanosaeta*). Acetyl-CoA is subsequently bound to the carbon monoxide (CO) dehydrogenase complex, at which it is decarbonylated by cleavage of the carbon-carbon bond. The methyl-group is subsequently transferred via tetrahydromethanopterin (THMP) to coenzyme M, and CO is oxidized to CO_2, providing the reducing equivalents for the reduction of the methyl-coenzyme M to methane by the pathway shown in Fig. 11 (Thauer, 1998; Ferry, 1997; Ferry, 1999). It is interesting that the CO dehydrogenase complex, which catalyzes the decarbonylation of acetyl-CoA to methyl-THMP and CO and the oxidation of CO, also catalyzes the reactions mentioned in a reversible manner. In methanogens utilizing acetate, the direction of decarbonylation predominates; when, however, organisms such as *Methanobacterium thermoautotrophicum* grow with $H_2 + CO_2$, they use this enzyme system to synthesize acetyl-CoA from methyl-coenzyme M and CO for autotrophic growth (Zeikus, 1983; Fuchs, 1986; Shieh and Whitman, 1988; Huber and Wachtershäuser, 1997). Similarly, acetogenic bacteria such as *Acetobacterium woodii* and *Moorella thermoacetica* produce acetyl-CoA from methyl-tetrahydrofolate and CO (Wood et al., 1986; Ljungdahl, 1986; Shanmugasundaram et al., 1988; Menon and Ragsdale, 1999).

A number of sulfate-reducing bacteria are also able to oxidize acetate completely to CO_2 under anaerobic conditions:



$$CH_3\text{-}COOH + SO_4^{2-} + H^+ \rightarrow 2CO_2 + HS^- + 2H_2O$$

Most of them also take advantage of the described C1-pathway with the CO dehydrogenase complex for decarbonylating acetyl-CoA. The pathway is investigated in more detail in *Desulfotomaculum acetoxidans*, *Desulfobacterium autotrophicum* and in the archaeon *Archaeoglobus fulgidus* (Spormann and Thauer,

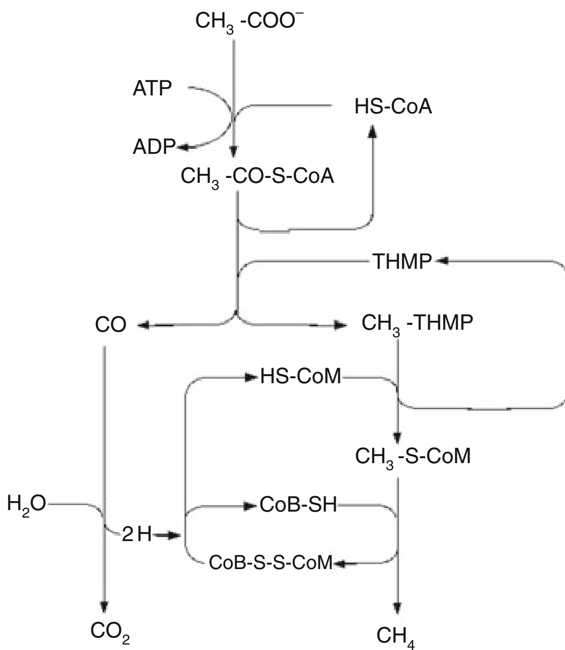
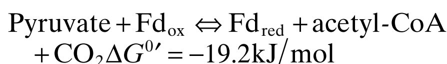


Fig. 11. Conversion of acetyl-CoA to methane and carbon dioxide. THMP, tetrahydromethanopterin; HS-CoM, coenzyme M.

1988; Hansen, 1994; Möller-Zinkhahn et al., 1989; Brüggemann et al., 2000). Only a small number of acetate oxidizing, sulfate reducers (e.g., *Desulfobacter postgatei*) use the tricarboxylic acid cycle to carry out acetyl-CoA oxidation (Brandis-Heep, 1983; Möller et al., 1987; Thauer, 1988; Thauer et al., 1989). Activation of acetate in *D. postgatei* occurs by a succinyl-CoA:acetate CoA-transferase; acetate kinase and phosphotransacetylase are lacking.

Many fermentation reactions are associated with the evolution of molecular hydrogen, H_2. This allows a shift from producing alcohols and lactate to acetate and butyrate, a shift beneficial to the organisms because the ATP yield is increased. Important precursors of H_2 are formate and reduced ferredoxin, and H_2 formation is catalyzed by formate hydrogenlyase and hydrogenase, respectively. There are two important reactions coupled to ferredoxin reduction and ultimately to H_2 formation:

1) The pyruvate-ferredoxin oxidoreductase reaction:



$$\text{Pyruvate} + \text{Fd}_{ox} \Leftrightarrow \text{Fd}_{red} + \text{acetyl-CoA} + \text{CO}_2 \Delta G^{0\prime} = -19.2 \text{kJ/mol}$$

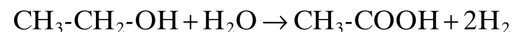
The reaction is exergonic so that it can drive H_2 formation even at a hydrogen partial pressure (PH_2) of 1.013 kPa. The enzyme was first purified from *Clostridium acidiurici* (Uyeda and Rabinowitz, 1971; Charon et al., 1999).

2) The NADH-ferredoxin oxidoreductase:

$$\text{NADH} + \text{Fd}_{ox} \Leftrightarrow \text{Fd}_{red} + \text{NAD}^+ + \text{H}^+ \Delta G^{0\prime} = +18.8 \text{kJ/mol}$$

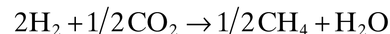
This reaction was discovered in *C. kluyveri* (Jungermann et al., 1969; Gottschalk and Chowdhury, 1969); it is endergonic and will only proceed at a largely reduced P_{H2}. In anaerobic habitats, the P_{H2} is kept as low as 10 Pa by H_2-consuming organisms such as the methanogenic archaea, and acetogenic and sulfidogenic bacteria. Hydrogen consumption by these microorganisms results in the phenomenon of interspecies hydrogen transfer, which has two consequences. First, the product patterns of saccharolytic fermentations as carried out by many clostridia are changed; for example, glucose can be fermented to acetate and CO_2. The second consequence of the generation of a low P_{H2} by the hydrogen-consuming bacteria is that it opens up an ecological niche for a fascinating group of anaerobes, the obligate proton-reducing bacteria. These organisms, were first described in 1967, when a culture called "*Methanobacillus omelianskii*" was found to consist of two different organisms carrying out two different fermentations (Bryant et al., 1967):

1) The "S" organism carries out ethanol oxidation:



$$CH_3\text{-}CH_2\text{-}OH + H_2O \rightarrow CH_3\text{-}COOH + 2H_2$$

2) A methanogenic archaeon consumes molecular hydrogen for methane production:



$$2H_2 + 1/2\,CO_2 \rightarrow 1/2\,CH_4 + H_2O$$

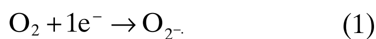
Cocultures of this type were termed "syntrophic" cultures, because the organisms involved mutually depend on one another. Molecular H_2 evolution allows fermentative growth of the "S" organism, but only if the PH_2 is kept low enough by the methanogenic bacterium. The term "interspecies hydrogen transfer" was coined for this kind of connection between H_2 evolution and H_2 consumption. Other examples for syntrophically ethanol-oxidizing bacteria known today are *Thermoanaerobacterium brockii* (Ben-Bassat et al., 1981), Pelobacter species (Schink, 1984; Schink, 1985), and in the absence of sulfate, *Desulfovibrio vulgaris* (Bryant et al., 1977). Not only alcohols but also organic acids can be oxidized to acetate and H_2 this way, such as propionate by *Syntrophobacter pfennigii* (Wallrabenstein et al., 1995) and butyrate by *Syntrophomonas* species (Roy et al., 1986; McInerney et al., 1981). As these oxidations are more endergonic than alcohol oxidations, PH_2 has to be decreased to significantly lower values (<10 Pa) than for example for ethanol (<100 Pa). Synthrophic degradation of aromatic compounds, amino acids and glycolate also

has been discovered (Mountfort and Bryant, 1982; Friedrich et al., 1991; Feigel and Knackmus, 1993). In addition to methanogens, H_2 consumption can also occur by sulfur- and sulfate reducers, homoacetogenic fumarate- or glycine-reducing bacteria (Schink, 1997). In this respect one species is of special interest, the homoacetogenic strain, named "acetate-oxidizing rod-shaped eubacterium" (AOR), which can either oxidize or synthesize acetate depending on the PH_2 (Lee and Zinder, 1988a; Lee and Zinder, 1988b; Zinder and Koch, 1984). Based on interspecies hydrogen transfers, metabolically different bacteria are connected in syntrophic relationships, which are a speciality of obligate anaerobes, and are the basis for the formation of consortia and aggregates in which the various types of microorganisms are in close physical contact (Schink and Thauer, 1987; Stams, 1994; Schink, 1997). One recent example is a marine consortium consisting of archaea and sulfate-reducing bacteria, which apparently mediate the anaerobic oxidation of methane (Boetius et al., 2000). However the elusive microorganisms responsible for this conversion have not yet been isolated and the pathway of anaerobic oxidation of methane is not characterized yet.

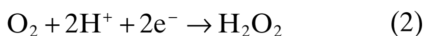
Anaerobes and Molecular Oxygen: Oxygen Sensitivity and Sensing

Oxygen Sensitivity

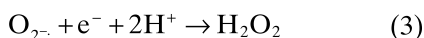
Metabolic reduction of oxygen results in the production of highly toxic and reactive oxygen species ("ROS"), such as superoxide anion ($O_2^{-\cdot}$) hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) (Fridovich, 1995; Cannio et al., 2000b). The latter is most likely responsible for the toxic effects of molecular oxygen. Its production can occur nonenzymatically, when superoxide reacts with hydrogen peroxide in the presence of transition metals (Haber-Weiss reaction [Eq. 4]; Haber and Weiss, 1934). The most probable sites involved in the production of superoxide and hydrogen peroxide at the level of the electron transport chain are cytochrome b_{566} and ubiquinone (Eqs. 1 and 2). In addition superoxide can be generated from O_2 by nonspecific oxidations of reduced flavines, catecholamines and tetrahydrofolates, or chemically.



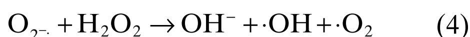
$$O_2 + 1e^- \rightarrow O_2^{-\cdot} \quad (1)$$



$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (2)$$

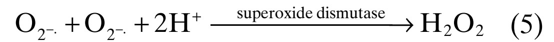


$$O_2^{-\cdot} + e^- + 2H^+ \rightarrow H_2O_2 \quad (3)$$



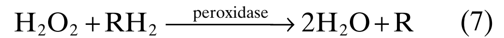
$$O_2^{-\cdot} + H_2O_2 \rightarrow OH^- + \cdot OH + \cdot O_2 \quad (4)$$

Most aerobes have developed appropriate protective mechanisms to overcome the toxic effects of hydrogen peroxide and the superoxide anion. Non-enzymic detoxification is effected by glutathione, which is present in high concentration in many bacteria. The main detoxification, however, involves the enzymes superoxide dismutase (Eq. 5), catalase (Eq. 6) and nonspecific peroxidases (Eq. 7) (Fridovich, 1995; Niimura et al., 2000).



$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 \quad (5)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (6)$$



$$H_2O_2 + RH_2 \xrightarrow{\text{peroxidase}} 2H_2O + R \quad (7)$$

Four classes of superoxide dismutases have been identified based on the metal cofactor, which can be either dinuclear Cu/Zn or monomeric Fe, Mn or Ni (Whittaker and Whittaker, 1998). Most bacteria contain only one superoxide dismutase with either Fe or Mn as cofactor. However, *E. coli* contains both types, the Mn-type only induced during aerobiosis and the Fe-type under both conditions, in the presence and absence of oxygen (Kargalioglu and Imlay, 1994). The novel type of superoxide dismutase with Ni as a cofactor has been recently discovered in several *Streptomyces* species (Youn et al., 1996a; Youn et al., 1996b). Interestingly, a few examples for extracellular superoxide dismutases are known, e.g., in *Sulfolobus solfataricus* (Cannio et al., 2000a) and *Streptococcus pyogenes* (Gerlach et al., 1998). Catalases are generally present in aerobic and facultative anaerobic bacteria.

Among the obligate anaerobic bacteria, organisms are found that are more or less aerotolerant. Many of these organisms (e.g., a number of lactic acid bacteria) have been shown to contain superoxide dismutase and lack catalase (Morris, 1976; Archibald and Fridovich, 1981). A number of obligate anaerobes, however, are extremely oxygen sensitive. Most noteworthy in this respect are the methanogenic archaea, clostridial species and sulfate-reducing bacteria. Cultivation of those anaerobes in the laboratory requires special precautions (Hungate, 1969). Simple exclusion of molecular oxygen is not sufficient to provide good conditions of growth. In addition, they require a low redox potential in their environment and growth media supplemented with compounds such as ascorbate, hydrogen sulfide, sodium thioglycolate or cysteine. Curiously, despite their catalytic capacity for producing molecular oxygen, Fe-containing superoxide dismutases have been discovered in the methanogenic archaea *Methanobacterium bryantii* (Kirby et al., 1981), *Methanobacterium thermoautotrophicum* (Takao et al., 1991; Meile et al., 1995) and *Methanosarcina mazei* Gö1 (G. Gottschalk, unpublished observation). Further

examples of Fe-containing superoxide dismutases in strict anaerobes have been reported for sulfate-reducing bacteria, e.g., *Desulfovibrio desulfuricans* (Hatchikian and Henry, 1971) and *Desulfoarculus boarsii* (Pianzzola et al., 1996). Interestingly, a superoxide reductase from the hyperthermophilic anaerobic *Pyrococcus furiosus* has been discovered, which reduces superoxide without the production of oxygen and therefore confers a selective advantage for anaerobes (Jenney et al., 1999). The physiological role of superoxide dismutases and superoxide reductases in anaerobes that supposedly evolved in ecosystems lacking oxygen, however, has to be elucidated.

Oxygen Sensing

Adaptation of facultative anaerobic microorganisms to anaerobic growth conditions is accompanied by dramatic changes in metabolic gene expression. To make these adaptations, those microorganisms have to be able to sense changes in the environmental oxygen availability. Various sensory and regulatory systems control the expression of aerobic and anaerobic metabolism in response to oxygen. Most of the oxygen sensor proteins known today contain heme, iron sulfur clusters or iron as cofactors, e.g., FixL from *Sinorhizobium meliloti* (Gilles-Gonzalez et al., 1995), the fumarate nitrate regulator (Fnr) and SoxR from *E. coli* (Hidalgo et al., 1995) and rhizobial NifA-proteins (Fischer, 1994; Fischer, 1996), respectively. The Fnr from *E. coli*, which is one of the prominent examples for oxygen sensing and redox control of gene expression in prokaryotes, will be briefly discussed here.

The global regulator Fnr controls transcription of genes, whose functions facilitate adaptation to growth under oxygen limitation (Spiro, 1994; Bauer et al., 1999). Under anaerobic conditions, it contains a [4Fe4S]-cluster, which is required for the oxygen-sensing function. Recent data suggest, that this [4Fe4S]-cluster is sufficiently unstable towards oxygen and apparently mediates the sensitivity of the transcriptional activator to oxygen (Khoroshilova et al., 1997; Kiley and Beinert, 1998; Beinert and Kiley, 1999). The presence of the [4Fe4S]-cluster favors dimerization of Fnr, which is correlated with increased site-specific DNA binding of the transcriptional activator Fnr. Upon the presence of oxygen, the [4Fe4S]-cluster is disrupted, resulting in the conversion of transcriptionally active Fnr-dimers into inactive monomers (Lazazzera et al., 1996; Melville and Gunsalus, 1996; Beinert and Kiley, 1999). Homologues of Fnr have been identified in several facultative anaerobic bacteria, some of which differ with respect to the cysteine residues and the coordination of the iron-sulfur clusters (reviewed in Spiro, 1994; Cruz Ramos et al., 1995; Saunders et al., 1999; Vollack et al., 1999).

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Prokaryotes (2006) 2:102–139
DOI: 10.1007/0-387-30742-7_5

CHAPTER 1.5

Bacterial Behavior

JUDITH ARMITAGE

Introduction

It is apparent that the majority of prokaryotic species move around their environment, either actively swimming using flagella, or gliding over surfaces. Although a lot less is understood about gliding, we know that swimming uses the most complex organelle identified in prokaryotes. The flagellum is the product of the controlled expression of up to 50 genes, producing an organelle which has components in the cytoplasm, the cytoplasmic membrane, the outer membrane and externally. This flagellar structure can rotate at speeds of up to 350 Hz to move a bacterium at well over 20 μm/sec through its environment. Incredibly, if driven by sodium rather than protons the flagellum can rotate at over 1,300 Hz, moving cells at speeds as great as 150 μm/sec. Unlike gliding, which may have evolved independently several times during evolution, flagellar driven motility may have only evolved once, as all bacteria and archaea seem to have flagella built along similar patterns, although the archaeal flagellum has some differences that may become more significant as more is discovered about motility in that kingdom. Interestingly, control of both flagella- and gliding-based motility is common to all prokaryotes, again suggesting development early in bacterial evolution.

The synthesis and control of motility can be metabolically expensive, therefore if a species is motile, it is because motility provides it with a survival advantage. This chapter will deal with the mechanisms involved in the synthesis and operation of flagella, and to a more limited extent, gliding and twitching. The control of responses by environmental gradients and the possible role of chemotaxis in reaching sites for symbiotic or pathogenic growth, or maintaining positions in natural environments will also be discussed. It has recently become apparent that motility and tactic responses may be important in physiological phenomena such as biofilm formation and social behavior and the possible role of motility in these will be described.

Reynold's Number and Brownian Motion

The environment in which a bacterium lives is very different from that experienced by larger organisms. At the very small size of a bacterium, the dominant physical force is due to viscosity, with almost no measurable force of inertia. A bacterium therefore does not move by displacing liquid, as a larger organism does, and does not glide or coast when the flagellum stops rotating; indeed, a bacterium will stop within the diameter of a proton when its motor stops rotating (Berg, 1983). The equation linking the physical environment to cell size is $R = Lv\rho/\eta$, where R is the Reynolds number, L the length of the organism, v the velocity of the organism, ρ the density, and η the viscosity of the liquid. While a large organism, such as man, may have a Reynolds number of over 100, a bacterium has one of about 10^{-6}. At these low Reynolds numbers, fluid flow is smooth and streamlining unnecessary; bacteria do not displace liquid, but carry a shell of medium with them.

In addition to the problems involved in swimming through a highly viscous environment, bacteria also have to cope with the buffeting that comes from the movement of the molecules in the water, i.e., Brownian motion. Nonmotile cells observed with a microscope are seen to be constantly moving. Swimming bacteria are subject to the same forces and therefore cannot swim in a straight line or stay on course for more than a few seconds. This combined with a gentle curve which results from the rotation of the cell body means that a sensory system has evolved to frequently reset the swimming direction and allow movement in a positive direction despite the constant bombardment.

The majority of bacterial species are also too small to sense a stimulus gradient along their body length. This means that unlike eukaryotic microbes, which can have a head and a tail and swim directly towards the source of an attractant, prokaryotes cannot sense spatially, but must make temporal comparisons (Berg and Brown,

1972; Berg and Turner, 1995). They compare the strength of a stimulus now with that a few seconds before. This means that their patterns of swimming must be different from eukaryotes: rather than steering towards an attractant, prokaryotes must change direction regularly to "check" whether they are going in a positive direction. (See Patterns of Swimming in this Chapter)

Flagella

The structure of the flagellum was thought to be generally conserved amongst all swimming prokaryotes, but recently it has become apparent that the motor structures of bacteria and archaea may be somewhat different, leading to the suggestion of two separate evolutionary events or distant divergence. However, both swim by rotating semi-rigid helical flagella filaments using the electrochemical ion gradient (usually the proton motive force, but in some species, a sodium motive force) across the cytoplasmic membrane to drive that rotation.

Patterns of Flagellation

An early method of classification relied on whether the bacteria swam, and if they did, the pattern of flagellation. We now know that whether a bacterium swims may depend on the medium in which it is grown, and many only swim under limited growth conditions or at certain phases of the growth cycle. The pattern of flagellation along with cell size and shape can still be a reasonable start in identification, however. Some species have single flagella, which may be polar as in *Pseudomonas aeruginosa* or subpolar as in *Rhodobacter sphaeroides*. Many species have flagella arising from apparently random sites all over the cell surface. These peritrichous flagella tend to come together in bundles to cause the cells (such as *Escherichia coli* or *Sinorhizobium meliloti*) to swim. Bundles of flagella are also found at the poles of some species, and these might be at both poles (as in *Rhodospirillum rubrum*, *Helicobacter pylori* or *Halobacterium salinarium*) or at one pole (as in *Chromatium vinosum*). Some species even have internalized filaments, a characteristic of the spirochetes. Interestingly, several species alter patterns of flagellation, dependent on their physical environment. *Vibrio alginolyticus* swims using a single flagellum when in an aquatic environment, but when it reaches a surface, a new set of flagella are synthesized and the hyperflagellate cells are now able to move over the surface (Atsumi et al., 1992; McCarter et al., 1988). This is not an uncommon phenomenon, having been known for many years as a characteristic of *Proteus* species and now recognized in *Bacillus*, *Salmonella*, *Rhodospirillum* and many other species (Harshey, 1994a). What makes the *V. alginolyticus* particularly interesting is the switch between sodium and proton motors that occurs when cells switch from free-swimming to surface movement (see below).

Patterns of Swimming

All flagella appear to be passive helices, rotated at their base by a transmembrane motor which is driven by either the electrochemical proton or sodium gradient. The different patterns of swimming seen in different species arise because of different patterns of motor switching. The best studied swimming pattern is that of the peritrichously flagellate bacteria. The individual flagellar motors can rotate either clockwise (CW) or counterclockwise (CCW) and switch between the two states (Khan and Macnab, 1980; Macnab, 1976; Macnab, 1977). When the majority of flagella are rotating CCW, the helical filaments come together as a bundle, rotate together and push the cell forward. Periodically a number of motors switch to CW rotation, causing a polymorphic transition in the flagellar helix such that the handedness and wavelength changes. The bundle of flagella is forced apart and the cell "tumbles" on the spot. The bundle reforms when the majority of motors return to CCW rotation and the cell resumes swimming, usually in a new direction (Berg and Anderson, 1973; Turner et al., 2000). This pattern of periods of smooth swimming interspersed every few seconds with a short tumble results in a three-dimensional random pattern of swimming. Other species achieve the 3D swimming pattern by different motor behavior. *Rhodobacter sphaeroides* has a single flagellum which only rotates CW. Every few seconds the flagellar motor stops rotating and the flagellar filament changes conformation to a large amplitude, short wavelength coil against the cell body. The cell is reoriented partly by Brownian motion and partly by the slow rotation of this coiled form, and when a functional helix reforms, the cell is usually pointing in a new direction (Armitage et al., 1999b; Armitage and Macnab, 1987). Other species with a single flagellum, such as *Ps. aeruginosa* change direction by briefly switching to CW rotation, the flagellum then pulls rather than pushes the cell for a brief period. Recent studies into the efficiency of using brief reversals rather than tumbles as a mechanism for moving through different media suggested that while tumbling is efficient in liquid, brief reversals worked better at moving bacteria through particulate media.

Sinorhizobium meliloti swims using a bundle of flagella. Rather than stopping or switching

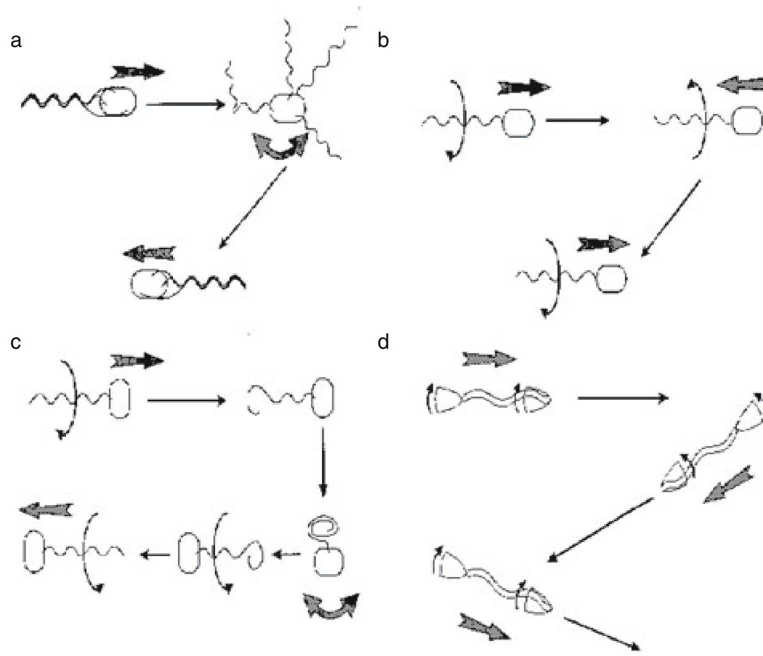

Fig. 1. This cartoon shows four different patterns of flagellation and the corresponding mechanisms of direction changing. a) *Escherichia coli* swims by rotating a bundle of flagella counterclockwise. Periodically, a number of motors switch to clockwise rotation, and the bundle flies apart and the cell tumbles. When the majority of filaments return to counterclockwise rotation and the bundle reforms, the cell is usually pointing in a new direction. b) *Pseudomonas aeruginosa* swims by rotating its single polar flagellum pushing the cell forwards. Periodically, the motor reverses and pulls the cell. During this time, Brownian motion tends to reorient the cell. The motor again reverses and pushes the cell, usually in a new direction. c) *Rhodobacter sphaeroides* has a single flagellum that rotates clockwise, pushing the cell. Periodically, the motor stops and the flagellum relaxes into a short wavelength large-amplitude filament. This is rotated slowly, reorienting the cell. The motor resumes normal rotation and the functional filament reforms to push the cell in a new direction. d) *Rhodospirillum rubrum* has two polar tufts of flagella that rotate to move the spiral-shaped cells through the medium. The bundles switch rotational direction synchronously to change swimming direction.

rotational direction, a number of flagella slow their rotational speed. This results in disruption of the bundle, and the cell changes direction without either stopping or tumbling (Sourjik and Schmitt, 1998a). This again results in a random swimming pattern. Spiral-shaped species such as *Rhodospirillum rubrum* have polar tufts of flagella that rotate in opposite directions, spiralling the cells through the medium. Periodically both bundles simultaneously switch rotational direction and the cell changes direction. In spirochetes these polar bundles of flagella are internalized, but they still rotate. Evidence indicates that the rotation of the filament lying between the outer cell wall and the cell body causes one to rotate against the other, and the spiral-shaped cell body to move through the viscous environment (Berg, 1976; Charon et al., 1992). These species all move far more efficiently through viscous media, and as many are pathogens, this is thought to aid their invasion of, for example, mucous membranes (Kaiser and Doetsch, 1975). Periodic direction changing occurs when the flagella at the two poles first stop rotating in the opposite direction and begin rotating in the same direction, and the cell body then flexes, causing the cell to change orientation.

Few archaea have been investigated in detail, but *Halobacterium salinarium* has polar bundles of filaments. When these change direction of rotation, the bundle does not separate but rotates smoothly in either CCW or CW directions, suggesting a slightly different flagellar structure (Alam and Oesterhelt, 1984; Marwan et al., 1991).

Some species swim differently under different growth conditions. Species from a wide range of genera have been shown to differentiate into highly flagellate swarmer cells when inoculated onto surfaces. In most cases it is thought that the expression of the genes involved in flagella synthesis is increased in response to some surface stimulus, either cell density or increased viscous drag (Alberti and Harshey, 1990; Givskov et al., 1998; Harshey and Matsuyama, 1994b; Kohler et al., 2000; McCarter et al., 1988).

Many members of the γ subgroup of Proteobacteria can be induced to swarm, including

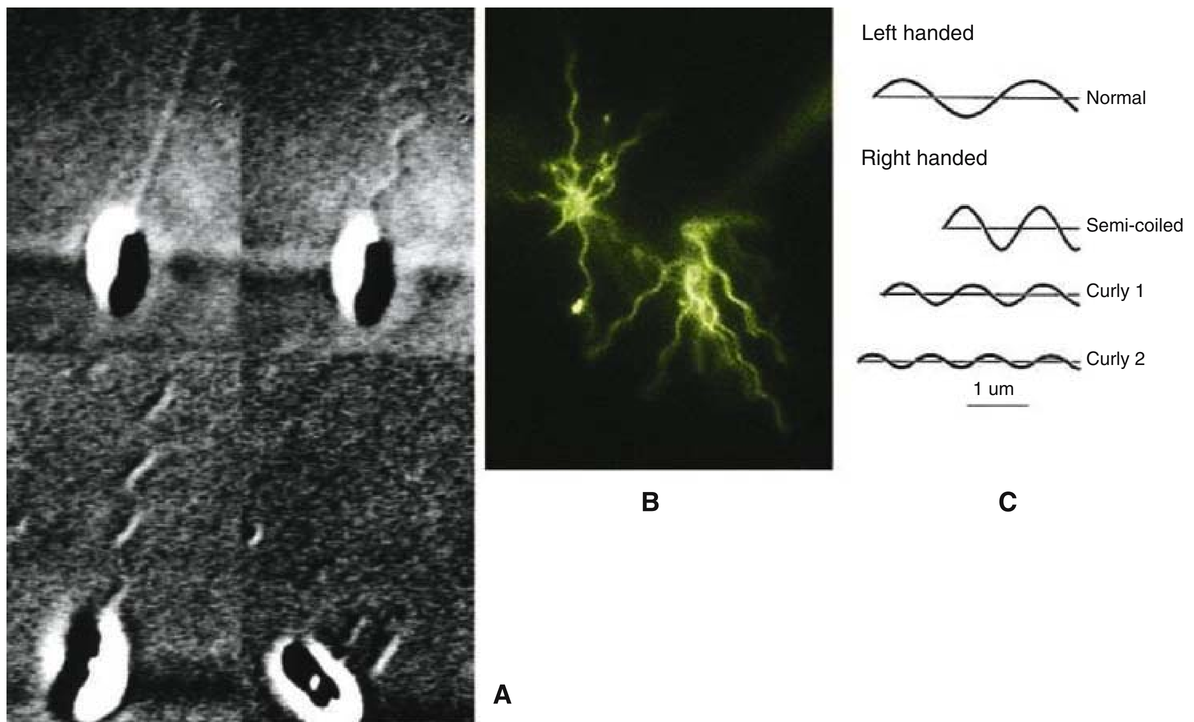

Fig. 2. Changes in shape and motion of flagella during swimming, tumbling and reversal of motor rotation. a) Still differential interference contrast (DIC) microscope images taken from a video film of swimming *Rhodobacter sphaeroides* showing a polymorphic transformation of the flagellar filament. The functional filament can switch to a longer wavelength (apparently straight form) or change to a coiled large amplitude (short wavelength form) when rotation stops. From Armitage et al. (1999b)) Still image taken from a video of tumbling *Escherichia coli* with filaments labelled with fluorescent dye. From Turner et al. (2000). c) Polymorphic shapes taken on by flagella during changes in the direction of motor rotation. (Figures 2a and 2b are courtesy of H.C. Berg and L. Turner using the Rowland Institute DIC microscope.)

E. coli, *Salmonella typhimurium*, *Serratia marscesens* and *Yersinia* species. The best studied are the swarmer cells of the marine vibrio, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* and the enteric *Proteus* species, *P. mirabilis* and *P. vulgaris*. *Proteus* species swim in liquid medium in much the same way as *E. coli*. However, when inoculated onto agar plates, the cells differentiate into long filamentous cells with a several fold increase in flagella numbers per unit cell surface. The highly flagellate filamentous cells move together as rafts over the surface, the increase in cell surface allowing them to overcome the surface tension. The marine vibrios, *V. haemolyticus* or *V. alginolyticus* actually synthesize completely new flagella motors when grown on surfaces, switching from the fast, sodium-driven motors (controlling polar flagella when free swimming) to large numbers of slower proton-driven motors when grown on surfaces (Atsumi et al., 1992). Another species which increases lateral flagella synthesis on surfaces is *Rhodospirillum centenum* (Ragatz et al., 1995). In this case, rather than some of the hyperflagellate cells moving away as rafts from the colony, the whole colony of photosynthetic bacteria moves over the agar surface towards red light. *Agrobacterium tumefaciens*, another α-subgroup bacterium can also become hyperflagellate on surfaces.

Swarming is not confined to Gram-negative genera, as both *Bacillus* and *Clostridium* species have been shown to swarm. The increased viscous drag on the polar motor appears to trigger the expression of the peritrichous proton-driven motor genes in *V. alginolyticus*, but this does not appear to be the signal for all switches to peritrichous expression, as the artificial increase in external viscosity only causes peritrichous expression in some species. Obviously, the increased expenditure of energy required to synthesize large numbers of flagella indicates that moving over a surface must provide a survival advantage for these species. There have been suggestions that motility may increase infectivity of *Proteus* spp. (Allison et al., 1992; Gygi et al., 1995; Mobley and Belas, 1995) and may help marine *Vibrios* maintain themselves on nutrient-rich surfaces, but evidence is still limited. Interestingly, many bacterial species found in biofilms move. Depending on the species, this

may be by twitching, gliding, or multiflagellate swarming, each of which can move a cell through a highly viscous environment and presumably provide survival advantages. Detailed analyses of gliding and twitching are starting to suggest that these may use common mechanisms (Semmler, 1999; Watnick and Kolter, 2000). A free-swimming *Synechococcus* cyanobacterial species has been isolated which swims without any obvious means of propulsion (Brahamsha, 1996; Pitta et al., 1997; Waterbury et al., 1985). Dark field and differential interference contrast (DIC) microscopy, and attempts at flagella isolation using classical techniques such as pH reduction or shearing, have all failed to identify any extracellular means of propulsion. Jet propulsion has been ruled out and the current hypotheses suggest Ca^{2+} is involved in movement along with very short, fine cilia-type structures, but the mechanism remains unclear.

Flagella Structure: Flagellin

Bacteria The majority of species investigated have flagellar filaments made from a single protein, flagellin. Flagellins vary in size from species to species, but all have a conserved C- and N-terminal domain, which allows the flagellum to polymerize into a helical structure (Fedorov et al., 1984; Macnab and DeRosier, 1988; Mimori-Kiyosue et al., 1997; Vonderviszt et al., 1991). Unusual amino acids are often found in flagellin proteins and many flagellins are glycosylated, but the significance of this has not been identified. The C- and N-terminal domains are on the inside of the hollow filament structure and allow the individual subunits to interact as protofilaments along the axis of the filament, with 11 protofilaments making up the complete structure of many thousand flagellin subunits (Morgan et al., 1995; Namba and Vonderviszt, 1997; Namba et al., 1989; Trachtenberg and DeRosier, 1987a; Trachtenberg and DeRosier, 1991). The flagellin subunits can interact with the neighboring subunits in two defined ways, which allows the protofilaments to be long or short. The association of short with long protofilaments creates the twist in the cylinder and hence the helical shape, and the relative numbers of short to long protofilaments defines the wavelength and handedness of the structure. The protein regions involved in the bi-stable switching have recently been putatively identified in the crystal structure of a flagellin fragment (Namba, 2001; Samatey et al., 2000; Yamashita et al., 1998). The change in helical shape results from the torque imposed on the filament by the rotating motor being transmitted through a junction protein connecting the flagellar filament to the hook (Fahrner et al., 1994). The helical filament can be several times longer than the cell body, for example filaments as long as 7 μm have been seen on *R. sphaeroides*, which is only 2 μm long. The filament itself is between 15 and 20 nm in diameter, but the wavelength is constant for the whole length of the filament. Analysis of cells tethered to glass slides by antibody bound to either the cell filaments or hook region, which connects the filament to the cell, suggests that the filament is one of the most rigid proteins in biology, certainly more rigid than actin (Block et al., 1989).

The central domain of the flagellin protein is highly variable and it is this region that is exposed on the outside of the flagellar filament. This provides the highly antigenic domain, the H-Ag, used for many years to type, for example, *Salmonella* strains. This region is not required for assembly, and mutants can be created lacking most of the central domain, but still capable of functional flagella assembly. *Salmonella* is known to "phase shift" flagellin expression, and by inverting genes, to express different flagellins under different conditions. It has been suggested this phenomenon may help the bacterium overcome the immune system of the host, although little research has been carried out to identify whether this is indeed the case (Iino and Kutsukake, 1983). This phase variation was used to show that flagella assemble from the distal end of the growing flagellum, not the base (Iino, 1969).

Some species have flagella made up of more than one flagellin, for example *S. meliloti*, *Treponema pallidum* and *Caulobacter crescentus*, have flagellar filaments composed of several related flagellins. The reason for this is unclear, although *S. meliloti* filament is more rigid (known as complex flagella) than other flagellar filaments, and these filaments are thought not to undergo polymorphic transitions (Trachtenberg et al., 1987b). The flagellins are expressed and incorporated sequentially rather than randomly mixed in the structure, and though mutants in the individual flagellin genes tend to still assemble functional filaments, these are often not as efficient as wild-type filaments.

Archaea The flagellins that have either been sequenced or identified from genome sequences of the archaea suggest slightly different flagellins than those of the bacteria. Unlike most bacterial flagellin, the archaeal flagellins appear to be glycosylated. The bundle of flagella rotate CCW and CW without disruption of the flagellar bundle, and it is thought the glycosylation may allow the filaments to slide past each other during the change in rotational direction, without getting tangled (Alam and Oesterhelt, 1984).

Motor Structure

Bacteria The semirigid helical flagellum is connected to the cell body via a short region, the hook, with a protein structure similar to the filament, but composed of a different protein (Uedaira et al., 1999; Wagenknecht et al., 1982). The role of the hook is not certain, but it is less rigid than the filament and may be involved in allowing the filaments to come together as a bundle and/or it may be involved in transmitting the changes in torque from the motor to the filament, allowing the polymorphic transformation needed for direction changing (Block et al., 1991). There are two proteins between the hook and the filament, known as hook-associated proteins (HAPs). Mutations in the gene coding for HAP3 of *E. coli* result in a filament that changes conformation far more frequently that the wild type. The HAP3 protein may hold the protofilaments in specific conformations and only allow changes when the torque transmitted through the hook from the motor changes significantly (Fahrner et al., 1994).

The hook then connects to the rotor via a rod that passes through the layers of the bacterial membrane. In Gram-negative species, the rod passes through a pair of rings in the outer membrane, the L and P rings, which probably act as a grommet to allow rotation of the rod without disrupting the outer membrane. In Gram-positive species, the rings are replaced by a collar-like structure. The motor consists of a rotor and a stator. The rod is connected to the rotor, which itself is a series of rings located in the cytoplasmic membrane and cytoplasm of the cell (Fig. 3). Early work suggested that the actively rotating region was the MS-ring in the cytoplasmic membrane, but that is now known to be a passive structure made of the protein product of a single gene (Ueno et al., 1994). On the cytoplasmic face of the MS ring is the active part of the rotor. It is estimated that about 26–30 copies of the FliG, M and N proteins form the C-ring (Mathews et al., 1998; Sockett et al., 1992; Thomas et al., 1999; Toker and Macnab, 1997; Zhao et al., 1996a; Zhao et al., 1996b). The movement of protons through the membrane cause FliG to rotate, rotating the rod and the helix (Blair and Berg, 1990; Blair and Berg, 1991). The FliM and FliN proteins are thought to interact with the cytoplasmic sensory signalling pathway to cause the motor to switch the direction of rotation (Bren and Eisenbach, 1998; Sockett et al., 1992; Sourjik and Berg, 2000; Zhao et al., 1996b). To allow rotation, there must be not only a rotor, but also a stator. The stator consists of a ring of about eight complexes of MotA and MotB proteins. The MotB protein has a single transmembrane domain and a large periplasmic region, which has a terminal peptidoglycan-binding domain (Muramoto and Macnab, 1998). This is thought to anchor the proteins to the cell wall. In addition, MotB is linked to MotA, which has four transmembrane domains and is the proton channel through the membrane (Zhou et al., 1998b; Zhou and Blair, 1997a). Protons, about 1000 per revolution, pass through MotA and interact with FliG, and this flow causes rotation to occur at about 300 Hz (Meister et al., 1987). Each of the Mot complexes is an independent force-generating unit (Block and Berg, 1984; Ryu et al., 2000). Crystal structures of MotA fragments and second-site suppressor mutations indicate a number of essential amino acids on FliG and MotA are essential for torque generation (Braun et al., 1999; Lloyd et al., 1999; Mathews et al., 1998; Zhou et al., 1998b). All bacterial motors investigated have homologues of the motor proteins, suggesting that the mechanism of driving rotation is common. Sodium motors, which work much faster, also have MotA and MotB homologues, but also have additional proteins (PomA, PomB, MotX and MotY; Asai et al., 1997; McCarter, 1994a; McCarter, 1994b). The role of these is still not understood, but chi-

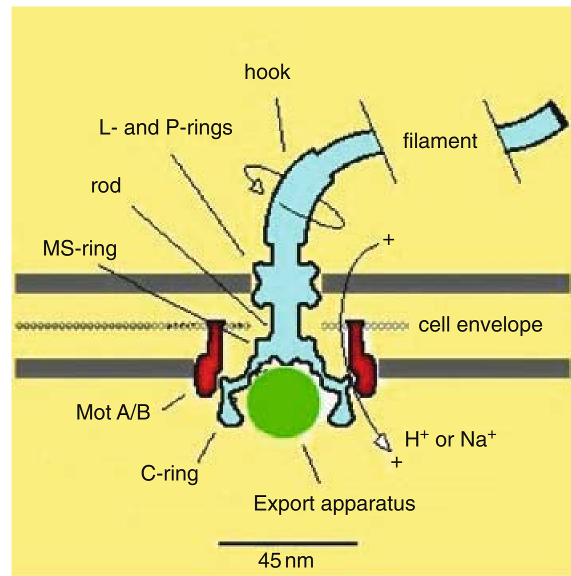

Fig. 3. Structure of the flagellar motor. Cartoon of the flagellar motor shows the L- and P rings (which act as a grommet through the outer membrane of Gram-negative bacteria), MS ring, (which provides the scaffold for the C-ring [the rotor part of the motor]). The C-ring is comprised of FliG, which interacts with the stator (MotA/B) and the electron chemical ion gradient, and FliM/N, which form the switch component and interact with the chemosensory pathway. A ring of 8–12 MotA/B proteins make up the stator, anchored in the membrane by MotB. Binding to the peptidoglycan, the ions move through MotA to interact with FliG on the C-ring to drive rotation. From Berry and Armitage (1999).

meric motors have been made with MotB from a proton-driven motor and MotA from sodium motors, which function as sodium motors.

Also, on the cytoplasmic side of the membrane and associated with the C-ring is the export apparatus. Recent studies on the toxin-exporting type III secretion pathway of species such as *Yersinia pestis* and *Shigella* has identified structures remarkably similar to the central flagellar motor and components which are remarkably similar to the flagellar-specific export pathway, suggesting a common evolutionary origin (Kubori et al., 1998; Macnab, 1999; Minamino and Macnab, 1999). While the proteins that make up the outer membrane L and P rings have classical signal sequences, the rod, hook and flagellin proteins lack these sequences and are exported through the 30 Å central channel of the forming flagellum, before polymerization at the distal end of the growing structure (Minamino and Macnab, 1999; Suzuki et al., 1998). It is assumed that the proteins are exported in an unfolded form and must therefore be held in an unfolded form before export. Export depends on FliI, a protein with significant sequence similarity to the B-subunit of ATP synthase, and thought to be a flagellar-specific export protein (Dreyfus et al., 1993; Vogler et al., 1991). Many of the other proteins involved in the export process have homologues in the Type III secretion system. For the components of the flagellar filament to be assembled across the inner cytoplasmic and outer membranes of a bacterium, the rod protein must pass through the peptidoglycan layer. A component of the flagella regulon, FlgJ has been found to digest a hole through the peptidoglycan to allow assembly of the motor components (Nambu et al., 1999).

Motor activity needs to be controlled by environmental signals for swimming to have any environmental advantage, controlling either the switching of rotational direction or causing the motor to transiently stop. As with the mechanism involved in rotation, that involved in switching is unclear. However, a cytoplasmic signalling protein, CheY is known to interact, when phosphorylated, with the FliM component of the C-ring via a sensory signalling pathway. The interaction of CheY-P with FliM is thought to cause conformational changes in the FliG proteins, such that the direction of rotation is reversed, even though ion flow remains inward (Welch et al., 1994).

ARCHAEA Much less is known about the motor proteins of the archaea, but the classical membrane ring structure of the bacterial motor is not evident in electron micrographs. A large cytoplasmic structure is however evident, but the composition of this is still very uncertain (Kupper et al., 1994).

Interestingly, large disk-like structures have been found associated with the eubacterium *Wolinella succinogenes*, and their role is uncertain, but structurally the whorl-like structures formed are very different from those seen in the archaea (Engelhardt et al., 1993; Kupper et al., 1989; Schuster and Baeuerlein, 1992; Stahlberg et al., 1995).

Gene Expression and Assembly

It takes about 40–50 genes to construct a functional flagellum, and as components are located in the cytoplasm, across both membranes and extracellularly, it is extremely important that flagellar assembly is highly ordered (Macnab, 1992; Macnab, 1996). There are only a specific number of flagella per cell, and therefore expression of the flagellar genes must be regulated by the growth rate (except when some species are induced to become hyperflagellate). In *E. coli*, a master operon, encoding FlhC and FlhD, under the control of the catabolite repression system, initiates expression of a series of operons in a highly coordinated manner. There is a flagellar specific sigma factor, σF or σ28, which controls expression of later proteins, whereas any assembly errors feedback and inhibit further operon expression. An anti-σ factor, FlgM, binds to σF until exported from the cell, which happens after rotor assembly. This is the signal that the motor has been correctly assembled, and the flagellin proteins can now be expressed and exported (Brown and Hughes, 1995; Iyoda and Kutsukake, 1995; Kutsukake and Iino, 1994). Although the majority of research has been on *E. coli* and *S. typhimurium*, other motile species studied in detail appear to have highly organized expression of the flagellar genes, and usually a σ28 homologue. Some species may have σ-54-dependent control of the initial operons rather than σ70, suggesting environmental regulation of motility.

Control of gene expression is more complex in species such as *C. crescentus*, which is only motile during certain stages of its life cycle. A sessile, stalked cell divides to produce a motile swarmer cell, which is unable to grow or divide but swims to a new location to settle and produce a stalk for attachment at a surface. In this case, the timing of flagellar gene expression is critical. In this species a response regulator protein, CtrA, controls both methylation of DNA and flagella synthesis. Phosphorylated CtrA inhibits cell division and DNA replication, and activates DNA methylation and flagella synthesis. Localized proteolysis of the protein in stalked cells appears to allow the cell cycle to continue in stalked cells while inhibiting division in swarmer

cells (Brun et al., 1994; Newton and Ohta, 1990; Reisenauer et al., 1999; Wheeler et al., 1998; Wu et al., 1995).

Gliding

Gliding is considered to be movement over surfaces that is produced without obvious motility organelles. It seems possible that there is more than one mechanism involved in gliding because the patterns of movement vary far more than flagellate motility. Gliding is the most common form of motility amongst cyanobacteria. In some genera, all the cells are motile, whereas in others, only differentiated structures such as hormogonia glide, probably helping dispersal. Interestingly, many species also have gas vacuoles, but these allow dispersal over large distances in species living in open water and allow diurnal movement in response to light and nutrient levels. Genera as distantly related as *Flavobacteria*, *Myxobacteria* and *Mycoplasma* also glide on surfaces.

Rates and patterns of gliding vary dramatically from species to species. *Flavobacterium johnsoniae* (*Cytophaga*) can move across surfaces at rates of 10 μm/sec, almost as fast a free-swimming bacterium, periodically changing direction by dramatically up-ending on the pole of the cell and then falling to point in a new direction (Beatson and Marshall, 1994; Gorski et al., 1993; Pitta et al., 1993). *Myxococcus xanthus*, on the other hand, moves at a maximum of 5 μm/min, very slowly gliding over surfaces and changing direction by simply reversing gliding direction (Hartzell and Youderian, 1995; Ward and Zusman, 1997). Extracellular polysaccharide has been implicated in gliding motility, supported by genetic evidence from *F. johnsoniae* (Agarwal et al., 1997; Gorski et al., 1993).

The best studied system of gliding is that shown by *Myxococcus xanthus*. This species undergoes a complex life cycle in which colonies of bacteria glide over surfaces, releasing antibiotics to kill other bacterial species, which then provides the cells with their nutrient source. Under nutrient limiting conditions, large numbers of cells (up to 100,000) aggregate to form fruiting bodies in which spores are formed. A complex system of cell-cell signalling controls this process, which depends on the ability of the cells to glide (Hartzell and Youderian, 1995). Two patterns of gliding have been characterized in *M. xanthus*: "adventurous" motility in which single cells move away from the central colony and "social" motility in which the colony moves as a unit. Little is known about the mechanisms involved in "adventurous" gliding, but "social" gliding requires cell-cell contact and trail formation (Rodriguez and Spormann, 1999). Cell-cell contact appears to occur at the poles of the gliding cells and requires type IV pili or fimbriae (Kaiser, 2000). Extracellular, peritrichous filaments known as "fibrils" have also been shown to be required for social motility (Dworkin, 1999).

Although the mechanisms involved in movement are unknown, it has been suggested that twitching motility, which also requires type IV pili and is important in biofilm formation, and gliding may be closely related, if not in fact the same phenomenon (Sager and Kaiser, 1994; Spormann, 1999; Wall and Kaiser, 1999; Ward et al., 1998a).

The wall-less pathogenic genus, *Mycoplasma*, also glides. Unlike all other prokaryotic species studied so far, these bacteria have a true polarized morphology, with a "head" and a "tail," which appears to help in attachment. *Mycoplasma mobilis* glides at speeds of up to 4 μm/sec and always leads with the head, which has a smaller diameter than the rest of the cell. Direction changing results from the cells turning. In the case of this gliding species, it has been suggested that there are adhesion proteins which flow along the cell surface, moving the cell forward, to be recycled to the front of the cell when they reach the rear (Neimark, 1983).

Twitching

Twitching has become the focus of increasing attention. Type IV pili turn out to be essential for twitching motility, for surface attachment and biofilm formation (O'Toole and Kolter, 1998). As Type IV pili are also essential for social gliding in *M. xanthus*, it suggests that twitching and at least some forms of gliding may in fact all be the result of Type IV pili. The role of motility, adhesion and biofilms in surface colonization makes this of particular interest to both medical and environmental research. Twitching motility has been identified in a wide range of bacterial species, but is best studied in *Ps. aeruginosa* where it can result in speeds of up to 5 μm/sec (Darzins and Russell, 1997). Twitching was defined as the intermittent twitching movement of cells, which can occur singly or as groups, although it seems likely that some contact is required with neighboring cells for movement to occur. Twitching results in a thin-spreading zone from a bacterial colony, which can occur between the Petri dish and agar surface or over the surface of solid agar.

Type IV pili are about 6 nm in diameter, therefore less than half the diameter of flagella, but can be several μm in length. They are made of pilin, which form helical strands along a

hollow tube. Pilin has a conserved N-terminal region which appears to be involved in assembly and is located on the inner surface of the pili, whereas a more variable C-terminal domain forms the outer surface and is thought to be involved in adhesion. Type IV pili are found at the poles of the cells, and over 30 genes are involved in their synthesis and assembly (Alm and Mattick, 1997). Recent data suggest that they may only be expressed in the cells at the actively moving bacterial front. Unlike flagellar assembly, pili assemble from the cell body outward. A family of proteins involved in protein secretion, DNA transfer, and the morphogenesis of filamentous bacteriophage are related to pilin and are all initially synthesized with a short, positively charged characteristic leader sequence. Prepilin is processed by a cognate prepilin peptidase to produce pilin, with an unusual first amino acid residue, *N*-methyl phenylalanine. Assembly occurs from the cytoplasmic membrane outwards and three additional proteins have been identified, PilC and PilB, in the cytoplasmic membrane and PilQ in the outer membrane. Their role in pilin assembly or function is currently unknown. Twitching direction appears to be controlled, and homologues of the chemosensory system which control flagellar activity have been identified (Alm and Mattick, 1997; Darzins and Russell, 1997; Watson et al., 1996). It is to be expected, therefore, that a number of cytoplasmic proteins remain to be identified which control the activity of the pili.

The mechanisms involved in twitching are still a mystery. Early research suggested that movement was the result of the pili retracting and extending. Extension and retraction have also been suggested as a mechanism for cell-cell interaction during social gliding. The helical nature of the fibrillar strands in the pili has lead to the suggestion that rotational forces coupled to retraction may be important in twitching. Recent studies using laser tweezers to measure the force of retraction of pili showed that the forces of retraction could exceed 80 pico-Newton (pN), moving cells at about 1 μm/s (Kaiser, 2000; Merz et al., 2000b). As protein synthesis and the *pilT* gene are both required, it is thought that Type IV pili attach cells to surfaces or to other cells, and then retract the pili pulling the cell forward. The subsequent resynthesis and reattachment of the pili allows cells to pull themselves along surfaces as the result of pilin retraction. Electron micrographs of *Neisseria gonorhoeae* twitching colonies show extensive pili interconnecting cells in the colonies, suggesting that these pili may be involved in colony movement (Merz and So, 2000a).

Intracellular Movement by Mobilizing Actin Filaments

Several pathogenic bacterial species (including *Salmonella* and *E. coli*) have developed mechanisms for usurping and exploiting the eukaryotic cells' own actin-based cytoskeleton machinery to aid invasion (Frischknecht and Way, 2001; Goosney et al., 1999; Zhou et al., 1999). Several species have taken this ability further and have evolved mechanisms for taking over the eukaryotic cytoskeleton both for the process of invasion and for cell-to-cell spreading. Species such as *Shigella flexneri*, *Listeria monocytogenes* and *Rickettsia rickettsii* produce toxins that cause actin polymerization and cell ruffling close to the bacterium, inducing uptake into the eukaryotic cell. Once inside the cell, the bacteria produce a number of toxins allowing release of the bacteria from the phagocytic vacuole. They also produce surface proteins which act as nucleation centers for polymerization of the eukaryotic actin. The polar expression of these proteins results in the formation of actin "comet"-tails capable of exerting considerable mechanical force—proteins which, by polymerization, depolymerization and association with a number of actin-associated proteins (such as profilin and small GTPases), push the cells through the cytoplasm at speeds of about 12 μm/min and cause spreading of the bacteria both across the epithelium and from cell to cell (Cossart and Bierne, 2001; Dramsi and Cossart, 1998; Frischknecht and Way, 2001; Gerbal et al., 2000; Heinzen et al., 1999; Kuo and McGrath, 2000; Nhieu and Sansonetti, 1999; Rathman et al., 2000; Tran-Van et al., 1999; Tran-Van et al., 2000).

Behavioral Control

Motility can be divided into at least three types: free-swimming, swarming over surfaces using flagella, and gliding or twitching. In all cases, expression of the locomotory organelle requires a large number of genes, and expression is energetically expensive. In addition, while running, a proton motor may take less that 1% of the proton-motive force of a bacterium growing under rich conditions, it may take several percent under growth-limiting conditions. The observation that many species stop swimming in rich growth conditions and only start under limiting conditions, strongly suggests that motility provides a major advantage under most naturally occuring conditions. To help a bacterium reach or maintain itself in its optimum environment for growth, motility must be under the control of environmental sensing systems. In general, bac-

teria are far too small to be able to sense a gradient along their length. They therefore sample their environment in time, comparing the concentration or strength of a stimulus at one time with that a few seconds earlier (Berg, 1983). All bacteria tend to move in a random pattern, whether three dimensionally, as with free-swimming cells, or two dimensionally on surfaces. This random pattern is biased in a favorable direction by the cell changing direction more often when moving away from a positive stimulus and changing direction less often when moving in a positive direction (Berg and Brown, 1972; Block et al., 1982). Prokaryotes can sense a very wide range of stimuli, including light, oxygen and other terminal electron acceptors, extracellular chemicals, intracellular metabolic state, pH, osmolarity and even, in some cases, the Earth's magnetic field. All of these signals must be sensed and balanced to produce an overall response. Of course, different species will respond to different stimuli, and many will be repelled by the dominant attractants of other species. Over the past few years, it has become apparent that the rather straightforward chemosensory pathway identified in *E. coli*, while being the core of chemosensing in other species, is a much simpler system than that used by many bacteria outside of the γ subgroup.

To allow any kind of behavioral response, a number of sensory steps are required: 1) the stimulus must be sensed; 2) the signal must be relayed to the motor apparatus; 3) the signal must be rapidly terminated; and 4) the receptor must be reset to allow future changes to be sensed. The mechanisms involved in these stages is best understood in *E. coli* (Armitage, 1999a; Armitage and Schmitt, 1997c; Falke et al., 1997).

Chemotaxis in *E. coli*

The best understood chemosensory system is that of *E. coli* and the closely related, *Salmonella*. Transport and metabolism are not required for chemotaxis by *E. coli*. Transport mutants are still chemotactic, whereas specific chemotaxis mutants can still metabolize the chemoattractant (Adler, 1969). In addition, nonmetabolizable analogues are still attractants. Four chemoreceptors, Tsr (for serine), Tar (for aspartate and maltose), Trg (for ribose and galactose), and Tap (for dipeptides), have been identified (Stock and Surette, 1996). The Tap receptor is found in *E. coli*, but not *Salmonella* (Manson et al., 1986). *Salmonella* has a receptor, Tcp, for citrate, which is not a metabolite for *E. coli* (Yamamoto and Imae, 1993). This illustrates that even the pathways of two very closely related species have adapted to the metabolic requirements of that species. In addition, a related protein has been identified, Aer, which is involved in oxygen sensing (see Aerotaxis in this Chapter).

S-Adenosyl methionine is required for resetting the signalling state of the receptors, i.e., adaptation (Aswad and Koshland, 1975; Kort et al., 1972). Mutants lacking S-adenosyl methionine behave like mutants with deficiencies in the proteins of the adaptation pathway, and tumble constantly. Receptor methylation, the extent of which depends on whether an attractant is added or removed, resets the receptor protein into a nonsignalling state and allows gradient sensing. In *E. coli*, therefore, one of a limited number of attractants binds to transmembrane receptors, and as a result, a signal is generated which results in a change in swimming pattern, biasing the random swimming pattern in a favorable direction. Modification of the receptors stops signal generation, and if the concentration of stimulus remains unchanged, allows gradient sensing. Five genes responsible for encoding the proteins for general chemosensory signal transduction were identified in motile but non-chemosensing mutants of *E. coli*. These (*cheB*, *cheR*, *cheA*, *cheY* and *cheZ*) form the intracellular signalling sequence to the flagellar motor. Related schemes have been found in almost all other motile species, but in the majority of nonenteric species, the sensory pathways are more complex (see section on Chemotaxis in Nonenteric Species in this Chapter).

Chemoreceptors

The chemoreceptors of *E. coli* are all built on the same basic design (Falke et al., 1997; Mowbray, 1999; Mowbray and Sandgren, 1998). Sequencing of the chemoreceptors of a wide range of species from archaea through to bacteria has identified a common domain in all receptors necessary for sensory transduction. The conservation of the core of the chemosensory pathway between species has allowed identification of the likely chemosensory genes in many species whose genomes have recently been identified. One of the major surprises (along with the number of apparently parallel signalling pathways in many species, see later) has been the number of chemoreceptor genes, which can be over 25 in species such as *Pseudomonas palustris* and *Vibrio cholerae*, and seems to average 10–15 in other species. This suggests that bacteria faced with complex environments have receptors to sense a wide range of chemical stimuli, while others, such as *E. coli* use a pared down system suited to a world with limited changes.

The four *E. coli* chemoreceptors (533–553 amino acids in length) have two membrane-spanning domains (a large periplasmic domain and a large cytoplasmic N-terminal domain).

Recent crystal and nuclear magnetic resonance (NMR) structures of the periplasmic and the cytoplasmic domain suggest that the chemoreceptors are one of the longest proteins found in a bacterial cell, extending a distance of 40 nm from the chemoeffector binding domain to signalling domain (Djordjevic and Stock, 1998; McEvoy et al., 1998). There is very little homology between the periplasmic domains on a sequence level, nor is there obvious similarity between the transmembrane domains, however, the cytoplasmic domain is very highly conserved between transducers. It is this highly conserved cytoplasmic domain that is common to chemosensory transducers across the bacterial and archaeal world.

These chemosensory receptors are called "methyl-accepting chemotaxis proteins" or MCPs and form stable dimers in the cell's membrane. The periplasmic domains of the different MCPs "sense" different chemoeffector molecules. Interestingly, some receptors, for example Tar, can sense two completely different stimuli, in this case the amino acid aspartate and the sugar maltose, bound to its periplasmic binding protein (PBP; Gardina et al., 1992; Gardina et al., 1997; Gardina et al., 1998). This has interesting implications for the sensing of the two chemoeffectors acting through Tar. The Tar receptor itself is expressed as part of the flagellar and chemosensory regulon, and as aspartate interacts directly with Tar, interaction will be directly related to the extracellular concentration of the amino acid. Expression of the maltose PBP, on the other hand, is under the control of the maltose transport system, and the response will depend on the level of its expression. The size of the response to a given aspartate stimulus will always be the same, while that to the maltose stimulus will depend on the level of induction of the PBP. Sugars also can be sensed through the phosphotransferase transport system (see section on Phosphotransferase Sugar Sensing in this Chapter). As this is also an inducible system, it means that *E. coli* responds constitutively to amino acids, but its responses to sugars depend on growth conditions. The biological implications of this for *E. coli* have not been investigated.

Because Tar is able to respond to binding of both a small amino acid, aspartate, and a large protein (the maltose PBP), the mechanisms involved have been extensively investigated. The effector interaction appears to occur at the interface of the two MCP monomers, with steric hindrance preventing the binding of two MBPs at once and only one aspartate-binding site being accessible to solvent at any one time. Although both attractants bind at different sites, there is steric hindrance between the sites, and saturated concentrations of one attractant will inhibit responses to the second; although if both are added simultaneously, the response is partially additive (Gardina et al., 1992).

Transmembrane Signalling

Once the ligand has bound, this binding has to be signalled across the membrane to the cytoplasmic domain of the MCP. The MCPs form stable dimers, resulting in four transmembrane α-helices, two from each monomer (Milligan and Koshland, 1988). Extensive studies have been carried out on the possible mechanisms involved in signalling across the membrane, involving nuclear magnetic resonance (NMR) spectroscopy of isolated periplasmic domains and membrane-bound receptors, large-scale cysteine substitution to crosslink different regions of the α-helices, tryptophan fluorescence, and revertant and second site suppressor studies of signalling mutants to identify the interface of the transmembrane (TM) helices (Chervitz and Falke, 1995; Danielson et al., 1994; Falke and Hazelbauer, 2001; Falke and Koshland, 1987; Lee et al., 1995a; Lee et al., 1995b; Ottemann et al., 1999). The occurrence of crystal structures of the periplasmic domains that signal through to the TM α-helices suggests that there is a small vertical movement of the TM2 helix in the membrane and a slight tilt (Milburn et al., 1991; Scott et al., 1993). Binding of an attractant is therefore thought to signal through to the cytoplasmic domain by the movement of one of the TM2 transmembrane helices with respect to the relatively fixed central axis of the TM1s. However, these changes may be one component of signal transduction, as recent evidence suggests that effector binding may also bring together numbers of receptors in clusters, allowing the cytoplasmic domains to interact.

Cytoplasmic Domain

The change in receptor binding has to change the cytoplasmic domain of the chemoreceptor in such a way that the cytoplasmic signalling sequence as well as eventual adaptation of the receptor is set in train. The cytoplasmic domain of the MCPs is very highly conserved, particularly the central signalling region. Several hundred MCPs from different bacterial species (all eubacterial subgroups and from archaea) have now been sequenced, and all show extensive conservation of the cytoplasmic signalling domain. The diversity of species with this conserved signalling domain suggests that chemotaxis as well as motility has a very early evolutionary origin. The conservation of this domain in transducers (which are not only transmembrane, but may be cytoplasmic, as in *R. sphaeroides* and *H. salinarium*, or may sense oxygen via bound redox

groups) may argue that this domain is the early central component of the signalling pathway, and the sensing domains have been added according to the niche of the species.

The cytoplasmic domain is made up of 10 α-helices (5 from each monomer), which have been divided into domains on the strength of mutational and crosslinking studies (Ames and Parkinson, 1994; Bass and Falke, 1999; Borkovich et al., 1992; Spiro et al., 1997). There is a region called the "methylation domain," which contains the glutamate and glutamine residues methylated by the *S*-adenosylmethionine-dependent methyl transferase, CheR, during receptor adaptation (Kehry and Dahlquist, 1982a; Kehry et al., 1983; Kehry et al., 1985). Between these two regions is the highly conserved signalling domain.

The signalling domain is the site of interaction with CheW and CheA, the proteins involved in initiating cytoplasmic signalling to the motor, and is the region highly conserved across species (Liu and Parkinson, 1989; Morrison and Parkinson, 1997). Genetic studies show that mutations within this domain can result in cells that are either predominantly smooth swimming or predominantly tumbly, suggesting that this region controls the activity of the histidine protein kinase, CheA.

Cytoplasmic Signalling

A small protein, the 18-kDa CheW, links the signalling domain of the MCP to the histidine protein kinase, CheA (Conley et al., 1989; Gegner et al., 1992; Liu and Parkinson, 1989; Sanders et al., 1989b; Schuster et al., 1993). It has no known catalytic activity, but without it, signalling stops. Although it is thought to be a simple scaffolding protein, transmitting the conformational changes in the signalling domain to the kinase, its structure is fairly well conserved between species. The MCP-binding region of CheW has been identified by mutagenesis, but the region binding to CheA is less certain.

CheA is a soluble histidine protein kinase (HPK) homodimer. It belongs to the extensive family of HPKs identified in a wide range of bacterial species. Functioning as a dimer, CheA binds ATP to a conserved domain, which allows the protein to phosphorylate a conserved histidine, His-48, on the other monomer of the dimer, located near the N-terminus of the protein. The region containing His-48, known as P1, also has a structure conserved with other HPKs. Purified P1 has no enzymatic activity, but can be phosphorylated by another kinase, after which it can function alone to transfer phosphate to its substrates, CheY and CheB (Zhou and Dahlquist, 1997b; Zhou et al., 1995). The phosphoramidate bond of phospho-His is very unstable compared to, for example, phospho-Ser, inasmuch as the standard free energy of phosphotransfer from ATP to His is positive (Bilwes et al., 1999; McNally and Matsumura, 1991; Tawa and Stewart, 1994; Zhou et al., 1995). In vitro, phospho-CheA can phosphorylate ADP to ATP. It is assumed that in vivo, the high intracellular concentration of ATP and the rapid transfer of the phosphate to the substrate proteins keeps a high rate of CheA autophosphorylation operating with little back reaction (Bilwes et al., 1999). Between the conserved histidine and the domain involved in ATP binding is a domain, P2, which binds CheY and CheB, the eventual substrates of CheA (Shukla and Matsumura, 1995). The CheY-binding domain is flanked by two flexible regions not found in other HPKs. This domain may move rapidly and independently of the rest of the CheA molecule allowing fast inter-domain phosphotransfer, possibly resulting in an amplified signal.

CheA phosphorylates conserve aspartate residues on two competing response regulators. One is the small 14-kDa protein, CheY, which is the prototypical response regulator, having only the aspartate receiver domain of response regulators and no other domains (Stock et al., 2000). Mutants with *cheY* deleted or mutated are smooth swimming, i.e., they cannot switch the direction of flagella rotation. The CheY protein binds to the P2 domain of CheA, and phosphate is removed from His-48 in a reversible reaction to generate phospho-CheY (Welch et al., 1998). This is released from P2 and diffuses through the cell to the motor, binding to FliM of the flagellar switch and increasing the probability of the motor switching to CW, probably by reducing the energy barrier between CCW and CW rotation (Barak and Eisenbach, 1992a; Ravid et al., 1986).

CheY, being only 14 kDa, has been crystallized and also studied by solution NMR spectroscopy under a range of conditions, including recently in a phosphorylated conformation. The structure of CheY is probably common to the phosphorylation domains of all response regulators. It has 5 α-helices surrounding a 5-stranded parallel β-sheet structure, with the phosphorylation site on aspartate 57. In addition, there is another group of conserved acidic, usually aspartate, residues that are located close to the active site forming an acidic pocket (Bourret et al., 1990; Cho et al., 2000; Sanders et al., 1989a; Stock et al., 1989; Volz and Matsumura, 1991; Zhu et al., 1997a). Phosphorylation depends on Mg^{2+} and involves CheY itself acting as a phosphotransferase. Indeed, Che Y can take phosphate from several metabolic phosphodonors such as carbamoyl phosphate or acetyl phosphate, although whether this has a role under natural conditions

is unknown. The presence of Mg^{2+} probably serves to stabilize the transition state. Dephosphorylation of CheY-P occurs autocatalytically with a half time of under 10 s. This is in contrast to many other response regulators that remain phosphorylated for many tens of minutes, and reflects the need for a fast signal and signal termination in chemotaxis. The majority of HPK and response regulator systems identified are involved in controlling transcription, and therefore rapid signal termination is not required. The natural rate of autodephosphorylation of *E. coli* CheY-P is still not fast enough for signal termination in chemotaxis, and a second protein (CheZ) increases the rate in enteric species. Not well characterized, CheZ has been isolated as a dimer, as well as a high order oligomer in the presence of phospho-CheY, but not of CheY. Also, CheZ appears to compete with FliM for CheY-P, but not for CheY (Blat et al., 1998; Blat and Eisenbach, 1994; Kuo and Koshland, 1987; McEvoy et al., 1999; Stock and Stock, 1987; Zhu et al., 1997b). The oligomer is thought to increase the rate of hydrolysis.

CheZ homologues have not been found in species outside the γ-subgroup. Bacteria from other subgroups do, however, usually have two copies of CheY, one often fused to CheA. It seems possible that signal termination occurs through the activities of these two CheYs, with both CheY homologues being phosphorylated, but only one able to bind the flagellar motor. As CheY-P can phosphotransfer to CheA, the second non-FliM binding CheY-P could operate as a phosphate sink, particulalry if the two CheYs had different phosphotransfer kinetics (Armitage and Schmitt, 1997c; Sourjik and Schmitt, 1998a).

Adaptation

CheA not only transfers phosphate to CheY, it can also phosphorylate another response regulator, CheB. Another unusual response regulator, CheB has a catalytic methyl esterase domain, in addition to the regulatory domain of a standard response regulator (Lupas and Stock, 1989; Stewart and Dahlquist, 1988). The activity of the methyl esterase is controlled by whether the aspartate-containing domain is phosphorylated, and the phosphorylation increasing methyl esterase activity by an order of magnitude. Adaptation is an essential part of chemotaxis. If the receptor were not reset after encountering a change in receptor occupancy, the receptor would continue to generate a signal and the cell would be unable to respond to future changes. In fact, mutants in the adaptation mechanism are either smooth swimming, CheR mutants, or constantly tumbling, CheB mutants. Therefore, although the signalling pathway is intact, without the ability to reset the receptor, a gradient cannot be sensed.

The cytoplasmic domains of most MCPs have two regions which have conserved glutamate residues that serve as substrates for the two enzymes involved in receptor adaptation (CheR, the methyl transferase and CheB, the methyl esterase). In *E. coli*, there are three methylation sites on one domain and one on the C-terminal domain (Kehry and Dahlquist, 1982b). The glutamates may be transcribed as glutamines, but CheB can posttranslationally deamidate the amino acid to form glutamate. It appears that newly translated MCPs have glutamines as well as glutamates to prevent inappropriate signalling. Mutants deleted for CheR cannot methylate the receptors and constantly swim, whereas CheB mutants have overmethylated receptors and tumble constantly. The decrease in attractant bound to a receptor changes the conformation of the cytoplasmic domain of the receptor and increases the autophosphorylation rate of CheA. In addition, CheY and CheB compete for binding to P2, and phospho-CheB becomes an active methyl esterase (West et al., 1995). This can then remove methyls from the conserved glutamates of the relevant MCP. The methyl groups are released generally as methanol (Kehry et al., 1985). The change in methylation probably alters the packing of the methylation helices and reduces the signal through the signalling domain to CheA. The four methylation sites can be methylated independently to alter the helical configuration, allowing adaptation over a range of stimulus strengths. This process of posttranslational reversible carboxymethylation of glutamates appears unique to bacterial chemotaxis (Shapiro et al., 1995; Springer et al., 1982).

The methyl groups are added to the glutamates by a specific methyl transferase, CheR, which has been crystallized and the structure elucidated (Djordjevic and Stock, 1997). There is a specific CheR docking motif on the C-terminal end of some, but not all, MCPs (Barnakov et al., 1998). In *E. coli* and *Salmonella*, the chemoreceptors Tsr, Tar and Tcp have a CheR-binding domain but Trg and Tap do not; nevertheless, they are still methylated in response to attractant binding. However, Trg is not methylated if Tsr or Tar are not present in the membrane, suggesting that the CheR bound to Tsr or Tar can methylate the glutamate residues of Trg, and indicating that they must be physically close in the membrane (Feng et al., 1999).

Localization of MCPs

Early studies of MCPs suggested that they were randomly located around the cell. However, immunogold electron microscopy using antibody

raised to the highly conserved domain of MCPs showed that MCPs are in fact localized at the poles of *C. crescentus*, *E. coli* and *R. sphaeroides* (Alley et al., 1992; Harrison et al., 1999; Maddock and Shapiro, 1993). In addition, they are also found in a cytoplasmic cluster in this latter species. Green fluorescent protein (GFP) fusion of a membrane-spanning MCP (McpG) and a cytoplasmic MCP (TlpC) expressed from genomic constructs has identified specific chemoreceptors at the poles and in the cytoplasm of *R. sphaeroides* (Wadhams et al., 2000). Detailed measurement of the numbers found at the poles of *E. coli* and the fluorescence at the poles and cytoplasm of *R. sphaeroides* suggest that the MCPs are targeted after cell division, as one pole always has significantly larger numbers than the other, but during cell growth, the number appears to increase. If mutants lack either CheW or CheA, the MCPs are found dispersed over the surface of the cells, but deletion of CheR and/or CheB does not affect localization. The cytoplasmic proteins CheA and CheW are therefore essential for localization of MCPs. Figure 4 illustrates the localization of an MCP in *R. spheroides*.

There are several reasons why MCPs might be located at the poles. 1) Bacteria may in fact have a "nose," and clustering receptors may help in gradient measurement. This is very unlikely as almost all bacteria are too small to sense a gradient along their length, and there is no evidence (with the possible exception of gliding mycoplasmas) that they swim with a particular pole forward (Berg and Turner, 1995). Indeed *R. sphaeroides* has a flagellum which is subpolar, causing the cell to swim with the long side of the cell forward, but the MCPs are still at the two poles, and it has been shown that *E. coli* swims with either pole forward. 2) This is simply the default location. Many proteins appear to default to the previous septation site, and the several thousand MCPs, not required for transport or energy producing processes, may localize there. 3) Localization may allow methylation of Trg or Tap by CheR-bound Tsr or Tar (Beel and Hazelbauer, 2001). 4) Changes in the binding of a chemoeffector to the periplasmic domain of an MCP may alter the packing of the MCPs in the membrane, bringing together the cytoplasmic domains in a conformation that allows signalling (Duke and Bray, 1999; Levit et al., 1998; Stock, 1999). Thus, a repellent would alter packing such that CheA would become active, whereas the addition of an attractant would hold CheA in an inhibited conformation. The glutamate residues involved in adaptation would therefore cause signalling because of electrostatic repulsion between cytoplasmic domains. When methylated, the charges would be masked and the conformation become active. On the other hand, when demethylated, the glutamate charges would result in repulsion, conformational change and signalling. Higher order interaction between receptor dimers would be essential for signalling in this model, hence the polar clusters. A model based on signalling through the formation of receptor clustering has been proposed in which the dynamic range of sensitivity seen in bacterial chemosensing depends on controlling the sizes of the receptor clusters (the extent of lateral packing controlling the signal), allowing a bacterial cell to respond to a change of a few molecules over background concentrations of several orders of magnitude (Bray et al., 1998; Shimizu et al., 2000). The evidence that receptor clustering is required for this level of sensitivity is currently limited, but modelling and crystal structures of the cytoplasmic domain indicate that the chemoeffector dimers may pack as trimers of dimers, and it is possible to develop higher order packing models based on these dimer-trimers.

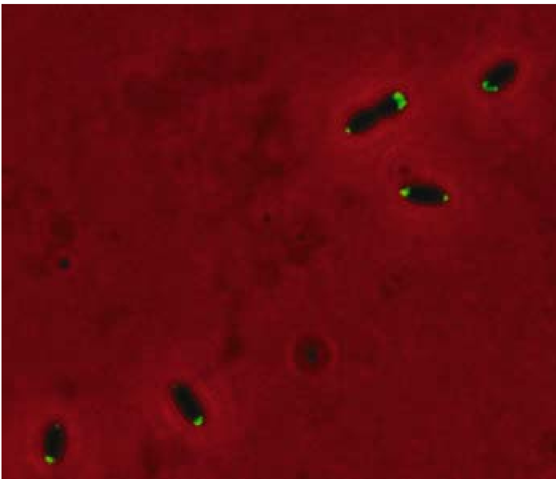

Fig. 4. Localization of a methyl-accepting chemotaxis protein (MCP) in *Rhodobacter sphaeroides*. The gene encoding McpG in *R. sphaeroides* was replaced behind its native promoter by a gene encoding McpG-GFP. The MCP was found to form clusters at the poles of the cells. These clusters depend not on the MCP itself but on the chemosensory proteins CheA and CheW. From Wadhams et al. (2000).

Phosphotransferase Sugars

Escherichia coli not only responds to the binding of sugar-binding proteins to MCPs, but also to sugars transported through the phosphoenolpyruvate-dependent phosphotransferase system (PTS) independently of the MCPs. *Escherichia coli* has at least 15 PTS systems (Nikaido and Saier, 1992). Membrane-bound substrate-specific transport proteins, enzymes II

or EII, accept phosphate from a nonspecific donor enzyme, enzyme I or EI, and phosphorylates the sugar as it is transported. Enzyme EI, a phosphoenol pyruvate (PEP)-dependent histidine kinase and a phosphohistidine carrier protein (HPr) are the phosphorelay to the EIIs. Although metabolism of the sugar is not required for a chemotactic response, the sugar must be transported to cause a signal. Methylation is not required for signalling to occur, but CheA and CheY are necessary. Enzyme EI of the PTS has been shown to interact directly with CheA, suggesting that EI when actively involved in transporting sugars suppresses CheA phosphorylation, resulting in smooth swimming (Lengeler and Jahreis, 1996; Lux et al., 1999; Pecher et al., 1983). Interestingly, fructose is transported via a rather different PTS system, where phosphorylation relies on FPr, a fusion between an HPr-like protein and EI. This protein does not cause a chemotaxis signal, and *E. coli* transporting through this pathway is not chemotactic to fructose. *Rhodobacter sphaeroides* has only an FPr PTS, and its chemotactic response to fructose depends not on PTS transport, but on metabolism of the sugar (Jeziore et al., 1998).

Thermotaxis

Escherichia coli not only responds to changes in chemical concentration, it also responds to changes in temperature (Maeda et al., 1976). Cells swim towards their growth temperature, biasing their swimming pattern from either a lower or higher temperature. Mutants in the serine receptor, Tsr, no longer respond to temperature and neither do cells incubated in saturating levels of serine. Temperature probably alters the conformation of the cytoplasmic signalling domain of Tsr and causes a sensory signal, which is inhibited if the receptor is fully occupied and a conformational change cannot be induced (Imae, 1985). Although Tsr seems to be the major thermosensory receptor, the other receptor proteins, particularly Trg and Tar, have also been shown to respond to temperature changes. Thus, Tsr, Tar and Trg appear to be "warm" sensors, while Tap acts as a "cold" sensor. However, though unmethylated Tar receptor acts as a "warm" receptor, when completely methylated, it will function as a "cold" receptor. Mutations in the adaptation sites or of the adaptation proteins alters the responses. Thermosensing may be caused by the temperature-dependent equilibrium between methylation and demethylation of the receptors, temperature-dependent alterations in this would result in a sensory signal to the motor (Nishiyama et al., 1997; Nishiyama et al., 1999).

Repellent Sensing

Chemotaxis studies on *E. coli* tend to use the removal of an attractant or addition of a repellent as the signal. Addition of a repellent results in a change in receptor methylation similar to the removal of the attractant, but the characteristics of the response have never been as clear cut. *Escherichia coli* shows repellent responses to heavy metals (such as Ni and Co), to changes in pH and amino acids (such as leucine and organic acids). The latter two can be growth substrates, indeed *E. coli* grows very well on acetate. Why *E. coli* should be repelled by good metabolites is not understood, and may indicate a more nonspecific mechanism involved in repellent responses than in the sensing of attractants (Kihara and Macnab, 1981; Repaske and Adler, 1981).

Tsr again appears to be a major receptor for repellents, although the other receptors may be involved, and changes in methylation may affect all receptors. The responses to organic acids and pH may all involve changes to the cytoplasmic signalling domain of the MCPs in a similar manner to temperature stimuli. Recently studies into the involvement of fumarate in receptor-independent control of flagellar switching suggested that several repellents may act by altering the activity of the fumarase enzyme, and any increase in cytoplasmic fumarate might cause motor switching directly, independently of the chemosensory pathway (Barak and Eisenbach, 1992b; Marwan et al., 1990; Montrone et al., 1998; Prasad et al., 1998).

The simultaneous release of "caged" positive and negative chemoeffectors by a flash of ultraviolet light, and the use of time-resolved motion analysis, suggested that while single receptors time-averaged different ligands, when effectors using two different receptors were used, a non-integrated response could result, with the cells initially tumbling and then smooth swimming (Jasuja et al., 1999; Khan et al., 1993). This suggests that the processing time for the signals through different receptors may not be identical and the signals may not be of the same strength ("caged" compounds are chemoeffectors chemically bound to a large molecule which can be released by photolysis to produce a step-up in effector concentration within a very short and measurable time frame).

Osmotaxis was one of the early responses measured by Pfeffer, with bacterial cells being shown to respond to their osmotic environment. The relationship between osmotaxis and chemotaxis is not really understood. *Escherichia coli* is attracted to its optimum concentration of an osmotic agent such as sucrose or ribitol and repelled by higher or lower concentrations.

These responses appear to be independent of the MCP receptors (Adler et al., 1988; Li et al., 1988; Qi and Adler, 1989).

Pattern Formation in Enteric Species

One of the earliest observations of *E. coli* chemotaxis involved the formation of moving rings of bacteria through soft nutrient agar. When the soft agar contained a number of metabolizable attractants, several moving rings of cells were seen, but if a mutant lacking a specific chemoreceptor was used, fewer rings would be present. This led to the realization that populations of bacteria were following gradients of nutrients, the gradients caused by their own metabolism. One ring was the population following, for example serines, while the second ring might be the population following the gradient of maltose. Several years later, much more elaborate patterns were seen in soft agar, with *E. coli* aggregating to form regular, complex but stable patterns on some substrates, particularly succinate. The complex patterns formed from the circular swarm rings described above, with of the formation of focal aggregates within the rings responding to secreted attractants, usually aspartate, to produce highly symmetrical complex geometric patterns (Brenner et al., 1998; Budrene and Berg, 1995; Emerson, 1999).

Chemotaxis in Nonenteric Species

While the majority of research has been carried out on *E. coli* and *S. typhimurium*, there is an increasing body of literature on a wide range of other species. This combined with the increasing number of complete genome sequences suggests that chemotaxis in the majority of other species is much more complex. Other species often have multiple copies of the chemosensory genes, and expression of the different genes may be controlled by the environment in which the cells are grown, that is they can fine-tune their chemosensory response to current growth conditions. In addition, many species have chemoreceptors that are only expressed under certain growth conditions, and some of these receptors are cytoplasmic and probably sense the metabolic state of the cell. Thus, unlike *E. coli* which uses only four constitutive receptors to sample the environment, and has no choice but to respond if there is a gradient of a specific chemoeffector, other species may only respond if they are currently limited for that nutrient. The Department of Energy (DOE) and the Intsitute of Genomic Research (TIGR) bacterial genome databases (http://www. jgi.doe.gov/JGI_home.html) suggest that these complex chemosensing systems may be common in bacterial species living in complex environments with flexible metabolic capabilities.

Alpha-Subgroup of Proteobacteria *Rhodobacter sphaeroides*, *Sinorhizobium meliloti*, *Rhodopseudomonas palustris* and *Caulobacter crescentus* are all members of the α-subgroup of Proteobacteria, and all now have sequenced genomes. This particular subgroup includes a very large number of free-living and symbiotic soil and water microorganisms. Analysis of their chemosensory system illustrates well the common themes that are being identified outside of the much studied γ sub-group. They also illustrate that chemosensing relies on a common central signalling pathway, related to that identified in the *E. coli*, but it has either become more elaborate in the α subgroup, or that of *E. coli* represents a pared-down system.

Rhodobacter sphaeroides has great metabolic flexibility. It is able to grow anaerobically as a photoheterotroph or using anaerobic respiration as an aerobic heterotroph. It also has a quorum-sensing system and may form biofilms (Puskas et al., 1997). It can fix nitrogen and carbon dioxide and can also ferment. *Sinorhinozobium meliloti* is an obligate aerobe, but it can fix nitrogen when maintained anaerobically in a symbiotic relationship with leguminous plants. *Caulobacter crescentus*, on the other hand, appears to have a restrictive metabolic lifestyle, living as a strictly aerobic organism in oligotrophic environments. However, it does go through a differentiation cycle with stalked cells dividing to release motile swarmer cells that cannot grow or divide but swim to new locations to settle down and differentiate into stalked cells and start the cycle again (Brun et al., 1994). The genomes of *S. meliloti*, *C. crescentus*, *Rps. palustris* and *R. sphaeroides*, while not all fully assembled, have provided details of the motility and chemosensory pathways.

A combination of the molecular genetic studies on the different species plus the genome sequencing of these species suggests they may have similar chemosensory pathways, much more complex than those of *E. coli* and *Salmonella*. They appear to have a large numbers of MCPs, between 12 and 28 (G.H. Wadhams and J.P.Armitage, unpublished observation; DOE Joint Genome Institute Sequence Database) (http://www.jgi.doe.gov/JGI_home.html). Deletion of these putative receptor genes does not result in the complete loss of chemosensory responses to a specific attractant, but usually only results in a reduction in a response, sometimes to a group of compounds. Not all of the genes

code for membrane-spanning proteins, but some code for cytoplasmic proteins. These still have the highly conserved signalling domains of MCPs, but no obvious transmembrane regions. These probably sense metabolites inside the cell, not in the extracellular environment. The chemosensory responses of *R. sphaeroides* are not identical under all growth conditions, and this species will respond to some carbon and nitrogen sources only when they are limiting for growth (Poole and Armitage, 1989). A large number of studies have shown that transport and metabolism are required for some responses to compounds as diverse as ammonia, sugars and amino acids, again suggesting a link to growth conditions.

The expression of the chemosensory systems and MCPs in *R. sphaeroides* has been found to be environmentally regulated. Immunogold electron microscopy and Western blot analysis using an antibody against the conserved region of MCPs showed a 17-fold increase in MCP levels under aerobic conditions compared to anaerobic conditions (Harrison et al., 1999). Under aerobic conditions, large clusters of MCPs are seen at both poles of the cell, and there are also clusters within the cytoplasm. But under anaerobic conditions, the polar clusters are greatly reduced in number. This supports the hypothesis that the cytoplasmic receptors may sense metabolic state and only respond if limiting for that particular metabolite. A GFP fusion to one specific *R. sphaeroides* MCP, McpG, shows that this receptor is expressed under both aerobic and anaerobic conditions, as is the cytoplasmic receptor TlpC (Wadhams et al., 2000). However, TlpC is expressed at a much lower level under all conditions, and segregation just before cell division suggests copy number is important for proper chemosensory behavior.

Genetic analysis and gene sequencing has shown that *R. sphaeroides* and *Rps. palustris* have three and *C. crescentus* and *S. meliloti* have two chemosensory operons coding for multiple homologues of the *E. coli* chemosensory genes. Interestingly, in all species, it seems that one operon may be dominant under laboratory conditions, mutations producing measurable phenotypes, and the roles of the other operons is less clear. This may indicate that these dominate under different growth conditions, e.g., in biofilms. All these species, and indeed every species studied outside of the γ-subgroup, lack a CheZ homologue, but instead have two or more CheY homologues, in the case of *R. sphaeroides*, seven *cheY* genes have been identified. Rapid signal termination is an essential component of chemotaxis, and CheZ is thought to increase the rate of CheY-P dephosphorylation in *E. coli*. In vitro studies examining the phosphorylation and dephosphorylation kinetics of the purified CheA and two of the CheY homologues of *S. meliloti* suggest that one of the CheY homologues function as a phosphate sink to terminate the chemosensory signal (Sourjik and Schmitt, 1998a). One CheY may be able to bind the flagellar motor when phosphorylated, and the other not. The motor-binding CheY may rephosphorylate CheA, the second (non-motor binding) CheY then acts as a phosphate sink and causes rapid signal termination (Shah et al., 2000a). However, *H. salinarium* and *B. subtilis* also have two copies of CheY, and experimental data on these species suggest an interaction of CheY-P with the MCP via another chemosensory protein (CheD) not found in the γ group, and this helps in receptor adaptation rather than signal termination (Kirby et al., 1999; Rosario and Ordal, 1996). As *S. meliloti* and *R. sphaeroides* also have copies of *cheD*, the role of the multiple CheYs remains somewhat uncertain. The role of the CheY homologues in *R. sphaeroides* might be even more complex as there are at least seven. Mutagenesis suggests that only two have a major motor-binding function, and the reason for the other five is unknown, although mutation alters the chemosensory phenotype by increasing response duration (Shah et al., 2000b). In vitro dephosphorylation studies of the *R. sphaeroides* CheYs suggests a much faster inherent dephosphorylation rate than *E. coli* CheY, perhaps indicating an alternative role for the multiple homologues in these species.

The role of the different chemosensory protein homologues has yet to be elucidated. Few major mutational studies have been undertaken in *C. crescentus*, while in *R. sphaeroides* deletion of one operon with two *cheY*, one *cheA*, one *cheW* and one *cheR* homologue(s) results in only minor changes under some growth conditions. Deletion of the second operon, which has a third *cheY*, second *cheA*, two more *cheW*, a second *cheR* and a *cheB* gene results in the loss of chemosensory and photosensory responses under all conditions (Romagnoli and Armitage, 1999). All the genes when expressed in *E. coli* interfere with *E. coli* chemotaxis, while not altering growth, suggesting a role in chemotaxis (Hamblin et al., 1997; Shah et al., 2000a). Only CheB and CheR from *R. sphaeroides* fully complement *E. coli* mutants, but the *R. sphaeroides* CheY and CheW proteins restore normal swimming. The role of the products of the third operon remains to be identified.

Two of the operons are expressed differently under different growth conditions, with the highest levels of expression under aerobic conditions, suggesting that the different cytoplasmic sensory-transducing proteins may be connected to different sensory receptors. Interestingly, expres-

sion of the *R. sphaeroides* Che operon 2 is under the control of the Prr (Reg) histidine protein kinase system, which also controls expression of the photosynthetic genes, CO_2 and N_2 fixation genes in response to changes in electron flow through the terminal cytochrome cbb_3 oxidase, indicating a tight interconnection between metabolic capability and chemosensory behavior under these conditions (Dubbs et al., 2000; Eraso and Kaplan, 2000; Oh and Kaplan, 2000). Many more species have now been found to have multiple copies of the chemosensory genes, and hopefully research on other species will identify whether the sensory pathways operate under different growth conditions or are linked to different sensory receptors.

Obviously, to be of any advantage to the survival of a species, bacteria must respond to metabolites that they have evolved to use. Chemosensory receptors have almost certainly evolved in parallel with the metabolic pathways of the species. For example, while amino acids are the dominant attractants for *E. coli*, they are minor attractants for *R. sphaeroides*, which shows the strongest responses to organic acids, their favored carbon source. *Pseudomonas putida* will grow on and has an MCP-like chemoreceptor for naphthalene. The conserved cytoplasmic signalling domain compared with those of *E. coli*, but the major differences in the periplasmic sensing domain is a good illustration of how the conserved chemosensory pathway has been adapted to the lifestyle of specific species (Grimm and Harwood, 1999).

There is evidence that some transport proteins may be able to cause signalling independently of MCP. A good example of this is in *Pseudomonas putida*, which grows on the aromatic acid, 4-hydroxybenzoate and shows chemotaxis towards this compound. The permease for 4-hydroxybenzoate (4HB), PcaK, has been found to be not only a member of the major-facilitator-super family of transporters, but also required for the chemotactic response to 4HB and several other aromatic acids (Harwood et al., 1994). The permease PcaK does not resemble any MCPs, but is a classical transport protein with 12 membrane-spanning helices. Mutations in PcaK result in the loss of taxis to the aromatic acids, even at pHs where the aromatic acids are able to diffuse into the cell and allow normal growth, suggesting a real signalling role for the transport proteins in chemotaxis (Ditty and Harwood, 1999; Parales et al., 2000). This is probably a very different process from the PTS signalling process in *E. coli*. Analysis of sequenced genomes indicates that several more species may have transporters that may also be involved in sensory signalling.

BACILLUS SUBTILIS Few species have had the same attention lavished on them as *E. coli*, and therefore the sensory pathway in other species is much less well understood. *Bacillus subtilis* has, however, been studied in some detail, and also has a complete genome sequence. While *B. subtilis* may not have multiple homologues of many Che genes, as seen in the α-subgroup, it does appear to have copies of all the proteins identified as being involved in chemotaxis across all species, and as such, has been suggested to represent the most complete chemosensory system of species investigated to date. *Bacillus subtilis* responds to a very wide range of amino acids and sugars, and a large and diverse number of repellents have been identified. As with many nonenteric species, *B. subtilis* has all the central components of the *E. coli* signalling pathway, except CheZ. It does not, however, have two free copies of CheY, but rather a fusion between CheW and CheY called "CheV." Whether this fusion is involved in signal termination as suggested for the CheA-CheY fusions is not known, but there are suggestions it may have a role in receptor adaptation rather than signal termination. The number of receptors is small, as in *E. coli*, but the large number of amino acids sensed suggests that the amino acids probably do not interact with the receptors directly, but may use binding proteins. These have not yet been identified. The responses to sugars may involve the PTS transport system and E-I, as in *E. coli*, but in the case of *B. subtilis*, MCPs are also required for a normal response (Garrity et al., 1998). It is possible, however, that the requirement for MCPs in PTS signalling is indirect rather than direct, with the MCPs providing the scaffold for the CheW/CheA proteins. A major difference from the *E. coli* sensing paradigm is that an increase in CheY-P concentration appears to be the response to an increase rather than decrease in attractant. An increase in CheA phosphorylation activity results in smooth swimming in *B. subtilis* rather that tumbling (Rosario et al., 1995; Rosario and Ordal, 1996). Two proteins CheD and CheC seem to be involved in both CheB/CheR-dependent adaptation and signal termination. The MCPs of *B. subtilis* seem fairly similar to those of *E. coli* in that deletion causes the loss of responses to amino acids. However, although the MCPs have the glutamate residues required for methylation during receptor adaptation, the extent of their methylation shows little change after stimulation. What does change is the rate of turnover of methyl groups on all receptors, with methyl groups apparently moving from one MCP to another (Kirby et al., 1999; Zimmer et al., 2000). Also, CheC inhibits CheR-dependent methylation of MCPs with mutations in *cheC* resulting in smooth swimming and increased

methylation. Mutations in the other unusual gene, *cheD*, cause tumbly phenotypes and low levels of MCP methylation. This has led to the hypothesis that CheD binding to MCP is required for CheR to bind and methylate the MCP, and the binding of CheD is negatively controlled by CheC. The addition of an attractant would thus produce high levels of CheY-P, which would allow CheD and CheC to interact, and this would reduce their binding to the MCP and thus reduce CheA activity and the subsequent level of CheY-P.

In *E. coli*, methyl groups released from MCPs following demethylation are released as methanol only after the addition of a repellent or removal of an attractant. In both *B. subtilis* and the archaeon *H. salinarium*, methanol is released when either attractants or repellents are added, rather than when attractant is removed or repellent added. The methanol is not, however, released immediately, as in *E. coli*, but after several cycles of stimulation. There are data suggesting that methanol is not released from the MCPs directly, but from a protein to which the methyl groups are transferred, probably via CheB. The CheB protein does not seem to cause the release of the methyl groups as methanol as in *E. coli*, but to transfer the methyls to a carrier (Kirby et al., 1999).

The pattern of methylation and methanol release is very similar to that seen in the archaea, suggesting an early origin for this mechanism. Interestingly, sequence analysis of chemotaxis operons of *S. meliloti* and *R. sphaeroides* suggests that a *cheD* homologue is present in these Gram-negative eubacteria. Mutations in this gene result in abnormal behavior, with a change in the pattern of methanol release, but its role has not been fully characterized (Rosario and Ordal, 1996; Sourjik et al., 1998b). It may be relevant that patterns and the extent of methanol release in *S. meliloti* and *R. sphaeroides* are not as simple as in either *E. coli* or *B. subtilis*.

MYXOCOCCUS XANTHUS *Myxococcus xanthus* is a Gram-negative, social, gliding bacterium that moves very slowly in two dimensions over the leaf litter, preying on other soil bacteria. Over 100 genes control its gliding behavior, and these have been divided into two subsets, "social" motility and "adventurous," or single-celled, motility. Chemotaxis is involved in aggregation into multicellular spore-forming bodies, but there is disagreement about its role in other aspects of movement. There has been some controversy about whether slow gliding species, moving at 1–5 μm min^{-1}, can show chemotaxis using temporal gradients. However, there are genes in *M. xanthus* that are very similar to those coding for the chemosensory proteins of *E. coli* and mutations within these genes results in altered patterns of gliding, causing either smooth or reversing phenotypes. The *frz* genes code for proteins involved in controlling reversal frequency (Blackhart and Zusman, 1985; Blackhart and Zusman, 1986). An MCP homologue, *frzCD*, has the highly conserved signalling domain, but the putative protein sequence does not contain any transmembrane domains. The FrzA protein is the CheW homologue, and FrzG and FrzF, the CheB and CheR, respectively. The *frzE* gene encodes a CheA-CheY fusion, while *frzZ* encodes a CheY-CheY fusion. A CheA-CheY fusion has been identified in several other species, both Gram positive and Gram negative, and would fit with a model where one CheY was signalling and the other involved in signal termination. In *E. coli*, FrzB and FrzZ have no obvious homologues. What are the roles for these in the complex behavioral life of *M. xanthus*?

Myxococcus xanthus has a complex lifecycle, with all stages dependent on motility and influenced by the *frz* genes. The bacterium forms vegetative "swarms" when nutrients are plentiful, with the moving colonies of bacteria excreting complex mixtures of proteases, nucleases and lipases to digest macronutrients and other bacteria in their surroundings (Hartzell and Youderian, 1995). When nutrients become scarce, the cells form tighter aggregates and these become fruiting bodies with spores. "A" or adventurous motility, allows individual cells to move away from a group, and "S" or social motility requires cell-cell interaction, and the bacteria move as a group. This "S" gliding appears to involve a system closely related to the Type 4 pili-dependent system involved in twitching and discussed earlier (Sager and Kaiser, 1994; Spormann, 1999; Wall and Kaiser, 1999; Yang et al., 1998). Social motility also requires the presence of the extracellular organelles known as "fibrils" (Dworkin,1999). There are data implicating chemotaxis in at least two areas of these complex behaviors, but often in combination with a second independent sensory system which measures cell-cell contact or cell density. The MCP homologue, FrzCD has been found to methylate in response to increased nutrient levels, conditions under which reversals were also reduced (McBride et al., 1992). Also, FrzCD is cytoplasmic and this suggests that gliding cells can sense a change in an intracellular nutrient via FrzCD, and this signals, via FrzA (the CheW equivalent) and FrzE (the CheA-Y fusion), to control reversal frequency. Interestingly, only groups of cells respond; individual cells isolated from a group that showed a response, no longer respond to changes in nutrient level. This suggests that some form of cell-cell signalling is

involved in controlling movement of well-fed cells; the swarm would therefore be sensing not only the nutrient gradient, but also cell density (Shi et al., 1996; Ward et al., 1998b). Isolated single cells can, however, respond to repellents through this pathway and, while single cells do not respond to nutrient gradients (including extracts of *E. coli*), if a single cell comes into physical contact with a colony of *E. coli*, its prey, it enters the colony and stops gliding until the colony has been digested. Again, cell contact must be important in stopping the cell movement. Also, *frz* mutants do not show this stop response, again suggesting a connection between gliding behavior and cell-cell contact. While *M. xanthus* may not be attracted to *E. coli*, *E. coli* is attracted to *M. xanthus*. In limiting conditions, *M. xanthus* excretes what is probably an amino acid and this "lures" bacteria, presumably via their chemosensory systems, to their death (McBride and Zusman, 1996).

The Frz system therefore appears to be involved in both the scavenging behavior of *M. xanthus* swarms, and in development. Experiments in which the movements of tetrazolium-stained single cells were followed during fruiting body formation showed that large rafts of cells formed and reversed very infrequently, and this behavior required S-motility, *frz* genes and a cell-density dependent signal. The cell-density signal, C-signal, is sensed through the Frz chemosensory pathway as a type of "autoattractant." The reversal frequency, and thus the movement of cells within a developing fruiting body would therefore be controlled by the aggregation signal being sensed by the chemosensory pathway. For a general review of motility and development in *M. xanthus*, see Ward and Zusman (2000).

A great deal more remains to be understood about the sensory pathway, about the role of methylation, the uncharacterized proteins, the response regulator fusions and the significance of responses to nutrient gradients in well-fed cells, and the switch to cell aggregation and differentiation during starvation. *Myxococcus xanthus* also illustrates the interconnection of the chemosensory pathway with the other sensory systems in bacteria. It seems probable that this is not the only case in which chemosensing and "quorum" sensing operate together to control bacterial behavior, and it should be remembered that for species in natural environments, the final response of a cell is probably the result of several chemosensory inputs balancing with other sensory pathways.

ARCHAEA The photoresponses of the archaea have been well studied, but the chemosensory responses less so. The majority of research has centered on *H. salinarium*, which has homologues for all the chemosensory proteins found in *E. coli*, except CheZ, and again has some additional proteins. At least 13 *mcp* homologues have been identified encoding classical transmembrane receptors, cytoplasmic receptors, receptors with many transmembrane helices and one apparently linked to a heme-containing protein and thought to be involved in oxygen sensing (Hou et al., 2000). The major attractants are amino acids and the sensory signals are transduced through the chemosensory pathway to the flagellar motor. This is the first species in which a chemoattractant, arginine, has been shown to be sensed not by transmembrane MCPs, but by a cytoplasmic receptor (Storch et al., 1999). An additional sensory molecule, fumarate, has been identified in *H. salinarium*, which may also play a role in other sensory systems (Montrone et al., 1993; Montrone et al., 1996; Montrone et al., 1998).

Fumarate was identified as a signalling molecule that causes switching of flagellar rotation independently of the chemosensory transduction pathway. It is thought to be released from membrane-bound sites during stimulus responses in *H. salinarium*. Investigations using motile sphaeroplasts of *E. coli* suggest it may also have a role in chemosensory transduction in this species. Mutants in the Krebs cycle with altered fumarase activity show altered responses to some stimuli, particularly repellents, leading to the suggestion that repellent signalling may involve fumarate release (Prasad et al., 1998).

Several complete genome sequences are now available for thermophilic archaea. The genome sequences show that while the flagellar genes are all present, no chemosensory genes have been identified in *Methanococcus jannaschii* (Andrade et al., 1997). In all cases studied to date, motile prokaryotes have been found to be able to sense some sort of environmental gradient, the expense of synthesizing and operating flagella would make it difficult to contemplate a bacterium in which swimming is not used to reach an optimum environment (Kostyukova et al., 1992). The mechanisms controlling the behavior of this species is unknown, but it has been suggested that thermosensing may be the most important sensory pathway in these species, maintaining the bacteria at the optimum temperature between the superheated waters of the hydrothermal vents and the cold water of the ocean bottom; a thermosensory signal may directly control the motor. As fumarate has been found to directly control motor activity in bacteria, possibly the archaeal motor is controlled directly by metabolic signals. There is certainly some evidence for chemosensing in some related species and obviously more research

needs to be conducted to identify the transduction pathway in the archaea.

Phototaxis

Most motile bacterial species respond to the electron acceptors required for generation of an electrochemical proton gradient, and many that are capable of photosynthetic growth also respond to light. Light sensing in most other species is linked to photosynthetic electron transfer. Nonphotosynthetic bacteria will, however, respond to a flash of blue light by tumbling, and prolonged exposure can lead to a complete loss of motility. This is almost certainly the result of the photo-oxidation of porphyrins, which then act as repellent, signalling through the classical MCP system.

Some photosynthetic eubacteria, for example, *Ectothiorhodospira halophila*, *Chromatium salexigens* and some strains of *R. sphaeroides*, have been found to contain a 4-hydroxycinnamic-acid-dependent soluble protein, the photoactive yellow protein (PYP), which undergoes a photocycle in blue light, and many of these species do respond to flashes of bright blue light by stopping or reversing (Hellingwerf et al., 1998; Hoff et al., 1999; Sprenger et al., 1993). However, the role of this protein in these responses is uncertain. For example, in *Rhodospirillum centenum*, PYP appears to be involved in transcriptional control, and though the absorption spectrum of PYP fits the response spectrum of *R. sphaeroides*, deletion of the gene does not result in the loss of the blue-light response (Jiang et al., 1999).

Most photosynthetic bacteria respond to light by reversing when swimming over a light/dark boundary. In fact, reversing or stopping seems to be the most common mechanism for direction changing in photosynthetic species; few have been described that tumble like *E. coli*. Observations suggest swimming pattern changes little when photosynthetic bacteria swim over a dark boundary into light (Armitage, 1997a; Pfennig, 1968). They therefore appear to sense and respond primarily to a reduction in light intensity, rather than an increase. However, if *R. sphaeroides* is tethered by its flagellum and given subsaturating increases and decreases in light, the cells respond both to an increase and a decrease (Armitage, 1998; Romagnoli and Armitage, 1999). They respond to a decrease by stopping and then adapting to the change. and to an increase by reducing the stopping frequency and then adapting. The response to the increase is less obvious than to the decrease, because the natural bias (stopping frequency) of the motor is 0.8, which means that it is swimming more than it is stopped (that of *E. coli* is closer to 0.5), making the response when moving up a gradient stronger than that seen in *R. sphaeroides* (Berry and Armitage, 2000). The motor biases of other species has not been measured, but it would be interesting to identify whether a high bias is linked to a specific environment or way of changing direction.

Whether bacteria can respond to a gradient of light is arguable and may depend on their environment and the type of gradient formed in that environment. Free-swimming cells are unlikely to be able to move far enough in a given time to experience the 1% drop in light intensity required to cause a step-down response, and experiments with free-swimming cells support this. However, in environments where intensities fall rapidly, such a microbial mats or dense colonies, the response could be different. Indeed, a bacterium *Rhodospirillum centenum* has been shown to respond as a moving colony to a light gradient, the whole colony moving across an agar plate towards infra-red light and away from white light. If presented with light from two directions, the colony will move along the averaged path (Jiang et al., 1997; Jiang et al., 1998; Ragatz et al., 1995). When cells from a moving colony were resuspended in liquid medium, they did not respond to light gradients, suggesting the response is dependent on the cell density within the colony (Sackett et al., 1997). Oxygen electrode measurements showed a large oxygen gradient within the colony (Romagnoli et al., 1997). It is therefore possible that the colony movement is directed by a combination of negative-oxygen and positive-light sensing.

In all cases of positive responses to light by photosynthetic eubacteria, photosynthetic electron transport has been shown to be essential. Inhibitors of photosynthetic electron transport inhibit photoresponses, as do mutations within the reaction centers which leave pigments intact (Grishanin et al., 1997; Packer et al., 1996). The sensory signal is probably a change in electron transport rate rather than a change in Δp, as low concentrations of uncouplers which cause a step-down in Δp do not alter responses. However, electron transport inhibitors which alter the rate of electron flow but not the size of Δp do cause a response. The sensory-receptor protein signalling the change in electron transfer has not been identified, but mutants deleted for the cbb_3 oxidase-sensing protein, PrrB, involved in controlling both expression of Che operon 2 and aerotaxis in *R. sphaeroides*, still respond to changes in light level, indicating another light-sensing system (J. P. Armitage, unpublished observation). The signal from the unknown sensor is transmitted through the cytoplasmic chemosensory signal transduction pathway in both *R. sphaeroides* and *R. centenum*. Mutants in the *che* genes of *R. centenum* lost the ability to

swarm towards red light (Jiang et al., 1997). In *R. sphaeroides*, the situation is more complex as there are two chemosensory operons. Deletion of operon 1 has no effect on photoresponses, but deletion of operon 2 leads to the loss of photoresponses. The response requires both $CheA_2$ and $CheW_2$. As the only known role of CheW is to link MCPs to CheA, this indicates the involvement of an MCP-like protein in signalling (Romagnoli and Armitage, 1999). This is supported by the measurement of methanol release after the reduction in light intensity, indicating adaptation requires changes in receptor methylation, just as in *E. coli* chemotaxis. Photosensing in eubacteria therefore probably involves a redox sensor linked to an MCP-like receptor signalling through $CheW_2$ to $CheA_2$ and thus to the flagellar motor, with adaptation requiring methylation of the receptor to reset the structure.

The mechanisms involved in responding to light by the archaeon *H. salinarium* is probably the best understood system. It combines the mechanisms used for proton translocation by bacteriorhodopsin (BR) with the conserved signalling domain of an MCP. *Halobacterium salinarium* grows in saturating salt concentrations and when the oxygen levels are low, it induces BR, a retinal-based light-driven proton pump. In addition to BR, it has three other retinal based light-absorbing pigments: one halorhodopsin (HR) uses light to drive a chloride pump, but the other two are sensory rhodopsins (Hoff et al., 1997; Spudich, 1998). A constitutive retinal-containing sensory protein, SRII, absorbs blue light and undergoes a fast photocycle that generates a signal to control the flagella bundle, and increasing blue light causes the cells to reverse and keeps the cells out of damaging blue light. Under anaerobic conditions when BR is induced, an additional sensory rhodopsin (SRI) is also induced. SRI acts to produce both a positive signal to orange light and a negative repellent signal to blue light. The photopigment undergoes a fast transformation to a blue-absorbing form in orange light. If blue light is present. there is a fast transformation back to the orange-absorbing form, but if blue light is not present the transformation is slow. Therefore in orange light, which is useful for BR activity, the SRI is in the blue form and sends a positive signal, but when blue light is present the orange form predominates and this sends a negative signal. Each SR protein has an accompanying sensory protein, HtrI and HtrII (Bogomolni et al., 1994; Krah et al., 1994; Spudich, 1994). These proteins have cytoplasmic domains homologous to those of the highly conserved domains of MCPs. If the Htrs are deleted from *H. halobium*, the SR proteins are able to pump protons (Sasaki and Spudich, 1999). Absorption of the appropriate wavelength of light probably leads to a conformational change in the retinal and a charge change. The latter is transmitted to the Htr proteins through the transmembrane helices and alters the conformation of the signalling domain, and in turn the activity of CheA and CheY.

Aerotaxis and Electron Acceptor Taxis

As would be expected, the responses to oxygen vary from species to species, and these are some of the earliest responses seen in bacteria (Armitage, 1997b; Engelmann, 1881). Obligate aerobes will swim towards oxygen, while obligate anaerobes are repelled. Microaerophiles respond positively to low concentrations, but are repelled by atmospheric levels. In some cases, the responses are different under different growth conditions. *Rhodobacter sphaeroides* is attracted to oxygen when growing as a heterotroph, but repelled when growing as a photoheterotroph. *Escherichia coli* responds to oxygen concentrations that correspond to the Km values of the cytochrome oxidases. and as with the photoresponses, inhibition of electron transport caused a loss of response (Laszlo et al., 1984; Laszlo and Taylor, 1981). This suggests that again there may be a redox sensor responding to changes in the rate of respiratory electron flow. This type of behavior has been called "energy taxis" (Stock, 1997; Zhulin et al., 1997).

A redox sensor has been identified in *E. coli*. A flavin adenine dinucleotide (FAD)-binding protein, Aer has a cytoplasmic domain homologous to the highly conserved domain of an MCP (Bibikov et al., 1997; Bibikov et al., 2000; Rebbapragada et al., 1997). It is thought to sense the change in the rate of electron flow through the quinone region of the respiratory electron transport chain, rather than oxygen itself, and the change in redox state of the FAD is thought to signal to the conserved signalling domain through a redox sensing PAS domain, which has been identified in a wide range of sensory proteins, including those of higher eukaryotes (Repik et al., 2000). This change is then transmitted to CheA and CheY and thus to the motor. Mutants in Aer do not accumulate around air bubbles, but overexpression of Aer results in an increased sensitivity to oxygen. Mutants in Aer, however, still show some response to changes in oxygen level and there is some evidence that the MCP, Tsr, may be able to sense changes in Δp (electrochemical proton gradient) directly, but again the mechanism is not understood. It has been suggested that a balancing between signals from electron transport and responses to changes in Δp could account for both positive

responses to optimum oxygen concentrations and the repellent responses shown to potentially toxic high concentrations. Whether this would have a role under physiological conditions is not known.

There seem to have been several independent mechanisms evolved for sensing oxygen levels. Two of the 13 MCPs identified in *H. salinarium* appear to be oxygen sensors, but in this case it uses a cytochrome oxidase attached to the highly conserved domain of an MCP or myoglobin-like receptors (Hou et al., 2000; Zhang et al., 1996). This suggests that *H. salinarium* can sense molecular oxygen directly. *Desulfovibrio vulgaris*, a strict anaerobe, on the other hand, has a sensory protein, DcrA, with a *c*-type heme attached to a domain with homology to the highly conserved cytoplasmic signalling domain of an MCP (Dolla et al., 1992; Fu et al., 1994). The ecology of a strict anaerobe is obviously different from the facultative bacteria in which aerotaxis has been studied. Thus, DcrA could sense either oxygen or redox potential, as the heme irons of *D. vulgaris* have been shown to be directly oxidized by oxygen. Under anaerobic conditions, DcrA would be reduced and oxygen would lead to either the direct oxidation of bound heme or allow heme to bind to the signalling domain; this would alter the conformation and signalling through the highly conserved domain. Unlike Aer, which does not apparently have glutamate residues that could be methylated to allow adaptation, DcrA has been shown to be methylated in response to changing oxygen concentrations.

All the different sensory modules identified to sense changes in oxygen levels are linked to the highly conserved signalling domain of MCPs, suggesting that the highly conserved signalling domain was an early component in the evolution of chemosensing, and different sensory domains have been grafted on by different species to serve their specific niche.

Electron transfer is involved in both light and oxygen sensing, and in many species, electron transfer components are shared between the different pathways, which could result in competition between the sensory signals. This appears to be the case. Most bacteria that grow using respiratory electron transfer can also use other electron acceptors when oxygen is absent (anaerobic respiration), inducing expression of specific acceptors if an electron acceptor is in the environment. Thus *E. coli* will grow on nitrate in the absence of oxygen, and under these conditions, will show tactic responses to nitrate. *Rhodobacter sphaeroides* will grow on dimethyl sulfoxide (DMSO) in the absence of oxygen and again shows taxis towards DMSO (Gauden and Armitage, 1995). In both cases, if the responding cells are now exposed to oxygen, the response to nitrate or DMSO is reduced or lost and the cells now respond to oxygen. Electron transfer under these conditions is diverted from the alternative acceptor to oxygen. In *R. sphaeroides*, light inhibits or reduces responses to either DMSO or oxygen and oxygen reduces the size of the photoresponse in photosynthetically growing cells. Under all of these conditions, electron transfer components are shared and photosynthetic electron transfer has been shown to be faster than respiratory electron transfer, whereas electron flow to a terminal cytochrome oxidase will be dominant over flow to DMSO reductase (Grishanin et al., 1997). All of these data suggest that these bacterial species are not responding to light or oxygen or nitrate under these conditions, but responding to the change in electron flow through a common receptor which then signals through the chemosensory pathway. As suggested with Aer and Tsr for oxygen sensing, there may, however, be more than one receptor. In *R. sphaeroides*, deletion of $CheA_2$ results in a reduction in responses to oxygen. In addition, deletion of PrrB, the redox sensor of electron flow through the terminal cytochrome cbb_3 oxidase results in a loss of aerotaxis (J. P. Armitage, unpublished observation). This suggests that the histidine protein kinase, PrrB, not only controls the activity of the transcriptional activator, PrrA, but can directly control the chemosensory pathway, perhaps interacting with $CheA_2$ by a mechanism related to EI-CheA interactions in PTS chemotaxis. Little research has yet been carried out into competition between electron transport-dependent signals and the chemosensory signals.

The Role of Taxis in Natural Environments

Only *E. coli* and *Salmonella* have had their chemosensory pathways analyzed in any great detail, but the role of taxis in their natural environment has not been examined. Indeed there have been very few detailed studies on the effect of chemotaxis in natural gradients. There have been some attempts to model behavior, but few of these have used experimental data. Several models have been produced that develop patterns similar to those produced by bacteria in gradients, but because few are linked to experimental measurement, it is hard to estimate their true significance.

There have been a number of individual studies of the possible role of motility and chemotaxis to the natural history of a wide range of species, from environmentally important species to pathogens. The depth of investigation varies enormously, but it suggests that chemotaxis plays an essential part in the survival of a very wide range of species and in allowing surfaces to be

reached for colonization, making chemotaxis an important virulence factor for some pathogens and important in symbiotic interactions. It may also be important for the initial colonization of surfaces for biofilm formation. Some species thought to be nonmotile when originally isolated are now suggested to be motile in some environments. Many bacterial species lose motility when cultured, particularly in rich conditions. *Shigella* was considered a nonmotile species, until examined directly from the gut flora, where it appeared to be actively motile. It is therefore dangerous to define a species as nonmotile unless the species is cultured under a number of conditions and preferably examined directly in its natural environment. This emphasizes the problems of defining motility when growing bacteria only in rich media, which may repress flagellar synthesis. For example, when examined in situ, the majority of marine bacteria are actively motile, but motility is rapidly lost on cultivation. The only way to be sure a bacterium is nonmotile under all conditions is by gene sequencing.

Very little work has been undertaken to examine the role of taxis in situ. One of the situations where behavioral responses almost certainly play an important role in the natural ecology of bacteria is in magnetotaxis.

Magnetotaxis

A large number of bacterial species appear to be magnetotactic, although few have been isolated in pure culture. There are coccoid, spiral and rod-shaped species, but what they all have in common is intracellular membrane-bound magnetosomes, flagellate motility and negative aerotaxis. Magnetotactic species are usually found in estuarine or salt marsh mud, where they grow microaerophilically. The turbulent environment can result in the bacteria being displaced by tidal movement into the aerobic upper layers of the water. The chains of magnetosomes contain single domain magnetite, which orient the cells along the local magnetic field lines (Blakemore, 1982; Blakemore and Frankel, 1981; Mann et al., 1984; Stolz et al., 1986). This orientation combined with a strong negative aerotactic response results in a two- rather than three-dimensional swimming pattern, moving the cells back into the microaerophilic mud (Frankel et al., 1997).

Viscotaxis

There has been some limited research suggesting viscotaxis may help in the invasion of some spiral-shaped pathogens (Kaiser and Doetsch, 1975). The spirochetes all have a flexible spiral cell body. The helix of the spiral changes wavelength when moving into increasingly viscous environments, such as mucous membranes (Berg and Turner, 1979). The shorter wavelength causes increased swimming speed when moving into a viscous environment, and decreased speed when moving out. This would in effect help the bacterium to invade (Greenberg and Canale-Parola, 1977). The same physical enhancement of tactic ability has been suggested for the wall-less spiroplasmas when invading plant tissues (Daniels and Longland, 1984).

Biofilms

In natural environments, many bacteria are not found free living, as in the laboratory, but as biofilms, which may involve single or multiple species. Biofilms can be very stable and the bacteria within them are often metabolically very different from the same species growing individually in suspension (planktonic; Costerton et al., 1994; Costerton, 1995; Nickel et al., 1994). Biofilm formation by *Ps. aeruginosa* requires type IV pili possibly for attachment. Type IV pili are also the organelles required for twitching motility, but the role of twitching in biofilm formation has not been identified (Davies et al., 1998; O'Toole and Kolter, 1998; Wall and Kaiser, 1999). Biofilms cost oil and shipping industries large amounts of money, and the formation of biofilms on medical implants is a major problem for the medical profession. Biofilms on plant roots, on the other hand, are thought to protect plants from infection by pathogens and may be involved in nonsymbiotic nitrogen fixation (Davies et al., 1998; Kolter and Losick, 1998; Shapiro et al., 1993). It is likely that the interplay of at least two environmental responses is essential for biofilm formation, motility and quorum sensing, leading to the eventual process of extracellular matrix synthesis (O'Toole et al., 2000). Biofilm formation is a strategy not only for keeping a bacterial species in a particular location, but also for reducing overcrowding, nutrient limitation and toxin production by packing them at low density in a protective polysaccharide matrix. Secreted homoserine lactones, the bacterial cells' mechanism for sensing population density, is necessary for biofilm formation, but several studies have shown that motility is also essential for the very early stages of biofilm formation and perhaps for movement within the biofilm, which can project many tens of μm from the surface (Pratt and Kolter, 1999; Watnick and Kolter, 1999). Although the requirement for chemotaxis has not been shown conclusively, it may be required to direct the bacteria to surfaces on which they then form a biofilm. The local

charge and nutrient concentration tends to be higher at surfaces than in surrounding environments, and if it is a plant or animal tissue surface, there are probably local gradients of excreted compounds. Biofilms also slough off individual cells, and it is probable that this leads to population and biofilm spreading. This requires motility. *Rhodobacter sphaeroides* produces an *N*-acyl-homoserine lactone (HSL), but a mutant unable to produce it, forms large amounts of extracellular matrix and large flocculant colonies (Puskas et al., 1997). The addition of the *N*-acyl-HSL to a flocculant colony results in breakdown of the polysaccharide and an increase in swimming speed of the released cells. In this case, the interplay of motility, nutrient levels and quorum sensing may determine whether the cells are free swimming, planktonic, or form a protective biofilm-like matrix.

Symbiosis

There have been a number of studies on the role of chemotaxis in the colonization of legume roots, because of the economic and ecological importance of nitrogen fixation by both free-living and symbiotic species. Again there is no unequivocal data that a particular rhizobial species is attracted to its specific plant root hair as a result of any species-specific exudates, but rhizobia are highly motile and chemotactic and are attracted by root exudates (Ames and Bergman, 1981; Caetano-Anolles et al., 1988; Kurdish et al., 2001). Bacteria would therefore be attracted up the exudate gradient towards the root hairs, but the response may not be specific for the host. It seems probable that, as with most colonizations, the attachment to roots is a complex process with motility and chemotaxis taking bacteria to the surface, followed by adhesion and colonization, processes which combine quorum sensing and complex intracellular signaling pathways. Plants secrete *nod*-inducing flavonoids into the rhizosphere and these can be degraded by rhizobial species. It has been suggested that the degradative products of flavonoids in the rhizosphere could act as chemoattractants. While many rhizobial species have been shown to exhibit chemotaxis towards root exudates in general, the most most convincing evidence that chemotaxis is involved in colonization comes from studies on the free-living nitrogen-fixing bacterium, *Azospirillum brasilense* (Zhulin and Armitage, 1992). In in vivo studies, a number of mutants have been identified in this species that are nonmotile or nonchemotactic and are unable to colonize wheat roots (Vande et al., 1998). Polysaccharide production was found not to be important in primary colonization, but in later stages. Some studies have implicated flagella in the initial process of adhesion. Interestingly, the recent genome sequence of *Sinorhizobium meliloti* shows that this species has two complete sets of chemosensory genes, one organized on an operon located in the *sym* plasmid, which also encodes the genes essential for symbiotic development.

Bacillus megaterium is a root-colonizing bacterium, which has been shown to protect roots against fungal attack. This species shows a highly sensitive chemotactic response to soybean root exudates, particularly amino acids, over a wide range of temperatures and pHs (Zheng and Sinclair, 1996). This has led to the suggestion that chemotaxis leads to the successful colonization by the bacterium of plant roots, which serves to protect the roots from potential fungal pathogens.

Plant Pathogens

The role of motility and chemotaxis in plant wound infection is unclear. There are reports that wound secretions act as attractants for the pathogen *Agrobacterium tumefaciens*, causing behavioral responses at lower concentrations than required to induce the expression of pathogenic genes, suggesting that the bacterium can sense a wounded plant, swim towards it and then cause disease (Ashby et al., 1988; Hawes and Smith, 1989; Parke et al., 1987; Shaw et al., 1988). Wounded plants secrete specific inducers of virulence genes, acetosyringones, and sugars. A periplasmic protein, ChvE, is involved in both chemotaxis towards monosaccharides and virulence gene induction. *Erwinia*, *Pseudomonas* and *Xanthomonas* species are all motile, but whether that motility has a direct role in their pathogenicity has not been investigated. Mixed inocula of motile and nonmotile transposon mutants of *Pseudomonas fluorescens* did show that only the motile wild type could effectively colonize the rhizosphere around spinach roots, although when directly inoculated, there was no significant difference in the ability to colonize the roots (Dekkers et al., 1998). This finding suggests that motility may be an essential trait in reaching the roots for colonization, but plays no part in the actual process of invasion.

Animal Pathogens

A great deal more research has been carried out on the role of motility and taxis in pathogenic interaction by animal pathogens. As many virulence factors, quorum sensing, specific pilin development and toxin secretion pathways are similar in plants and animals, it seems likely that motility will have a role in moving plant as well as animal pathogens to their site of adhesion

and/or invasion. In animal pathogens, flagellar synthesis and virulence factors are often coregulated. Nonmotile or nonchemotactic mutants usually remain pathogenic if inoculated directly onto an invasion site, but if incubated with the host rather than inoculated onto the site of potential infection, infection is lost (e.g., a nonchemotactic *Vibrio anguillarum* will not infect trout when added to its water, although pathogenicity has not been lost; O'Toole et al., 1996). A mutation in the methyl transferase gene, *cheR*, resulted in a smooth-swimming phenotype, and although the mutants remained fully pathogenic when added to the fish directly, these could not cause infections when added to the fish tanks. This and a related fish pathogen, *V. alginolyticus*, have been shown to exhibit strong chemotactic responses to mucus from sea bream (Bordas et al., 1998). Mucus was isolated from different parts of the fish, and the pathogens were found to respond to and adhere to mucus from the skin and gills (the usual sites of infection) but showed little response to mucus from the intestine.

There have been many studies on the role of motility in human infection. *Vibrio cholera* is highly motile, and there is research suggesting that motility and expression of virulence factors are intimately linked: the correct environmental signals for pathogenicity switching off motility and switching on virulence genes. However, the initial role of motility in invasion has not been fully characterized (Klose et al., 1998; Postnova et al., 1996). Interestingly, flagellar mutants were not only inefficient at colonizing mouse intestine, they also had an altered colony morphology and biofilm structure, emphasizing the interconnection between this range of physiological phenomena, geared to optimizing survival under different growth conditions (Watnick et al., 2001). More detailed studies have been carried out on wound infection, urinary tract infections and intestinal mucus invasion. Research has concentrated on infections by *Campylobacter jejuni* and *Helicobacter pylori*, given their involvement in serious outbreaks of gasteroenteritis and in stomach ulcers and gastric cancer, respectively. Early studies of *C. jejuni* showed, surprisingly, that in vivo, this organism did not appear attached to the gut lining, but freely swimming in mucus filled pits of the large intestine (Grant et al., 1993; Lee et al., 1988; Nachamkin et al., 1993). *Campylobacter jejuni* is highly motile in viscous media that inhibit most flagellate species, suggesting that motility has evolved to enable this organism to operate efficiently in its niche in the intestine. There is some evidence that chemotaxis is essential for colonization, as nonchemotactic but motile mutants showed a limited ability to colonize intestines in animal models, whereas nonmotile mutants were unable to colonize birds or rabbits (Szymanski et al., 1995; Wassenaar et al., 1993; Yao et al., 1997). Also, CheY mutants failed to colonize mice. Similarily, flagellar mutants of *H. pylori* were less able to colonize gnotobiotic piglets. *Helicobacter pylori* has been found to respond chemotactically to urea and bicarbonate and negatively to oxygen (Eaton et al., 1992). The gastric epithelial cells secrete urea and bicarbonate, and hydrolysis of urea by urease is essential for colonization of the gastric mucosa (Foynes et al., 2000; Nakamura et al., 1998; Yoshiyama et al., 1998). The positive responses to these compounds strongly suggests that chemotaxis plays an essential role in colonization of the stomach by *H. pylori* and helps to maintain it in that niche.

Proteus mirabilis is a major cause of urinary tract infections. It is one of an increasing number of bacterial species that (when in liuide medium) have been found to swim using a small number of flagella; however, when growing on surfaces, the increased viscosity apparently induces increased flagella synthesis and the cells become hyperflagellate, and often elongated, allowing them to move as large rafts of cells over surfaces. In the case of *P. mirabilis*, it has been suggested that the hyperflagellate, swarming cells may be better able to colonize the urethra, bladder and kidneys (Allison et al., 1992; Mobley and Belas, 1995). Indeed, swarmer cells have been shown to increase expression of a large number of identified virulence factors, and mutations that cause the loss of motility and swarming also inhibit expression of virulence factors. In animal models, only swarmer cells were isolated from infected kidneys, even though the initial inoculum had only contained free-swimming non-swarmer cells, suggesting that swarmer differentiation does occur in vivo (Liaw et al., 2000; Mobley et al., 1996). Experiments designed to investigate the role of flagella in infection have however been equivocal, with one study showing a reduction in infection by nonmotile cells and the other showing no effect (Legnani-Fajardo et al., 1996). These results suggest that the link between motility and virulence may be very complex, and more sophisticated studies may be needed before we can be sure whether *P. mirabilis* swarms up the human urethra to reach sites of infection. *Clostridium septicum* is another pathogenic species with small free-swimming cells that differentiate into swarmers on surfaces. Adhesion to and invasion of cultured human epithelial cells was greatest if the short motile form was used rather than swarmers, suggesting that differentiation may play different roles in different species (Wilson and Macfarlane, 1996). A connection between swarming motility and quorum sensing has been identified in the occasional

human pathogen, *Serratia liquifaciens*, where the development of swarmers has been shown to require genes controlling not only flagella synthesis, but also the synthesis of the quorum sensing molecule, homoserine lactone. Again, this finding indicates that motility is just one of several environmental sensing systems determining the development and behavior of the cell (Eberl et al., 1996; Givskov et al., 1998; O'Rear et al., 1992).

It may be interesting that the so called "Type III secretion system," used by a range of important pathogens such as *Yersinia*, *Shigella*, *Salmonella* and *Bordetella* to secrete toxins, is very similar to the flagellar motor in structure (see earlier). This system is required for the direct release of toxic proteins without a classical signal sequence. For example, the Yops from *Yersinia* "inject" toxins directly into the eukaryotic cytoplasm after cell-cell contact, and there are suggestions of a common evolutionary origin for the export apparatus for flagellar proteins and Type III toxins. The majority of Type III-secreting pathogens are also motile. In several of these, including *Bordetella*, a link between motility and pathogenicity has been suggested (Akerley et al., 1995). The cells are flagellate and motile when not in a suitable colonization site, and one of the effects of the expression of virulence factors in response to extracellular signals is to switch off flagella synthesis. Mutants unable to switch off flagella synthesis showed a reduced ability to infect, even when the mutation was combined with one allowing expression of virulence factors. The antigenic ability of flagellin may be one reason, but it seems likely that in the case of this species, control of motility and colonization is essentially negatively connected. In these species, it has been suggested that the motile phase may be important for transmission rather than colonization.

Chemotaxis also may play a part in the survival of pathogens. *Escherichia coli* is repelled by concentrations of hydrogen peroxide, hypochlorite and *N*-chlorotaurine lower than the toxic concentrations (Benov and Fridovich, 1996). As these compounds are part of the "respiratory burst" produced by phagocytes in response to bacterial invasion, the ability of bacteria to use chemotaxis to evade the leukocytes could help in survival.

Aquatic Environments

In general, studies of the behavior of bacteria in aquatic environments have centered on the behavior of photosynthetic species in microbial mats and in stratified lakes. Often these species have gas vacuoles, which move bacteria over large distances. In mats, swimming motility may be important in local movements during the natural day-night cycle. Several species have been identified with both gas vacuoles and flagella (for example, *H. salinarium*), but few studies have been undertaken into the relative roles of the two systems under natural conditions. Indeed, the gas vacuoles proved a nuisance during phototactic studies in *H. salinarium*, therefore all the phototactic research is conducted in gas-vacuole-minus mutants.

There is some evidence that gliding motility is important in maintaining the vertical distribution of both the sulfur bacterium *Thioploca*, and cyanobacteria within sediments, and recent studies on the behavior of a mixed population of sulfate-reducing bacteria within a cyanobacterial mat showed extensive diurnal migration by the bacteria (Huettel et al., 1996). It has been suggested that diurnal oxygen stress and the balancing of chemotaxis towards oxygen, carbon and sulfate may maintain the different species, (whether facultative, microaerophilic or anaerobic) in their optimum environment. In experiments where microcores of natural microbial mats were replaced with small glass beads, highly motile species were found to concentrate and position themselves within a very distinct band within three days of regrowth. The sensory signal for maintaining the bacteria within the band was not identified, inasmuch as classical chemoattractants had no effect on positioning of the band; however, since the species were not identified, the dominant effectors may not have been identified, and oxygen could have played a major role.

Measurement of bacteriochlorophyll fluorescence of photosynthetic bacteria in a stratified lake in situ showed that they moved several centimeters during the day (Joss et al., 1994). The movement might be the result of a combination of photoresponses and responses to the changing sulfide and oxygen concentrations. In the day, the photosynthetic bacteria need to be in high light and sulfide, but low oxygen and balancing the signals may be the cause of the cyclic movement.

Clouds of highly motile bacteria are found close to surfaces in marine environments. These unclassified bacteria show strong aerotactic and chemotactic behavior, which may maintain them close to surfaces where nutrient levels are higher than in the open waters (Mitchell et al., 1995). Marine algae also have been found to secrete high levels of demethylsulfoniopropionate (DMSP), and there is evidence that marine bacteria may be chemotactic to DMSP, using it as a metabolite, and responsible for its turnover in natural environments. *Alcaligenes* M3A was found to be attracted to 10^{-6} M DMSP, which is similar the concentration measured close to phytoplankton (Steinbüchel and Schubert,

1989). Dimethylsulfide (DMS) gas is the major source of biogenic sulfur emissions from the oceans and is involved in climate regulation. This is therefore a situation where chemotaxis could be directly involved in the rate of DMS production and sulfur cycling between seawater and atmosphere.

Several marine isolates move differently when free living rather than on surface. Free-living *Vibrio alginolyticus* swims using a single sodium-driven flagellum, but on surfaces, the increased viscosity is sensed through the flagellar motor and induces the synthesis of large numbers of proton-driven lateral flagella, which allow the cells to move over surfaces (Liu et al., 1990). This type of transformation, from single or few flagella on free-swimming cells to large numbers of lateral filaments when the cells are on surfaces is now being found in all sorts of species of Gram-positive bacilli, suggesting that this is a common solution to the problem of small bacteria moving over surfaces. They tend to move as large rafts, and this multicellular movement probably helps to overcome the excessive viscous drag and tension of the surface.

An interesting behavioral response is shown by stable consortia of bacteria. Several phototrophic consortia have been identified in eutrophic fresh water, "*Chlorochromatium aggregatum*" and "*Pelochromatium roseum*" being two whose behavior have been investigated (Frostl and Overmann, 1998; Overmann et al., 1998). They are both consortia formed between a large central, motile but pigmentless bacterium and pigmented nonmotile cells that surround it (some have been described with several layers of cells around the motile central cell). These consortia are not rare and can make up as much as two-thirds of the biomass of the chemocline of a lake and may therefore be important in the general physiology of these ecosystems. Analysis of the vertical distribution of specific consortia shows that they are found at specific regions with maximum light intensity but very low oxygen levels. The behavioral response must balance the signals from oxygen, light, sulfide and iron to keep the consortia in these regions. The diurnal distribution also was found to change, with the consortia moving upwards at night. When the behavior of these bacteria were analyzed in the laboratory, it was found that although the motile member of the group was not photosynthetic, the consortia responded to changes in light intensity and accumulated in wavelengths that corresponded to bacteriochlorophyll *c* and *d*, the pigments found in the nonmotile members of the group. The nonmotile species therefore must signal the motile bacterium when the light intensity changes. The mechanism is unknown, but many motile species respond to changes in pH, and the change in extracellular pH that may accompany changes in photosynthetic activity could serve as a signal. In some cases, consortia only remain together when incubated photosynthetically and disperse in the dark. Given the predominance of these consortia in some freshwater lakes, it seems likely that this association and the phototactic and chemotactic behavior that goes with their formation is important for the colonization of these lakes.

Conclusion

It seems likely from the sequencing of bacterial genomes, that the majority of bacterial species move, either swimming or gliding to their optimum environment for growth. What has become apparent is that the motility and chemosensory pathways of the majority of bacterial species are built from the same basic skeleton as found in *E. coli*. However, different species have expanded and added to the basic system to tune it to their requirements. Therefore, different species not only sense different stimuli, but also the number of sensory signals varies greatly. Many species also seem to have several different chemosensory pathways, expressed under different conditions. The reason for this is unclear and may reflect the kinetic requirements of responses to different signals. What is really apparent, however, is that motility and chemotaxis do not stand alone as physiological traits, but are just part of a large, interconnected sensory network that makes a particular species robust for life in a current specific niche.

Acknowledgements. Research on *R. sphaeroides* in the Armitage laboratory is funded by the UK BBSRC.

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Prokaryotes (2006) 2:140–166
DOI: 10.1007/0-387-30742-7_6

CHAPTER 1.6

Prokaryotic Life Cycles

MARTIN DWORKIN

Introduction

Modern prokaryotic biology has focused much of its attention on understanding the nature and regulation of bacterial growth. This reflects Francois Jacob's memorable rhetorical question, "A bacterium, an amoeba . . . what destiny can they dream of other than forming two bacteria, two amoebae . . .?" (Jacob, 1973). It has been an unstated assumption that the primary approach to fulfilling this destiny is to optimize growth, exemplified by the remarkable efficiency and adaptability of the growth of enteric bacteria, pseudomonads, and other prokaryotes. However, the ability of many bacteria to go through a life cycle is another way (more common than is recognized) that prokaryotes increase the likelihood that one bacterium will eventually become two. It is the goal of this chapter to describe some typical life cycles found among the bacteria.

Prokaryotic life cycles can be divided into three general groups: 1) there are simple life cycles, during which the cells alternate between two states, each of which is optimal for a different set of environmental conditions; for example, cells may alternate between growing and resting, between the motile and sessile state, or between infectious and reproductive stages. 2) There are complex cycles where more than two developmental stages are involved; an example is the cycle found in the myxobacteria where cells may not only alternate between vegetative and resting stages, but also undergo a colonial morphogenesis and form multicellular fruiting bodies. 3) Finally, there are life cycles leading to the formation of truly differentiated populations; for example, a culture of the cyanobacterium *Anabaena* may simultaneously contain vegetative cells, nitrogen-fixing heterocysts, and resistant akinetes.

This chapter will describe representatives of each of these groups, but for a more detailed description of those prokaryotes that have been used as experimental model systems for studies in development, the reader is directed to the recent excellent monograph on prokaryotic development edited by Brun and Shimkets (2000a).

Simple Life Cycles: Alternation Between Two States

Some bacteria have a relatively constant habitat (i.e., they are not subjected to constantly changing environmental conditions). For example, an organism that is an extracellular or intracellular parasite of a higher organism is likely to be the fortunate inhabitant of an environment that is relatively constant with regard to such physiological parameters as temperature, pH, ion concentration, and rate of supply of nutrients. Other bacteria, however, are likely to be subject to environmental conditions that frequently change. The physiological makeup of an organism that must alternate, on the one hand, between inhabiting the gut of a warm-blooded animal while attached to an epithelial cell, and on the other hand, floating suspended in a body of fresh water, must have considerable flexibility. An organism that inhabits the soil must be able to deal with changing temperature, nutrient level, moisture content, and light-dark cycles.

Bacteria have evolved a number of different adaptive strategies. An organism like *Escherichia coli* has evolved an extraordinarily sophisticated network of regulatory mechanisms that allows it to adapt quickly and effectively to different substrates, levels of nutrient, osmotic environments and temperatures. Its modus vivendi allows it to grow extremely rapidly when conditions are optimal, more slowly when they are not, and when growth is not possible, to remain dormant until growth conditions are again available. However, an alternative strategy is in fact available, namely the developmental alternation between two morphogenetic states, each of which is optimal for a different set of circumstances.

This strategy of functional specialization allows the organism to exist in narrowly optimized alternative states rather than in a single, broad optimal state. The advantage of this may

be that the limits of the conditions that can be dealt with by each differentiated state are substantially greater than the limits contained in the repertoire of the undifferentiated, but more broadly versatile cell. In its simplest states, the alternation may take four different forms. These are described below and examples of each alternation are given.

Alternation Between Vegetative Growth and a Resistant, Resting Stage

This developmental pattern is characteristic of many genera but three exemplary types are discussed here: *Bacillus* endospores, *Azotobacter* cysts, and methylotroph exospores. (Some other types of resting cells whose formation is part of a more complex life cycle will be dealt with in subsequent sections. Also, see in Breznak, 2001, the description of *Sporomusa*, a Gram-negative endospore-former.)

The Life Cycle of *Bacillus* The processes of sporulation and germination in *Bacillus* have been intensively studied for over 50 years, with the result that we now know more about the details of these developmental processes than about any other (Sonnenshein, 2000; Slepecky and Hemphill, 1992). A variety of other genera of bacteria also form endospores, e.g., *Clostridium* (Hippe et al., 1992), *Sporosarcina* (Claus et al., 1992; Zhang et al., 1997), *Sporolactobacillus* (Claus et al., 1992; Kitahara and Lai, 1967), *Thermoactinomyces* (Cross and Goodfellow, 1973), and *Desulfotomaculum* (Widdel, 1992; Campbell and Postgate, 1965), and as far as is known, the morphological events leading to endospore formation in these organisms seem relatively similar. While it is tacitly assumed that the developmental events in these organisms are fundamentally the same, evidence to make that statement with any confidence is insufficient. (For a brief discussion of the diversity of bacteria able to form endospores, see Slepecky and Leadbetter, 1983, and Slepecky and Hemphill, 1992.)

The endospore of *Bacillus* is a metabolically quiescent cell, resistant to extremes of temperature, desiccation, chemical agents, radiation, and physical disruption. The events leading to its formation are customarily divided into seven stages, diagramatically represented in Fig. 1. The definition of these stages is based on discernable morphological changes and the isolation of mutants that are blocked in any one of these stages. (For

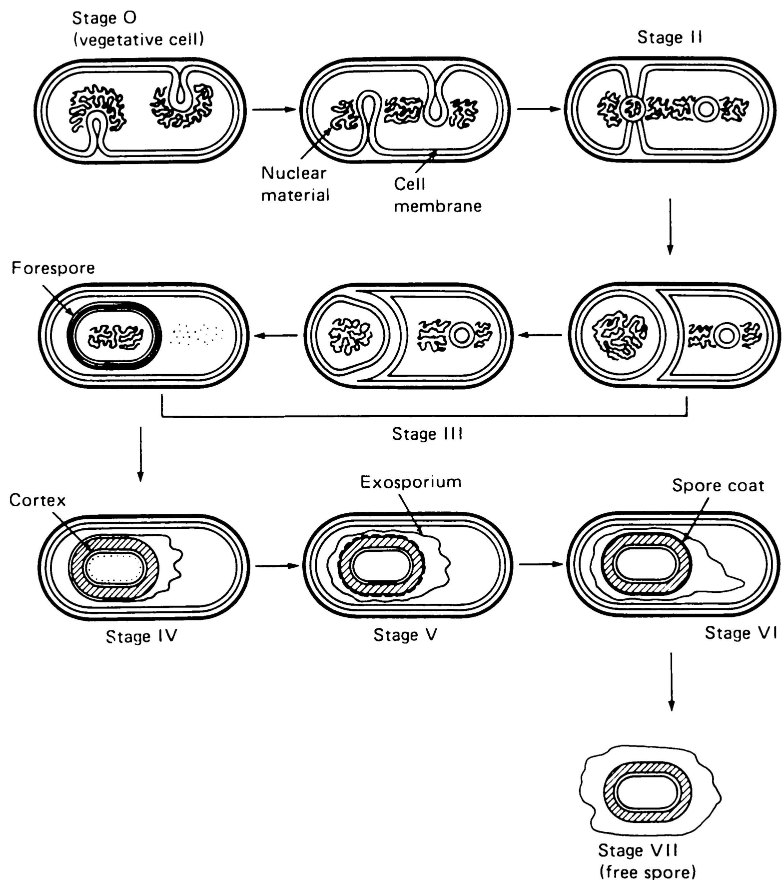


Fig. 1. The seven stages of sporulation in *Bacillus*. (From Dworkin, 1985a.)

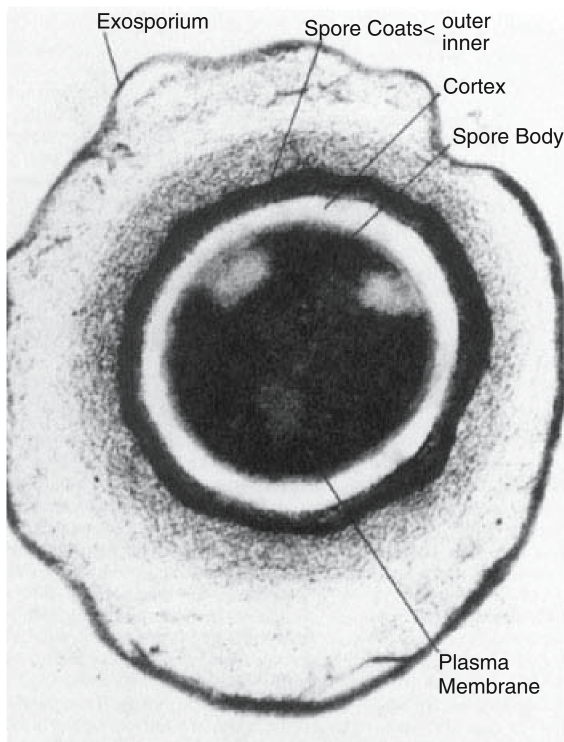


Fig. 2. Electron micrograph of a thin section of a spore of *Bacillus sphaericus*. (Courtesy of Dr. S. Holt.)

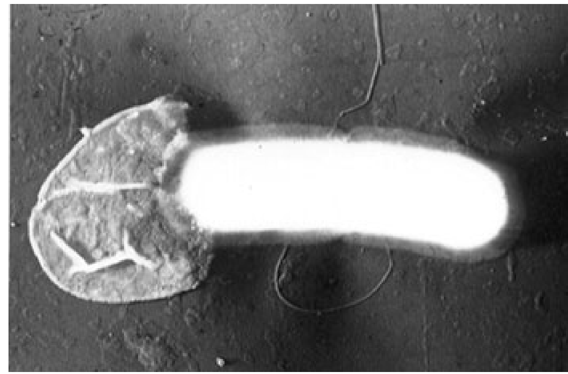


Fig. 3. Electron micrograph of a germinating cell of *Bacillus cereus*. (From Strange and Hunter, 1969.)

a detailed description of the genetic analysis of endospore formation in *Bacillus*, see Errington, 1993.)

When vegetatively growing cells of some species of *Bacillus* are subjected to a nutritional deficiency (shift-down), the nature of which has not been precisely defined, the cells cease the process of dividing by binary transverse fission and instead go through an asymmetrical division leading to the formation of a mother cell and a forespore (Fig. 1, stage III). It has been shown (Lencastre and Piggot, 1979; Losick and Stragier, 1992) that there is considerable biochemical and genetic cross-talk between these two cells, resulting finally in a mature spore (Fig. 2) and a mother cell that eventually lyses and dies.

Most of the emphasis on the mechanisms that control sporulation have focused on the transcriptional regulation of developmental gene expression (Stragier and Losick, 1996). Relatively little attention has been paid to that part of the process that completes the cycle-namely, spore germination (Fig. 3). Germination is perhaps an even more remarkable process than sporulation. The spore may exist for a considerable period of time (Dombrowski, 1963; Gest and Mandelstam, 1987; Cano and Borucki, 1995), in a state of metabolic quiescence, resistant to most external extremes, yet it is poised to return almost instantaneously to a state of metabolic competence and active vegetative growth. There is a considerable amount of information about the mechanisms of resistance of the *Bacillus* endospore and its DNA. The resistance of the endospore seems to be a result of the relative dehydration and mineralization of the spore coat (Marquis et al., 1994), whereas the resistance of the spore DNA is a result of the protective effect of a series of small, acid-soluble spore proteins (Setlow, 1995). Less insight is available regarding the mechanisms that allow the remarkable transformation of a resistant, resting cell to an actively growing one (Setlow, 1983).

THE LIFE CYCLE OF *AZOTOBACTER* *Azotobacter* is a large, Gram-negative, free-living, nitrogen-fixing rod that forms cysts as an alternative stage in its life cycle (Becking, 1992). Its life cycle is diagrammatically represented in Fig. 4. Figures 5 and 6 are electron micrographs of thin sections of a vegetative cell and a mature cyst of *Azotobacter vinelandii*, respectively. Unlike the endospore of a *Bacillus* species, the cyst is formed by the rounding up of the entire cell. *Azotobacter* cysts are not resistant to extremes of temperature, but are resistant to desiccation and to some deleterious chemical and physical agents (Socolowsky and Wyss, 1962). As is usually the case with sporulation or encystment, the developmental events are set in motion by a nutritional shift-down; encystment in *Azotobacter vinelandii* (the organism used for most of the developmental studies), is considerably accelerated by the addition of β-hydroxybutyric acid (Sadoff et al., 1971), which serves as a precursor of the electron-transparent poly-β-hydroxybutyrate granules that are characteristic of the cysts (Fig. 6). The cells shed their flagella, cease nitrogen fixation, gradually become rounded, and finally become optically refractile. The cyst is surrounded by a thickened, multilayered outer coat called "the exine," consisting

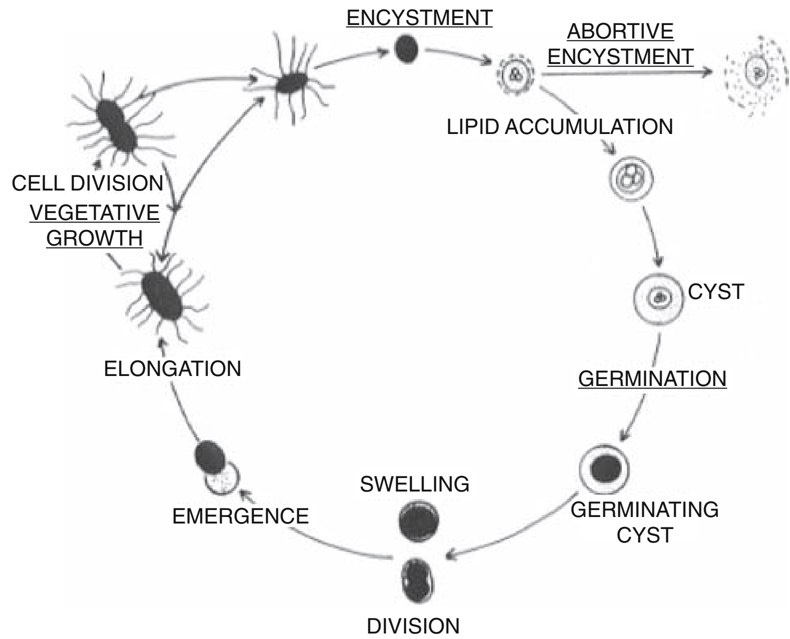


Fig. 4. A schematic of the life cycle of *Azotobacter vinelandii*. (From Sadoff, 1975.)

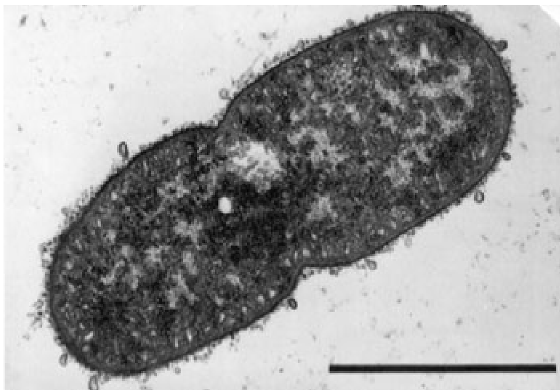


Fig. 5. Electron micrograph of a thin section of a dividing vegetative cell of *Azotobacter vinelandii*. Bar = 1 µm. (From Hitchins and Sadoff, 1970.)

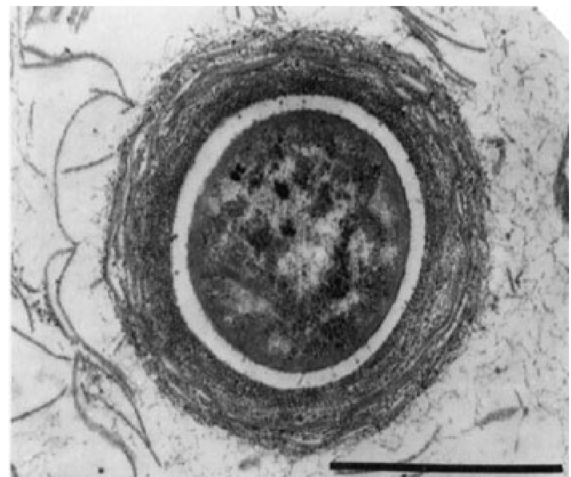


Fig. 6. Electron micrograph of a thin section of a mature cyst of *Azotobacter vinelandii*. Bar = 1 µm. (From Hitchins and Sadoff, 1970.)

of lipoprotein and lipopolysaccharide. A number of unique lipids have been shown to be synthesized and form part of the cyst structure (Reusch et al., 1981). Germination occurs when the cysts are placed in the presence of an exogenous carbon source such as glucose. This immediately induces respiration, macromolecular synthesis, and the conversion of the cyst to the vegetative cell.

THE LIFE CYCLE OF METHYLOTROPHS The methanotroph *Methylosinus trichosporium* (Bowman, 2000) forms an unusual type of resting cell called an "exospore" (Whittenbury et al., 1970). When cells of *M. trichosporium* reach the stationary phase of growth, a proportion of the cells elongate, become tapered, and bud off rounded bodies, which gradually acquire optical refractility. These events are illustrated in Fig. 7. The exospore is resistant to desiccation and to elevated temperatures of at least 78°C. Germination occurs slowly when the cells are placed once again under conditions conducive to growth. Figure 8 is an electron micrograph of a thin section of a mature spore of *M. trichosporium*, and Figs. 9 and 10 illustrate successive stages in the germination of an exospore. Relatively little is known about the biochemistry, physiology, regulation, and developmental biology of these unusual resting cells.

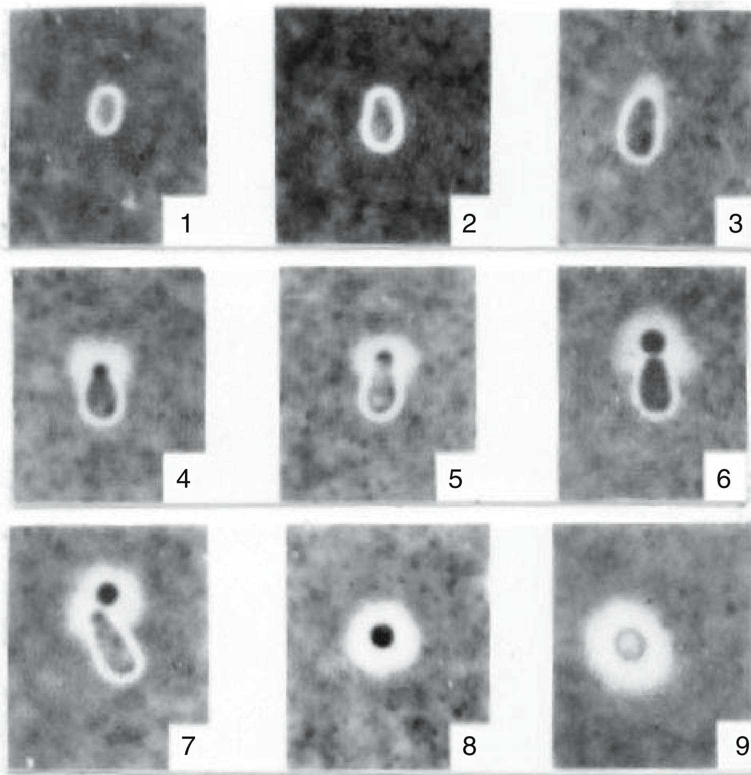


Fig. 7. Phase contrast photomicrographs of exospore formation by *Methylosinus trichosporium*. The capsulated, rod-shaped, vegetative cell becomes pear-shaped, and the tapered end buds off the cell that is eventually released as the refractile exospore. (From Whittenbury et al., 1970.)

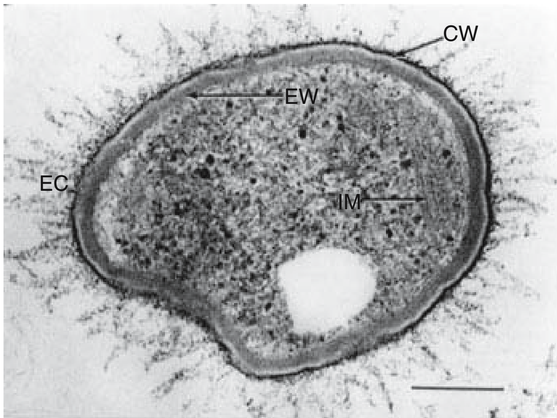


Fig. 8. Electron micrograph of a thin section of an exospore of *Methylosinus trichosporium*. Bar = 0.2 µm. CW, cell wall; EC, exospore capsule; EW, exospore wall; and IM, intracytoplasmic membranes. (From Reed et al., 1980.)

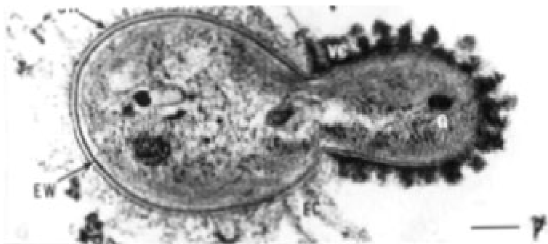


Fig. 9. Electron micrograph of a thin section of an early stage of a germinating exospore of *Methylosinus trichosporium*. Bar = 0.2 µm. VC; G; and other abbreviations are the same as those used in Fig. 8. (From Reed et al., 1980.)

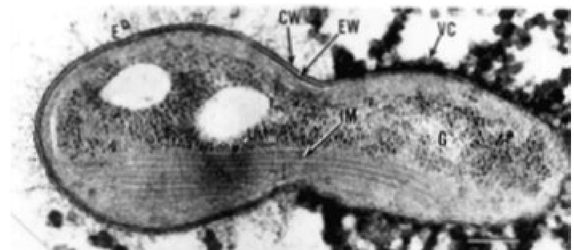


Fig. 10. The same as Fig. 9, but at a later stage. (From Reed et al., 1980.)

Alternation Between Sessile and Motile Stages

CAULOBACTER LIFE CYCLE; ALTERNATION BETWEEN STALKED AND SWARMER CELLS *Caulobacter crescentus* has been the subject of considerable attention on the part of prokaryotic developmental biologists (Brun and Janakiraman, 2000b; Gober and England, 2000; Ohta et al., 2000; Hung et al., 2000; also see the chapter on the Dimorphic Prosthecate Bacteria in Volume 5). This organism provides an excellent model system for asking two general types of developmental questions. The first question has a spatial quality and pertains to the cellular localization of the stalk and the flagellum; the second concerns the temporal aspects of development and is concerned with the regulatory relationship between the growth and the development of the cells. While neither of these may seem to bear any obvious relationship to the issue of sessile versus motile stages, the end result of the developmental events in *Caulobacter* is, in fact, an alternation between two states: the flagellated, free-swimming, nongrowing swarmer stage and the stalked, sessile, reproductive stage. Thus, *Caulobacter* exemplifies the temporo-spatial aspects of development and offers an almost unique opportunity to examine the epigenetic aspects of developmental regulation in the context of an extremely convenient and tractable experimental system.

The life cycle of *Caulobacter* is diagrammed in Fig. 11. Starting from the inexperienced stalk cell

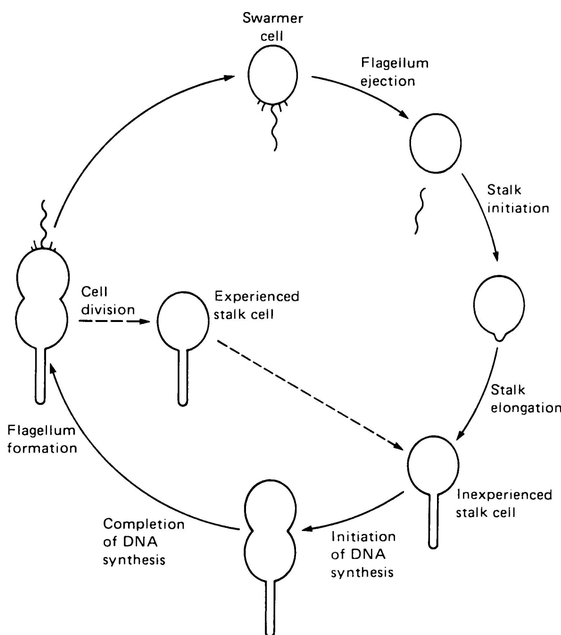


Fig. 11. Diagram of the life cycle of *Caulobacter crescentus*. (From Dworkin, 1985a.)

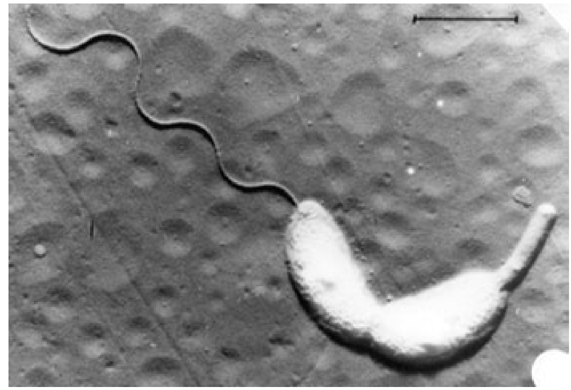


Fig. 12. Electron micrograph of a shadowed cell of *Caulobacter crescentus*. The stalked mother cell is in the process of dividing, leading to the formation of a flagellated swarmer cell. Bar = 1 μm. (From Poindexter, 1964.)

(i.e., a stalk cell that has not yet undergone a cell division) attached to a substrate, the cell will grow and produce a flagellated daughter cell (Fig. 12). This motile daughter cell apparently is involved in colony dispersal and will, at some point, attach to a surface via its holdfast, shed the flagellum, and in its place synthesize the characteristic *Caulobacter* stalk. At this point it again becomes a new, inexperienced stalk cell. The original stalk cell meanwhile continues to grow and produces new swarmer cells. In a sense, the stalk cell acts as an immortal stem cell; unlike the situation in conventionally dividing bacteria where one cannot distinguish between a mother and a daughter cell, in *Caulobacter*, the mother is theoretically immortal, continuing to bud off daughter swarm cells.

Figure 13 relates biochemical and developmental events to the cell cycle and further emphasizes the relationship between growth and development (Laub et al., 2000).

It is evident that, unlike those developmental cycles in which events (such as the formation of a resistant resting cell) is an alternative to growth of the cell, in *Caulobacter*, the two processes are intimately intertwined (Hung et al., 2000). The asymmetric localization of the stalk and subsequently the flagellum has been the model system for examining the process of spatial differentiation (Gober and England, 2000). What are the clues that determine the polar placement of the stalk and the flagellum? It appears that the spatial localization of polar structures in *Caulobacter* is a result both of cell pole-specific gene expression and protein targeting.

The questions of spatial morphogenesis in *Caulobacter* are essentially no different from the kinds of questions one can ask about the biosynthesis and placement of cellular structures in organisms that do not exhibit a developmental

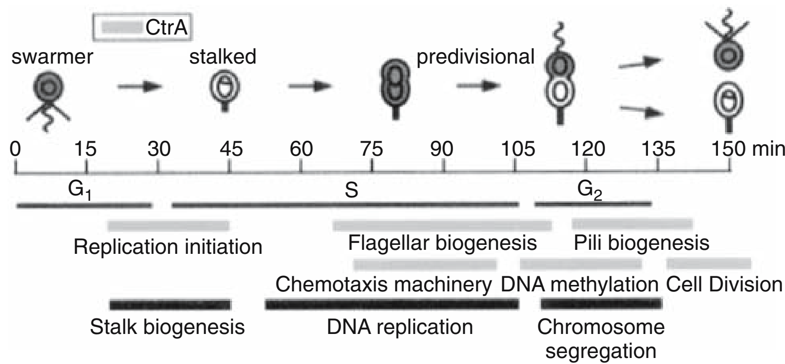


Fig. 13. A schematic illustrating various developmental and biosynthetic events as a function of the cell cycle of Caulobacter crescentus. Circles and "theta" structures in the cells represent quiescent and replicating chromosomes, respectively. A member of a two-component signal transduction family, CtrA, is present in the shaded cells, where it represses DNA replication and is cleared by proteolysis during the swarmer-stalked cell transition. (From Laub et al., 2000, with permission of the American Association for the Advancement of Science.)

life cycle, and it is an intriguing possibility that the answers may emerge from the investigations of *Caulobacter* rather than from work on these simpler systems. In fact, recent work with *Caulobacter* has taken the lead in examining development as an integrated regulatory system, and has shown that a single regulatory factor CtrA, a member of a two-component signal transduction family, is involved in the control of a quarter of all the cell-cycle associated genes (Laub et al., 2000).

THE LIFE CYCLE OF SPHAEROTILUS *Sphaerotilus*and the related genus *Leptothrix* (Mulder and Deinema, 1992) behave in a way that is analogous to *Caulobacter*. Both groups of organisms alternate between a motile and a sessile state. In the case of *Sphaerotilus*, however, the sessile state is represented by a filamentous sheath that encloses the rod-shaped cells (Fig. 14) and is attached by one end (the holdfast) to a surface (Fig. 15). As growth and division occur, the cells acquire polar flagellation (Figs. 14B and 16) and emerge from the sheath. Upon reaching an appropriate new site, the cells attach to a surface, shed the flagellum, and begin dividing within a new sheath. Neither the physiology nor the regulatory developmental events in *Sphaerotilus* have been extensively examined.

THE LIFE CYCLE OF THE ACTINOPLANETES The actinoplanetes are a group of nine genera within the actinomycetes (Kroppenstedt and Goodfellow, 1992) and represent another variation of a life cycle based on the theme of alternation between a sessile, feeding, and reproducing stage and a motile, nonreproductive one. In these organisms, the sessile stage is the vegetative mycelium, and the motile stage is represented by flagellated spores formed within a sporangium. (The actinomycetes will be further discussed later in this chapter as an example of a complex life cycle; at this point, only the alternation between the sessile and motile stages will be discussed.) Figure 17 is a diagram illustrating the characteristic sporangial morphology and the zoospores of various genera among the actinoplanetes. Figure 18 is an electron micrograph of a thin section of a sporangium of *Pilimelia* filled with spores.

Alternation Between an Extracellular, Infectious Stage and an Intracellular, Reproductive Stage

THE LIFE CYCLE OF BDELLOVIBRIO Bdellovibrio is a genus comprising three species of small, Gram-negative, predatory bacteria (Jurkevitch, 2000). They are obligate intracellular parasites of other Gram-negative bacteria such as the enteric bacteria, *Pseudomonas*, *Rhizobium*, *Spirillum*, *Photobacterium* and *Chromatium*. The cells alternate between a motile, polarly flagellated, nonreproducing, extracellular form and a nonmotile, reproductive form localized in the periplasmic space of the host cell. Figure 19 illustrates the morphology of the free-swimming, extracellular form. The anterior of the cell is differentiated into a distended tip that serves as a holdfast, with which the Bdellovibrio attaches to the surface of the host cell. The polar flagellum is unusual in that it is covered by a sheath, which is a continuation of the outer cell membrane (Fig. 20).

The encounters between the *Bdellovibrio* cell and its host appear to be random; no evidence for a chemotactic perception of host cells or their extracts has been found (Straley and Conti, 1977), despite the fact that *B. bacteriovorus* is chemotactic toward other compounds (Straley et al., 1979). There is an initial, reversible stage

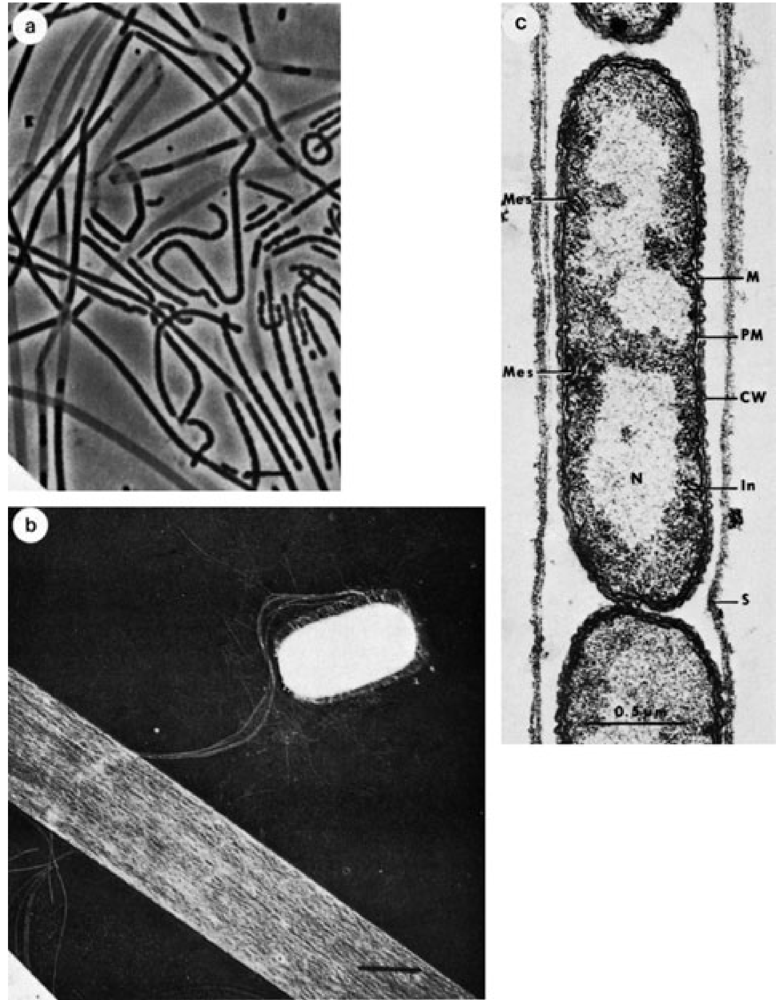


Fig. 14. *Sphaerotilus natans*. (a) Phase contrast photomicrograph illustrating free cells, sheathed cells, and empty sheaths. Bar = 1 µm. (From Mulder and Deinema, 1992.) (b) Electron micrograph of a swarmer cell and an empty sheath. Bar = 1 µm. (From Mulder and Deinema, 1992.) (c) Electron micrograph of a thin section of sheathed cells. S, sheath; CW, cell wall; P, peptidoglycan layer; M; PM; In, intrusion of plasma membrane; Mes, mesosome; and N, nucleoplasm. (From Hoeniger et al., 1973.)

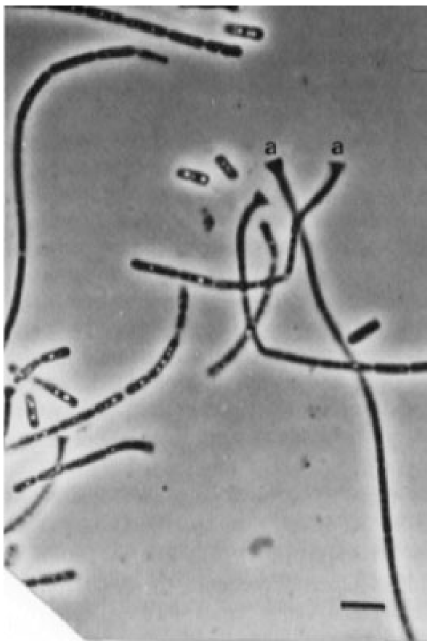


Fig. 15. Phase contrast photomicrograph of *Sphaerotilus natans* illustrating cells released from their sheath and holdfasts (a) at the end of the sheaths. Bar = 1 µm. (From Mulder and Deinema, 1992.)

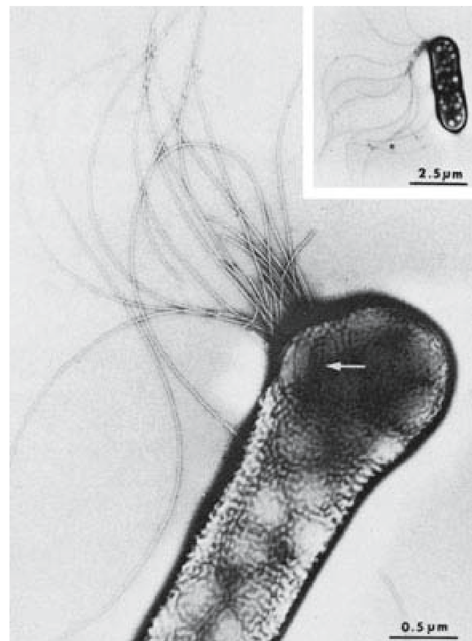


Fig. 16. The end of a swarm cell of *Sphaerotilus natans*. Inset: electron micrograph of a swarm cell of *S. natans*. (From Hoeniger et al., 1973.)

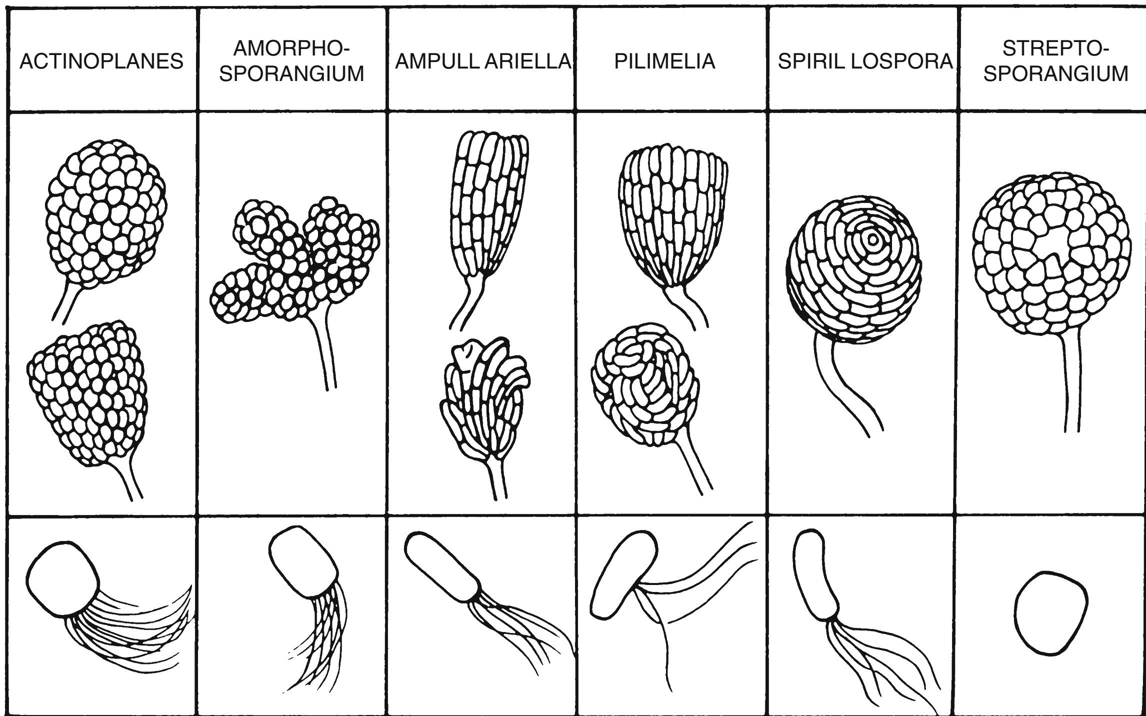


Fig. 17. A schematic showing the differing arrangements and morphologies of the spores and sporangia of the various genera of the Actinoplanaceae. (From Vobis, 1984.)

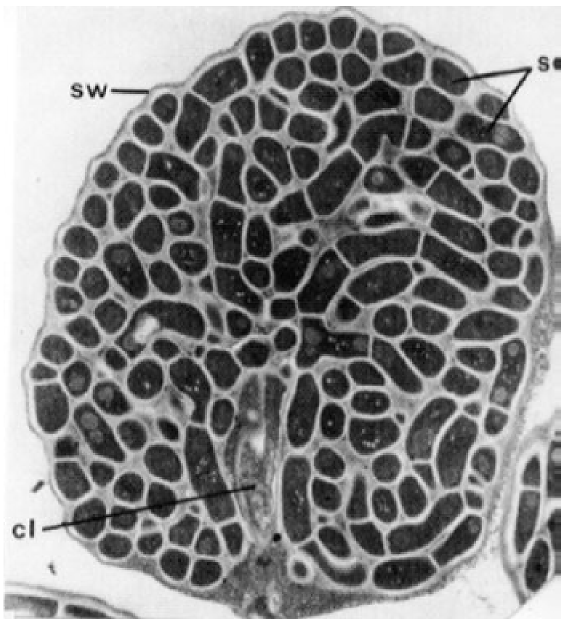


Fig. 18. Electron micrograph of a thin section of a sporangium of the actinoplanete genus Pilimelia, illustrating the arrangement of the spore chains. sw, sporangial wall; cl, columella; and sp, spore. (From Vobis, 1984.)

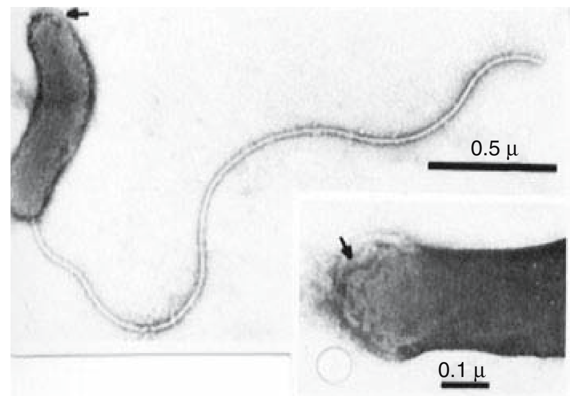


Fig. 19. Electron micrograph of negatively stained cell of *Bdellovibrio bacteriovorus* showing the thick, polar flagellum and the surface convolutions at the anterior end of the cell (arrow). Inset: distended anterior end that acts as a holdfast. (From Burnham et al., 1968.)

Fig. 20. Electron micrograph of a thin section of *Bdellovibrio bacteriovorus*; the sheath enclosing the flagellum is continuous with the outer membrane of the cell. (From Burnham et al., 1968.)

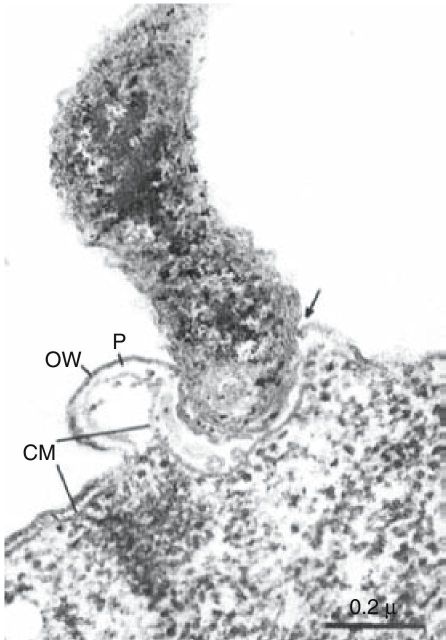


Fig. 21. Electron micrograph of a thin section of *Bdellovibrio bacteriovorus* at the early stage of its invasion of the host cell, *Escherichia coli*. P, periplasm; OW, outer wall; and CM, cell membrane. (From Burnham et al., 1968.)

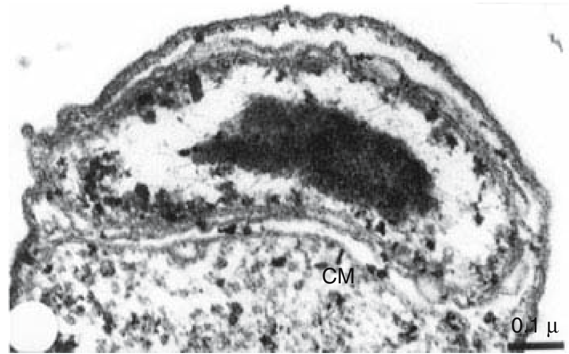


Fig. 22. Electron micrograph of the late stage of penetration of the host cell by *Bdellovibrio bacteriovorus*. The *Bdellovibrio* occupies the periplasm of the host cell. CM, cell membrane. (From Burnham et al., 1968.)

after contact, quickly followed by an irreversible stage (Fig. 21). The *Bdellovibrio* then penetrates the outer envelope by a mechanism that seems to involve its rapid rotation at speeds up to 100 rotations per second (Stolp, 1967). The parasite sheds its flagellum and enters the periplasm, between the inner cell membrane and the peptidoglycan layer (Fig. 22). It then grows into a septate filament, which, when growth ceases, fragments into flagellated, attack-phase progeny. These cells then lyse the host and swim off, ready for the next encounter with a susceptible host. These events are schematically illustrated in Fig. 23.

The attack-phase cells, which are free-living, are differentiated from the morphologically distinct intracellular reproductive cells by considerable physiological differences. The intracellular stage is able to carry out the biosynthesis of RNA, DNA and protein, in contrast to the free-living cells, which are biosynthetically inert. The cells have the unusual ability to transport ATP and phosphorylated nucleosides; however, the two cell types also differ in this trait. The attack-phase cells are characterized by a system with a high substrate affinity and a low V_{max}, whereas the transport system of their intracellular counterparts has just the reverse properties, i.e., a low affinity and high V_{max} (Ruby, 1985). The ability of *Bdellovibrio* to take up phosphorylated compounds is reflective of their general ability to utilize the high-molecular-weight components of the parasitized host cell directly for their own biosynthetic processes. This results in an unusually high level of growth efficiency; up to 65% of the host cell material is converted to *Bdellovibrio* (Rittenberg and Hespell, 1975).

It has been possible to obtain mutants of *Bdellovibrio* that are host independent, i.e., that can be grown axenically on conventional laboratory media (Seidler and Starr, 1969; Cotter and

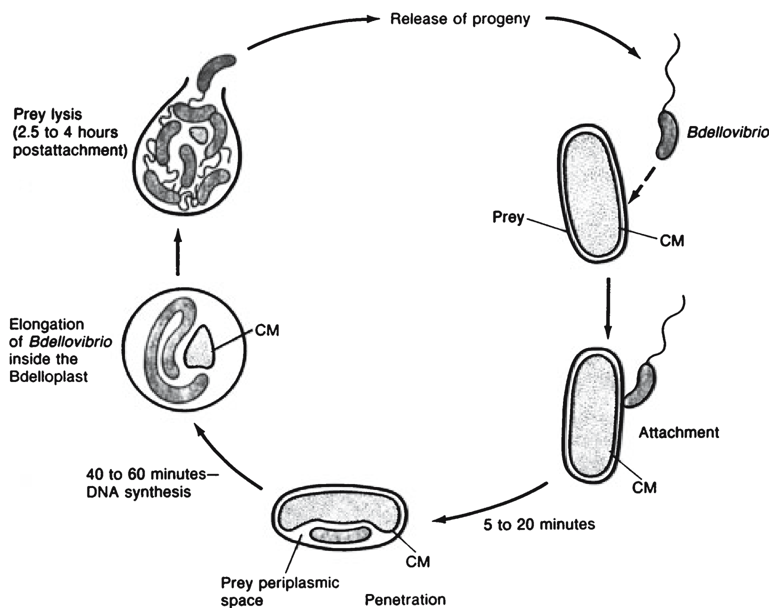


Fig. 23. Diagrammatic representation of the life cycle of *Bdellovibrio*. CM, cell membrane. (From Brock and Madigan, 1988, with permission of Prentice-Hall, Inc., Englewood Cliffs, NJ.)

Thomashow, 1992). However, more useful from an experimental point of view has been a technique that allows the release of intact, viable growth-phase cells, which can be induced to convert to the motile attack-phase cells (Ruby and Rittenberg, 1983). This has allowed a physiological and biochemical comparison of the two types of cells (Ruby, 1989).

There is relatively little information on the nature of the regulatory events that control the conversion from one cell type to the other. Gray and Ruby (1991) have proposed that there are likely to be at least two signals—one triggering the conversion of the attack phase cells into the growth phase cells, and the other initiating DNA replication in the growth phase cells.

The Life Cycle of Chlamydia *Chlamydia* is an obligate parasite that alternates between two states, extracellular and intracellular. Correspondingly, its life cycle consists of two alternating cell types, the elementary body and the reticulate body, each of which is specifically adapted to carry out one aspect of the organism's life cycle (Rockey and Matsumoto, 2000; Kalayoglu, 2001).

The elementary body is a resting cell, resistant to desiccation, unable to grow, and highly infectious. Its primary functions seem to be its resistance to adverse, extracellular conditions, and its infectivity. Its alternative state, the reticulate body is a larger cell, containing ribosomes and possessing the ability to use the biosynthetic capacities of the host cell for its own growth. Table 1 gives a comparison of the properties of the two cell types.

The life cycle of *Chlamydia* consists of the following series of events:

1. Infection of the host cell by the elementary body. It is likely that there are specific interactions between the chlamydial cell and that of its susceptible host, but that has not been demonstrated. The elementary body is immediately phagocytized by the host cell and engulfed in a phagosome, an organelle bounded by a membrane derived from the host membrane. Within the phagosome, the Chlamydia is protected from the normal lysosome-mediated destruction that would occur with other types of bacterial invaders.

2. Conversion of the elementary bodies to the reticulate bodies. Within 10–15 h, the chlamydial cells have synthesized ribosomes, reorganized

Table 1. Properties of the elementary body and the reticulate particle of the genus *Chlamydia*.

Property	Elementary body	Reticulate particle
Size (nm)	200–400	600–1,000
Rigid cell wall	+	–
Extracellular stability	+	–
Serotype-specific antigens	+	–
Species-specific antigens exposed	–	+
Organization of DNA	Nucleoid	Dispersed
RNA/DNA ratio	<1	3–4
Infective	+[a]	–
Induce phagocytosis	+[a]	–
Inhibit phagosome fusion	+[a]	–
Toxicity	+[a]	–
Metabolic activity	–	+
Replication	–	+

Symbols: +, property; and –, property absent.
[a]Properties apparently blocked by specific antibody.
From Schachter and Caldwell (1980), with the permission of *Annual Review of Microbiology*.

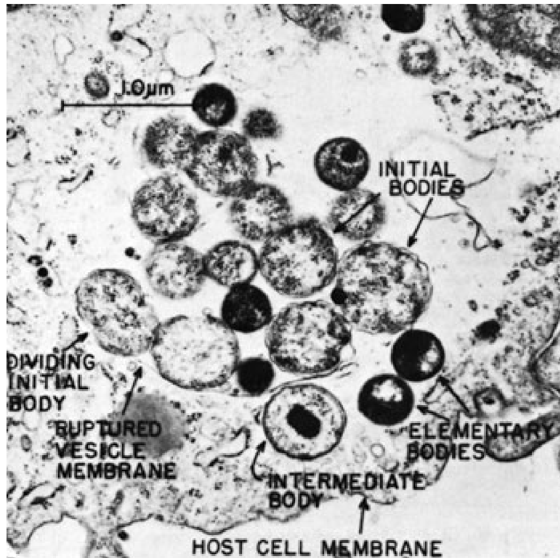


Fig. 24. Electron micrograph of a thin section of an animal cell infected with *Chlamydia psittaci*. The "Initial Bodies" are the reticulate bodies. (From Cutlip, 1970.)

their DNA, acquired a new and different cell wall, and begun to divide by binary fission.

3. Growth and replication proceed until the phagosome is filled with cells of *Chlamydia*.

4. The reticulate bodies convert once again to the elementary bodies, lyse the host cell, and either emerge to the outside environment or reinfect adjacent cells.

A phagosome filled with both types of cells and some intermediary forms is shown in Fig. 24.

The Life Cycle of *Flexibacter*

Flexibacter filiformis is one of a group of filamentous, gliding bacteria. It is distinguished from the others, however, by its ability to go through a cycle in which long, threadlike filaments alternate with short, fat, almost coccoid rods. Figure 25 illustrates the presence of both cell types in a heterogeneous population growing on an agar surface. This is probably a reflection of the fact that the conversion of the long, filamentous cells to the short cells occurs when the culture enters the stationary phase of growth (Simon and White, 1971). Poos et al. (1972) showed that the conversion also could be regulated by the growth temperature. When a culture of *Flexibacter* was grown at 30°C, the cells grew exponentially as long filaments and then divided in the middle; when the culture was shifted to 35°C, the cells fragmented into the shorter forms and continued to grow in that fashion. The authors suggested that the shift-up in temperature increased the rate of septum formation and induced cell separation, whereas the shift-down in temperature inhibited cross-wall formation but not growth.

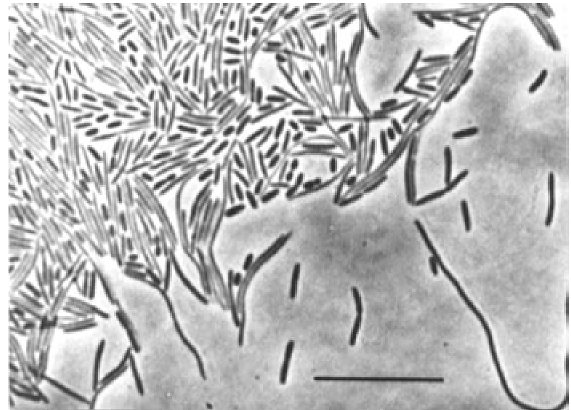


Fig. 25. Phase contrast photomicrograph of individual cells and long filaments of *Flexibacter filiformis*. (From Reichenbach, 1989.)

Complex Life Cycles: Multiple Alternative States

There are prokaryotes whose life cycles consist of multiple alternative states. These include alternations between growing and resting states, between sessile and motile states, and between unicellular and multicellular states. When the possibility of different modes of growth and the formation of structures such as fruiting bodies are added, the developmental tapestry becomes an even more intricately woven one.

The Life Cycle of *Myxobacteria*

The life style of the myxobacteria is at the interface between unicellularity and multicellularity. Their life cycle is characterized by pervasive cell-cell interactions during both growth and development. Their most distinguishing features are that they move by gliding over solid surfaces and their life cycle culminates in the formation of macroscopic, multicellular, often complex fruiting bodies (Dworkin, 2000; Reichenbach and Dworkin, 1992; see Figs. 29 and 30).

The myxobacteria share the property of gliding motility with a large, heterogeneous, essentially unrelated group of bacteria referred to as the gliding bacteria (Reichenbach and Dworkin, 1981; Spormann, 1999). While a number of theories have been proposed for the mechanism of gliding, none of these has emerged as a definitive explanation (Spormann, 1999).

The unique feature of the myxobacteria is their ability to go through a complex life cycle, during which the cells aggregate, form fruiting bodies, and within the fruiting bodies, convert to myxospores.

Most of the recent work on the myxobacteria has focused on two species, *Myxococcus xanthus* (Ward and Zusman, 2000; Kaiser, 2000; Shimkets, 2000) and *Stigmatella aurantiaca*

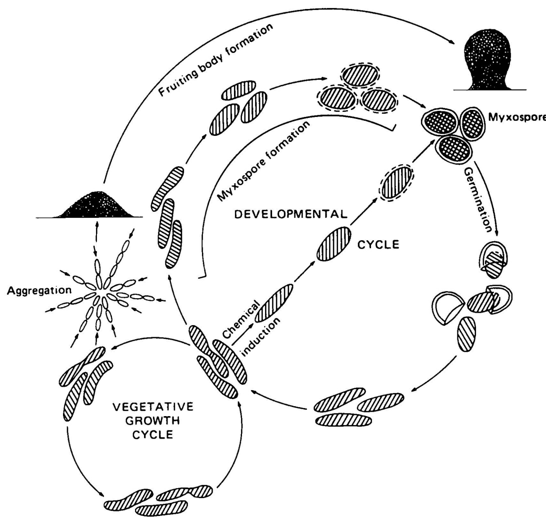


Fig. 26. Diagram of the life cycle of *Myxococcus xanthus*. The fruiting body is not drawn to scale, but it is a few hundredths of a mm in diameter, in contrast to the vegetative cells, which are about 5–7 × 0.7 μM. (From Dworkin, 1985a.)

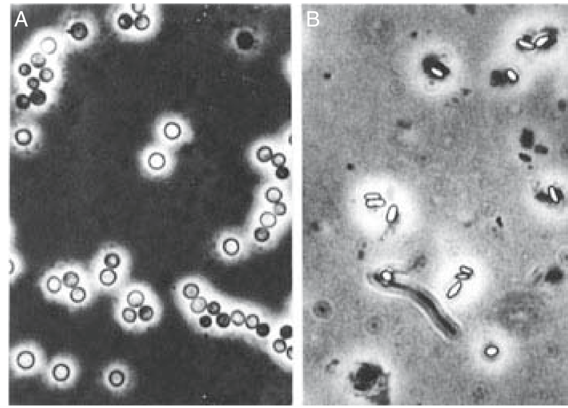


Fig. 28. Phase contrast photomicrographs of myxobacterial myxospores. (A) *Myxococcus xanthus*. (B) *Stigmatella aurantiaca*. (Courtesy of Dr. H. Reichenbach.)

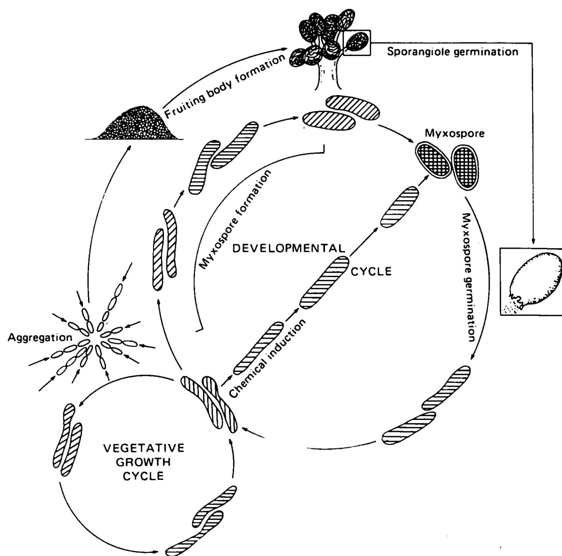


Fig. 27. Diagram of the life cycle of *Stigmatella aurantiaca*. (From Dworkin, 1985a.)

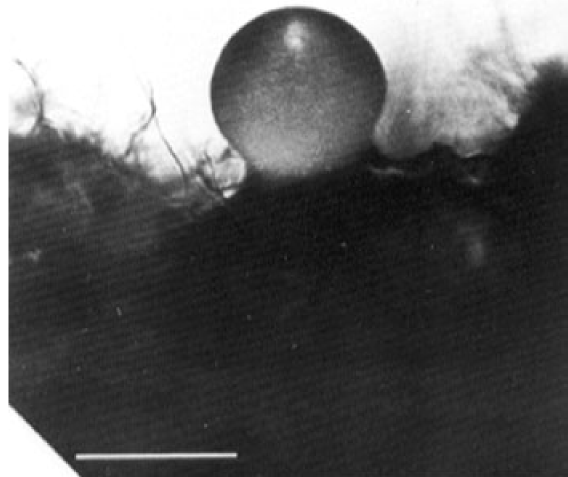


Fig. 29. Fruiting body of *Myxococcus fulvus*. Bar = 35 μm. (From Reichenbach, 1984.)

(White and Schairer, 2000). The life cycles of these organisms are illustrated in Figs. 26 and 27.

The myxospores of *M. xanthus* are optically refractile, resistant, resting cells (Fig. 28) that are able to germinate when placed under conditions that allow vegetative growth.

There are essentially two parts to the myxobacterial life cycle. *Myxococcus xanthus* uses proteins, peptides, or amino acids as its source of carbon, energy, and nitrogen (Shimkets, 1984), and under favorable conditions, the cells grow exponentially with a generation time of about 3.5 h. Three conditions must be satisfied for the cells to shift from the growth mode to the alternative developmental mode (Dworkin and Kaiser, 1985b): 1) The cells must experience a nutritional shift-down. 2) The cells must be on a solid surface. 3) The cells must be at a high cell density. If these three conditions are met, the cells cease growth and begin to aggregate into centers that eventually become the fruiting bodies. These fruiting bodies may be either more (Fig. 29) or less (Fig. 30) elaborate.

Myxobacteria feed by excreting a variety of powerful hydrolytic enzymes, which they use to degrade proteins, polysaccharides, lipids, and nucleic acids. They are thus at the mercy of the

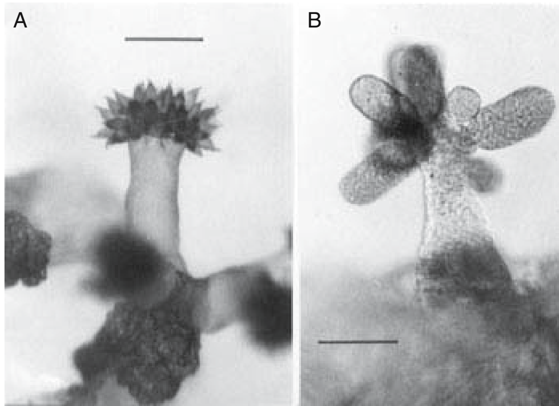


Fig. 30. (A) Fruiting body of *Chondromyces apiculatus*. Bar = 150 µm. (B) Fruiting body of *Stigmatella aurantiaca*. Bar = 40 µm. (Courtesy of Dr. H. Reichenbach.)

processes of diffusion—of the enzymes away from the cell and of the products of hydrolysis toward the cell. It has been shown that their feeding on macromolecules is a density-dependent process (Rosenberg et al., 1977), and it has been suggested that the function of the myxobacterial life cycle is to maximize feeding efficiency by maintaining a high cell density at all times; this has been referred to as a "microbial wolf-pack effect" (Dworkin, 1973).

A feature of the myxobacteria that is extremely unusual for prokaryotes, and that has been the central subject of many of the studies of their development, is the role of cell-cell interactions during growth, motility and development. These interactions involve both diffusible chemical signals exchanged between the cells (Kaiser, 2000) as well as contact-mediated signals (Dworkin, 1999).

The myxobacteria represent an unusual example of multicellularity among the prokaryotes, and as such, offer the opportunity to pursue questions about cell interactions and multicellular morphogenesis in the context of an experimentally tractable system.

The Life Cycle of *Streptomyces*

Streptomyces is one of the genera comprising the actinomycetes (Ensign, 1992), a group of Gram-positive soil bacteria, and is distinguished by a marked tendency toward mycelial growth. The life cycle of *Streptomyces* is characterized by the presence of two types of mycelium, aerial and substrate, by the alternation between vegetative cells and hyphal spores, and by the developmental autolysis of a substantial portion of the population (Champness, 2000). These stages are diagrammatically illustrated in Fig. 31.

The life cycle may be considered to begin with the germination of the hyphal spore, which

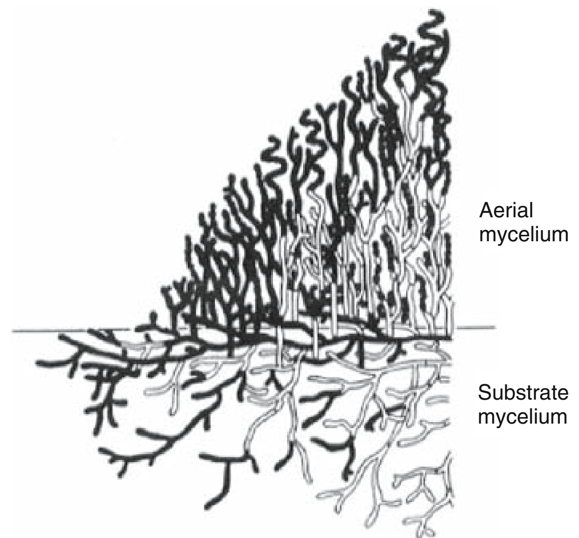


Fig. 31. Idealized diagram of a vertical section through the center of a sporulating colony of *Streptomyces coelicolor*. The black color indicates intact cells and the white color disintegrating or lysing cells. (From Dworkin, 1985a.)

occurs when the spore is placed in appropriate physical and nutritional conditions (Hirsch and Ensign, 1976). The germinated spore produces a structure morphologically analogous to a fungal germ tube; this gives rise to a radially spreading, branched mycelium that either penetrates into the substrate or travels along its surface. After a number of hours of substrate growth, the mycelia begin to develop vertically. These aerial mycelia are the forerunners of the hyphal spores and differ from the substrate mycelia physiologically as well as by being considerably more hydrophobic. (Their hydrophobic nature is possibly of value to a cell that must exist in the absence of water.) Presumably as a consequence of nutrient depletion, these aerial mycelia then begin to form spores. The formation of the spores and aerial mycelia give a colony of Streptomyces its characteristic fuzzy, powdery appearance. The process of sporulation is illustrated in Fig. 32. During this process of aerial mycelium and spore formation, the population undergoes massive autolysis (Fig. 31), the function of which is unknown. The spores are metabolically quiescent resting cells and are resistant to desiccation and to slightly elevated temperatures (i.e., 55°C). Their function is presumably to aid in dispersal of the organism and to allow the organism to withstand periods of nutrient depletion, desiccation, and the temperature extremes that might be encountered in the soil.

One of the most striking features of the *Streptomyces* is the ability of various species of the genus to produce antibiotics; over 8,000 have been discovered (Champness, 2000) and almost

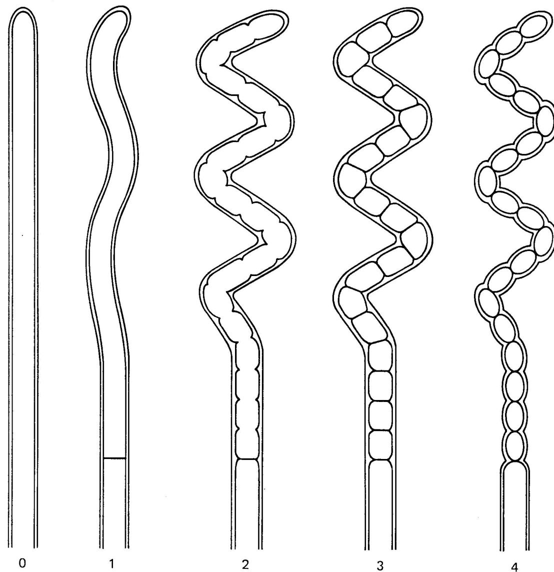


Fig. 32. Diagram of the four stages of sporulation in *Streptomyces coelicolor*. After a phase of vegetative growth, as shown in (0), the sporulating hyphae are divided into long cells by ordinary cross-walls, and the tips begin to coil (1). The apex is then partitioned into spore-sized compartments by sporulation septa (2). The cell walls thicken and constrictions appear between the young spores (3). As spores mature, they round off and separate (4). Some spores begin to germinate immediately after maturation. (From Dworkin, 1985a.)

2,000 have been characterized (Berdy, 1974). The function of an antibiotic for the producing organism is not clear. Historically, the two opposing arguments have been an ecological one and a developmental one. The ecological argument, originally suggested by Selman Waksman (Waksman, 1961), who discovered streptomycin, one of the first *Streptomyces* antibiotics, was that the ability of the organism to produce the antibiotic in the soil would reduce competition for nutrients and space. The developmental argument notes that the overwhelming majority of the organisms that produce antibiotics (e.g., fungi, actinomycetes, myxobacteria, and *Bacillus*) go through a life cycle during which spores are produced. It has thus been frequently suggested that antibiotics may be regulatory molecules that function to coordinate or regulate the developmental process or to maintain the dormancy of the spore. A combination of the ecological and developmental arguments has recently emerged; it suggests either that organisms with life cycles may be particularly vulnerable to predation at certain stages (e.g., during spore germination), or that massive lysis of portions of the population during development (e.g., aerial mycelial formation by *Streptomyces* or fruiting body formation by myxobacteria) may generate nutrient-rich areas, which other microbes could detect and enter chemotactically (Chater and Hopwood, 1989). The production of an antibiotic may thus enable the developing organism to ward off predation or reduce competition during a particularly vulnerable stage of its life cycle. For a recent review of the developmental genetics of *Streptomyces*, see Chater (2000).

The Life Cycle of *Rhodomicrobium vannielii*

Rhodomicrobium vannielii is a photoheterotrophic, budding bacterium. It combines in its life cycle the alternation of growing cells and resistant, resting cells characteristic of the sporeforming prokaryotes, and the alternation of sessile, reproductive cells and swarming, nongrowing cells characteristic of *Caulobacter*.

If one examines a batch culture of *R. vannielii*, one may find three types of cells simultaneously present. These are peritrichously flagellated rods, ovoid cells linked together by branched, mycelial connections, and heat-resistant, angular exospores. The understanding of the developmental relationship among these three types was facilitated by the finding that the swarmer cells and the budding, mycelial forms could be separated by passing the culture through a column containing glass wool. Thus, homogeneous populations of each cell type could be obtained and the sequence of developmental events connecting them determined (Whittenbury and Dow, 1977). Figures 33 and 34 illustrate the morphology of these three cell types. Whittenbury and Dow (1977) clarified the developmental relationship between these cell types, and this is illustrated in Fig. 35. The polarly flagellated swarmer

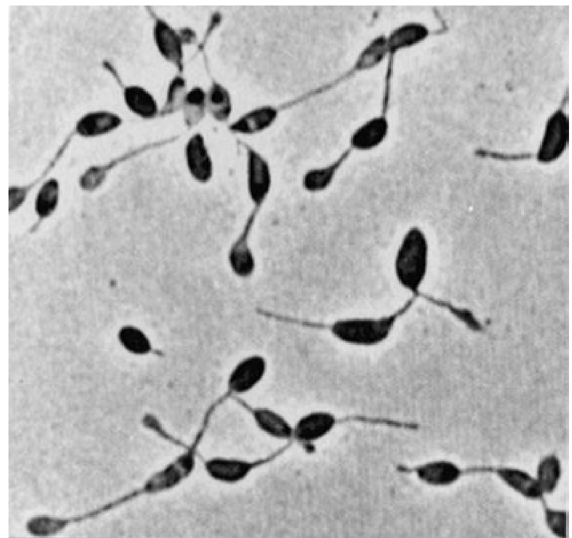


Fig. 33. Electron micrograph of cells of *Rhodomicrobium* in the growth phase. (Courtesy of Dr. P. Hirsch.)

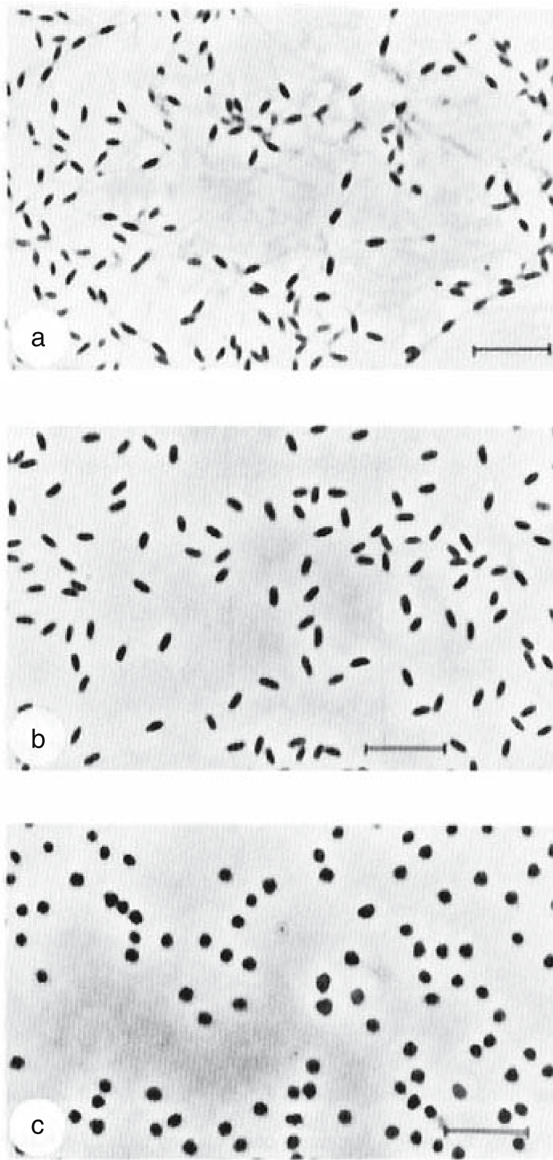


Fig. 34. Phase contrast micrographs of *Rhodomicrobium*. (a) Mycelial forms. (b) Homogeneous population of swarmer cells. (c) Exospores. Bar = 10 μm. (From Dow and Whittenbury, 1979.)

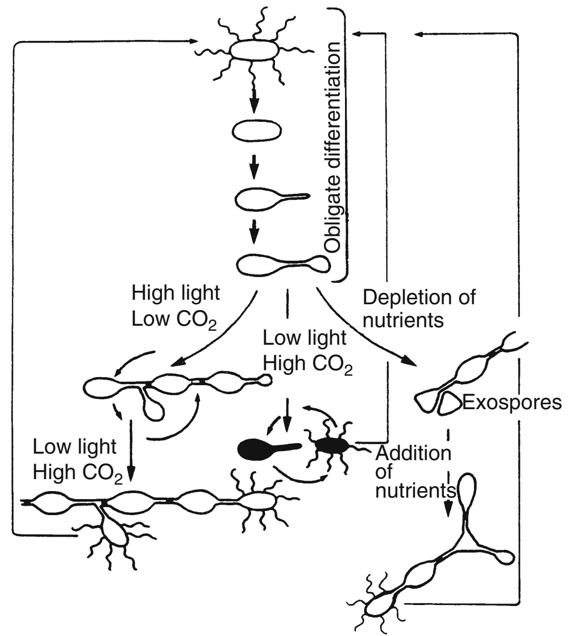


Fig. 35. Diagrammatic representation of the life cycle of *Rhodomicrobium* showing the developmental responses of the cells to environmental stimuli. (From Dow and Whittenbury, 1979.)

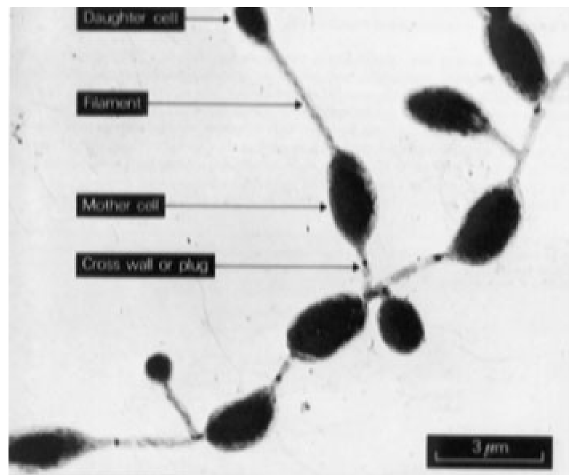


Fig. 36. Electron micrograph of shadowed cells of *Rhodomicrobium*. Bar = 2 μm. (From Dow and Whittenbury, 1979.)

cell is, like its swarmer cell counterpart in *Caulobacter*, a nongrowing cell, whose function seems to be to maximize dispersal. Its formation is induced by conditions of low light intensity and high levels of CO_2; both of these conditions would be generated by a high cell density—the low light intensity by shading of the cells and the high CO_2 by the respiratory activity of the cells. In the presence of optimal light intensity, the swarmer cells shed their flagella and begin to undergo the morphogenetic conversion to the budding, mycelial stage—the reproductive phase of the organism's life cycle. These budding cells then have three developmental options, depending on the circumstances of light intensity, nutrient concentration, and CO_2 partial pressure. Under conditions of high light intensity and low partial pressure of CO_2, the cells remain in the budding, mycelial mode (Fig. 36). As indicated earlier, when these conditions are reversed (i.e., low light intensity and high CO_2 partial pressure), the budding cells revert to the swarmer stage. Finally, when the nutrient level drops below a threshold, the budding cells begin to

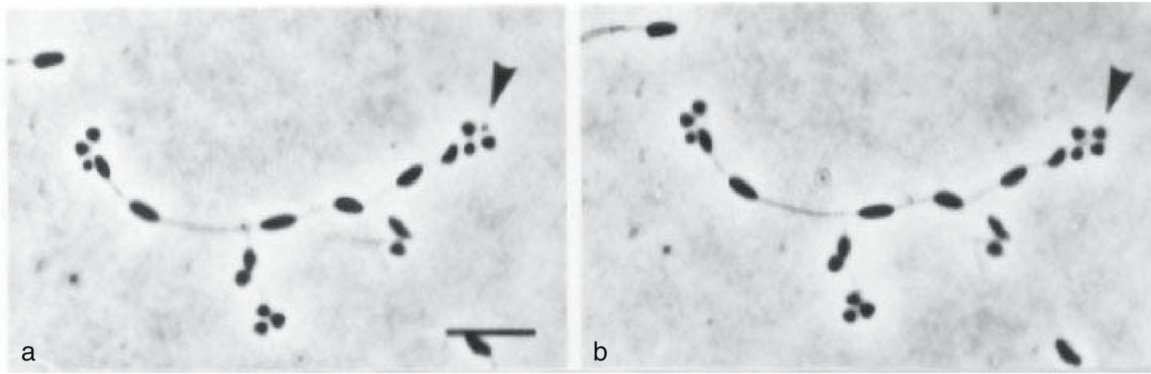


Fig. 37. Phase contrast micrographs of exospore formation in *Rhodomicrobium*. Figure 37b is at a later time than Fig. 37a. The exospores are formed terminally from the filament tip (arrowhead) and characteristically are arranged in clusters. Bar = 10 µm. (From Whittenbury and Dow, 1977.)

produce an unusual resting cell somewhat similar to the exospores formed by the methylotroph *Methylosinus trichosporium* (see "The Life Cycle of Methylotrophs" in this Chapter). The spores are budded off the mother cells and are formed at the tips of prosthecae. The process is illustrated in Figs. 37 and 38. Note that each prosthecal tip may give rise to multiple exospores. The exospores are bona fide resting cells and are resistant to desiccation and to elevated temperatures (e.g., 60°C). The exospores can germinate (presumably under conditions that are optimal for growth), and the sequence of events during their germination is depicted in Fig. 39.

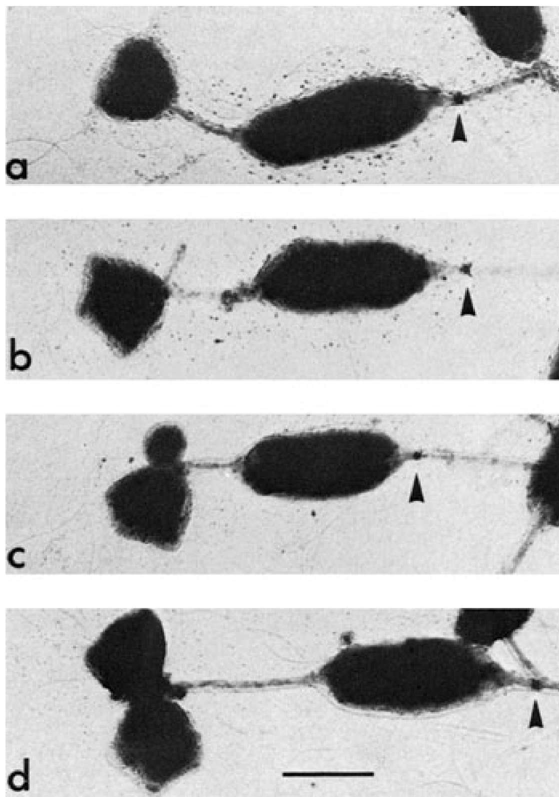


Fig. 38. Electron micrographs of shadowed, sporeforming cells of *Rhodomicrobium*. (a) First exospore formed. (b) Filament for second exospore is extended. (c) Beginning formation of second exospore. (d) Completion of second exospore. Arrowheads indicate filament plug separating the mother cell from the rest of the vegetative cells. Bar = 1 µm. (From Whittenbury and Dow, 1977.)

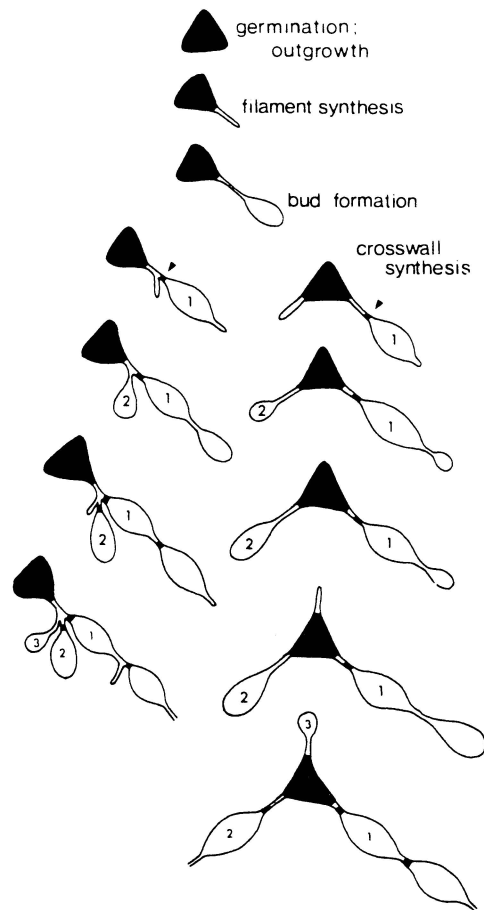


Fig. 39. Diagrammatic representation of exospore germination in *Rhodomicrobium*. The left and right sides show two different exospores. (From Whittenbury and Dow, 1977.)

As pointed out earlier, the strategy of alternating swarmer and stalked cells is strikingly similar to that manifested by *Caulobacter*. However, there is a considerable strategic difference. In *Caulobacter*, development is bifunctional whereas *Rhodomicrobium vannielii* is able to generate three developmental forms. Furthermore, in *Caulobacter*, swarmer cells and stalked cells must alternate with each other; a stalked cell cannot give rise to another stalked cell, but only to a swarmer cell. The sessile, reproducing cell of *R. vannielii*, on the other hand, has the option of either entering the dispersal mode, i.e., producing a swarmer cell, or it can continue to produce additional reproductive units. It can also generate a resistant, resting cell. In addition to this remarkable panoply of developmental options, it can metabolize either anaerobically as a phototroph or aerobically as a chemotroph, so it is clearly an extremely versatile organism.

Life Cycles Involving True Differentiation

The Cyanobacteria

In their classic review in 1971 on the Chroococcales, Stanier et al. (1971) pointed out that for the blue-green algae (as they were then called), the transition from natural history to biology required that the organisms be subjected to study in pure culture. That is what Stanier then proceeded to do for the Chroococcales (the unicellular cyanobacteria) (Stanier et al., 1971) and the Pleurocapsales (those cyanobacteria that reproduce by multiple fission; Waterbury and Stanier, 1978). Stanier's work also emphasized that the blue-green algae were indeed bacteria, and thus hastened the terminological conversion from "blue-green algae" to the present, generally accepted term, "cyanobacteria" (Stanier, 1982). The use of pure cultures has now led to an accurate portrayal of the life cycles of many of the cyanobacteria (Waterbury, 1992).

THE FILAMENTOUS CYANOBACTERIA *The Heterocyst* Among the filamentous cyanobacteria, Anabaena is the genus that has been subjected to the most scrutiny (Wolk, 2000). In addition to its photosynthetic properties, it is capable of fixing dinitrogen (as can many of the cyanobacteria), and it forms heterocysts, akinetes, and hormogonia. The heterocyst (Fig. 40) is Anabaena's solution to the problem of carrying out oxygen-sensitive dinitrogen fixation in an oxygenic phototroph. The heterocyst is the site of dinitrogen fixation; however, the absence of oxygen-generating photosystem II, and the presence of an outer coat that effectively insulates the cell from external oxygen, ensure that the oxygen-sensitive nitrogenase in the heterocyst will not be inactivated. The heterocyst exists in a syntrophic association with neighboring vegetative cells (Fig. 41), exchanging fixed nitrogen and reducing power (Fig. 42). This remarkable division of labor, resulting from the differentiation of the population into heterocysts and vegetative cells, was first suggested by Fay et al. (1968) and conclusively demonstrated by Wolk (1979).

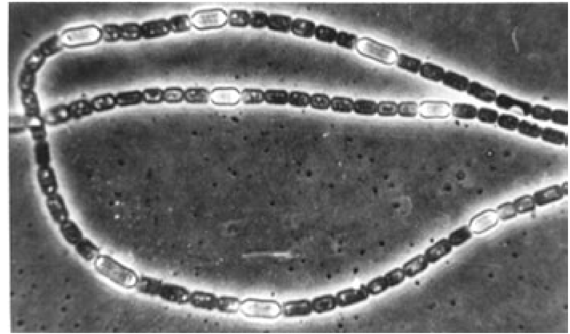


Fig. 40. Phase contrast photomicrograph of *Anabaena* filaments, showing the vegetative cells and the interspersed heterocysts. (From Wilcox et al., 1975.)

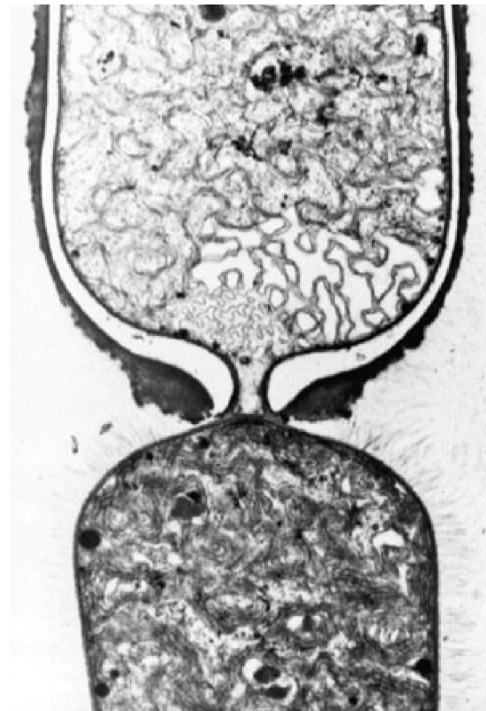


Fig. 41. Electron micrograph of a thin section of a heterocyst (top) and a vegetative cell (bottom) of *Anabaena*. (From Lang, 1968.)

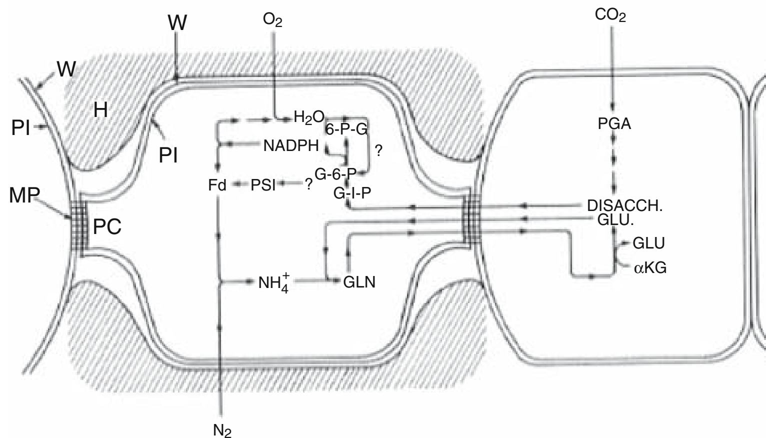


Fig. 42. Diagrammatic representation of the principal structural differences and metabolic interactions between a heterocyst (left) and a vegetative cell (right) of Anabaena. The wall (W) of the heterocyst is surrounded by a three-layered envelope consisting principally of a laminated glycolipid layer, a homogeneous polysaccharide layer (H) and an outermost fibrous layer. The plasma membranes (Pl) of the two cell types are joined by microplasmodesmata (MP) at the end of the pore channel (PC) of the heterocyst. A disaccharide formed by photosynthesis in the vegetative cells moves into the heterocysts and is then metabolized to glucose-6-phosphate and oxidized by the oxidative pentose phosphate pathway. Pyridine nucleotide reduced by this pathway (NADPH) can donate electrons to O_2 to maintain reducing conditions within the heterocysts, and can reduce ferredoxin (Fd). Ferredoxin can also be reduced by photosystem I (PSI). Reduced ferredoxin can donate electrons to nitrogenase, which reduces N_2 to NH_4^+. Glutamate (GLU) produced principally by vegetative cells reacts with the NH_4^+ to form glutamine. The glutamine moves into the vegetative cells, where it reacts with α-ketoglutarate and forms two molecules of glutamate. (Adapted from Wolk, 1979.)

Strictly speaking, the heterocyst is not part of a cycle; it is a dead-end cell (and as such, unusual in prokaryotes) that is not able to divide or germinate. It is formed in the absence of fixed nitrogen, i.e., fixed nitrogen in the medium will prevent heterocyst formation. The approximate timing of events is as follows: Four to five hours after a culture of *Anabaena* has depleted its supply of fixed nitrogen (NH_4^+) or has been transferred to a nitrogen-free medium, proheterocysts begin to appear along the filaments at regularly spaced intervals. After about 16 h, a maximum of 10% of the vegetative cells have been converted to proheterocysts, and after about 32 h, they have become mature heterocysts (Bradley and Carr, 1976).

The Akinete A number of cyanobacteria form cells that have some of the properties of resistance and metabolic quiescence characteristic of bacterial spores. These cells, called "akinetes," are usually larger and thicker-walled than the parent vegetative cells. In *Anabaena*, akinetes usually form immediately adjacent to a heterocyst (Fig. 43) and may form strings of such cells extending along the filament.

They are somewhat more resistant to desiccation and physical disruption than the corresponding vegetative cells, but the akinete's metabolic or resistance properties have not been the subject of a great deal of work. It is likewise not possible to make any easy generalizations as to the physical or nutritional conditions that induce their development. However, it has been shown that a culture of *Anabaena* strain CA, grown on a medium containing nitrate, will form filaments consisting entirely of vegetative cells; at the end of exponential growth, these become almost completely converted to akinetes (Fig. 44). Under optimal environmental conditions, the akinetes will germinate; the spore coat ruptures and the germling emerges and begins to grow and divide. When this happens to a chain of akinetes, it results in the pattern illustrated in Fig. 45. If germination takes place in the absence of fixed nitrogen, heterocysts appear at approximately every seventh cell (Fig. 45). Occasionally, germination will take place within the spore, without breaking the spore coat (Fig. 46). These

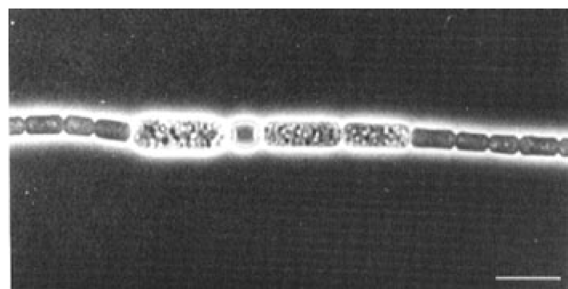


Fig. 43. Phase contrast photomicrograph of part of a filament of *Anabaena cylindrica*, showing a heterocyst with akinetes on either side. (From Nichols and Adams, 1982.)

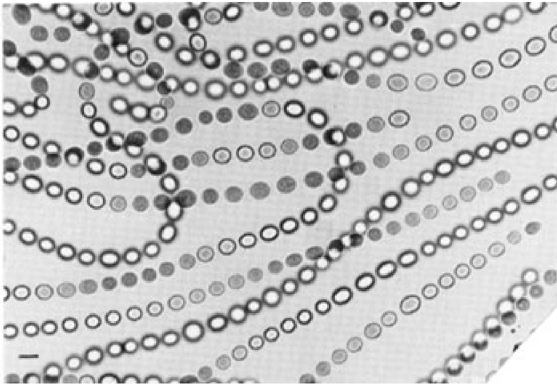


Fig. 44. Phase contrast photomicrograph of *Anabaena* grown in the presence of nitrate, showing filaments completely transformed into akinetes. (From Nichols and Adams, 1982.)

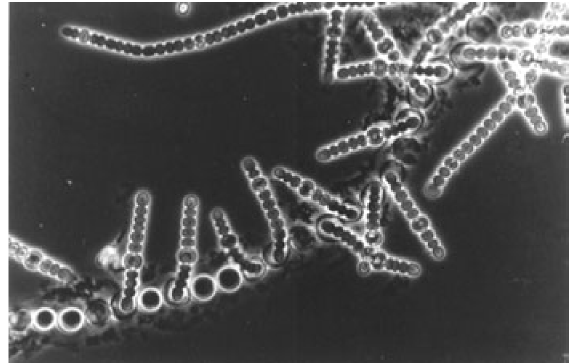
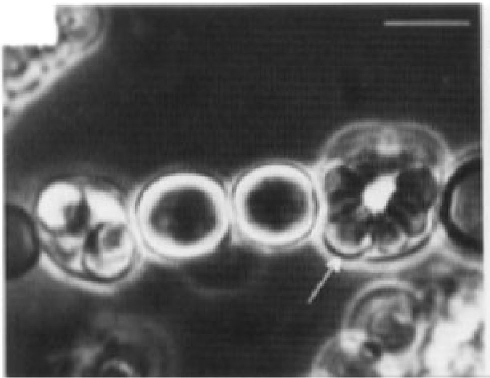


Fig. 45. Phase contrast photomicrograph of a filament of germinated akinetes of *Anabaena*. Short germlings, each containing a heterocyst, are emerging from many of these akinetes. Bar = 10 μm. (From Nichols and Adams, 1982.)



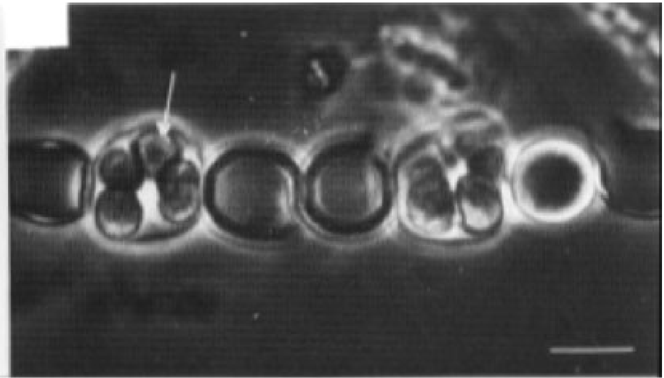


Fig. 46. Phase contrast photomicrographs of germinated akinetes of *Anabaena*. Here the strings of germlings, often containing heterocysts (arrows), have remained within the akinete coat. Bars = 10 μm. (From Nichols and Adams, 1982.)

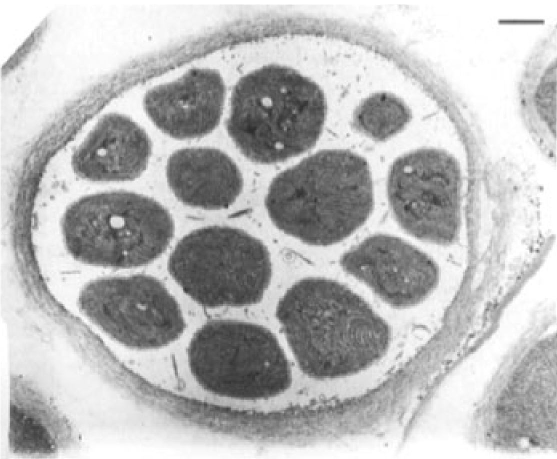


Fig. 47. Electron micrograph of a thin section of a *Dermocarpa* cell. The cell has undergone multiple fission and is filled with baeocytes, each of which is surrounded by layers of peptidoglycan and outer membrane. Bar = 1 μm. (From Waterbury and Stanier, 1978.)

too may contain periodic heterocysts (Fig. 46). Herdman (1987) has reviewed the properties of akinetes.

THE PLEUROCAPSALEAN *CYANOBACTERIA* The pleurocapsalean cyanobacteria characteristically reproduce by multiple fission. The initial cell, termed a "baeocyte," becomes covered by a thick, fibrous sheath and increases in size—in some species of *Dermocarpa* by as much as 1,000-fold. When the maximum size has been reached, the cell undergoes multiple fissions within the fibrous sheath (Fig. 47). The parental cell then ruptures, releasing the numerous small baeocytes. This is illustrated in Fig. 48, at 288 h. The baeocytes are phototactic and motile by gliding until the cells become covered by the fibrous sheath. At this point, they tend to become attached to a solid surface. Figure 49 is of a mass culture of *Dermocarpa*, showing the baeocytes and the parental cells either undivided or filled with baeocytes.

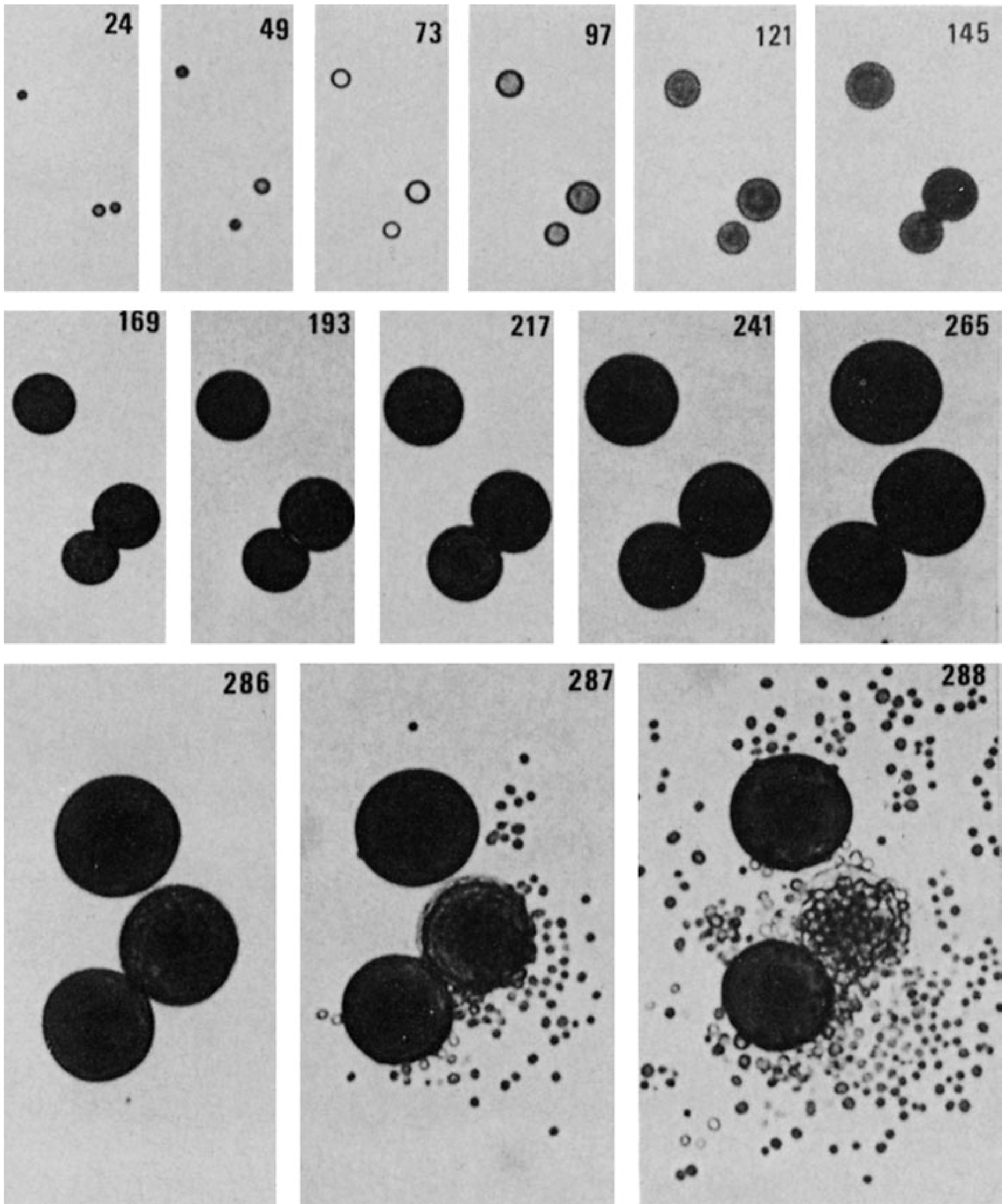


Fig. 48. Phase contrast photomicrographs illustrating the development of *Dermocarpa*. The number on each photo indicates the elapsed time in hours since the initial observation. (From Waterbury and Stanier, 1978.)

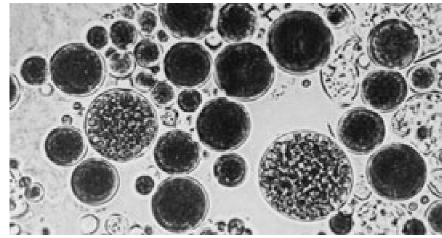


Fig. 49. Phase contrast photomicrograph of a mass culture of *Dermocarpa* illustrating the range of sizes of the spherical cells that either have not yet undergone fission or are filled with baeocytes. (From Waterbury and Stanier, 1978.)

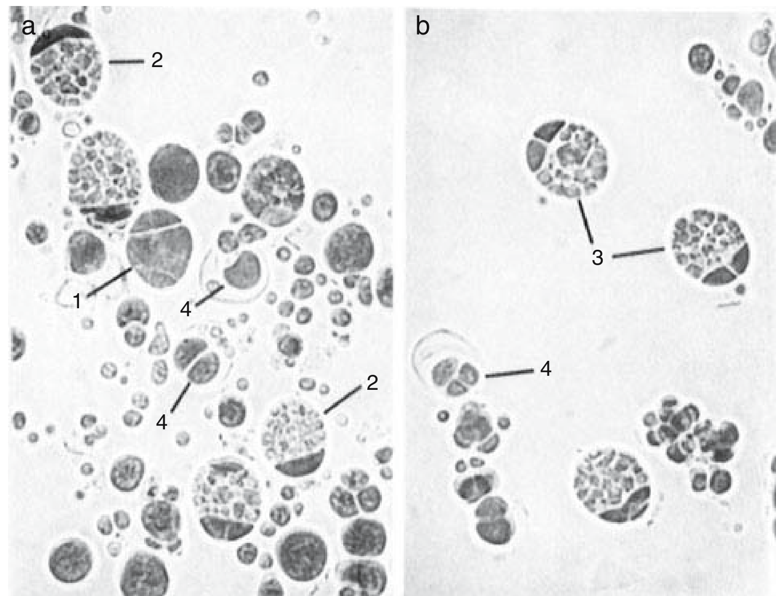


Fig. 50. Phase contrast photomicrographs a) and b) of *Dermocarpella* illustrating the various stages of division: 1) a cell that has just undergone transverse fission to form a small basal and a large apical cell; 2) individuals containing a single basal cell and an apical cell that has completed multiple fission; and 4) individuals in which baeocytes have been released from the apical cell, revealing the parental wall layer. (From Waterbury and Stanier, 1978.)

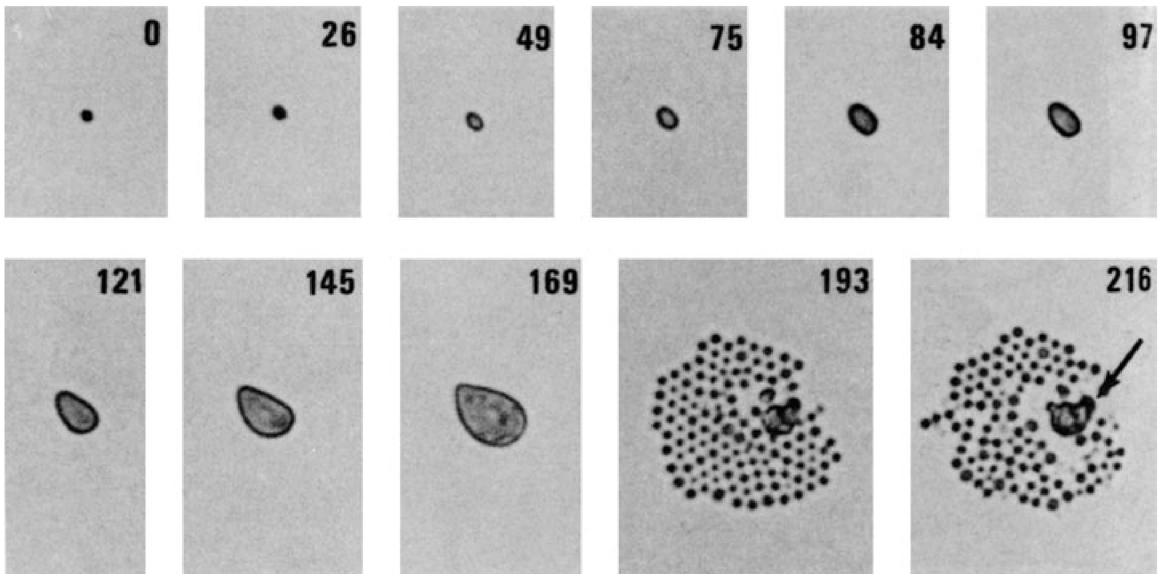


Fig. 51. Phase contrast photomicrograph of the growth pattern of *Dermocarpella*, beginning with a single baeocyte. The cell undergoes asymmetric enlargement, leading to a large ovoid cell, which goes through a binary fission, followed by multiple fissions. The arrow points to the basal cell remaining after baeocyte release. The numbers refer to the hours elapsed after the initial observation. (From Waterbury and Stanier, 1978.)

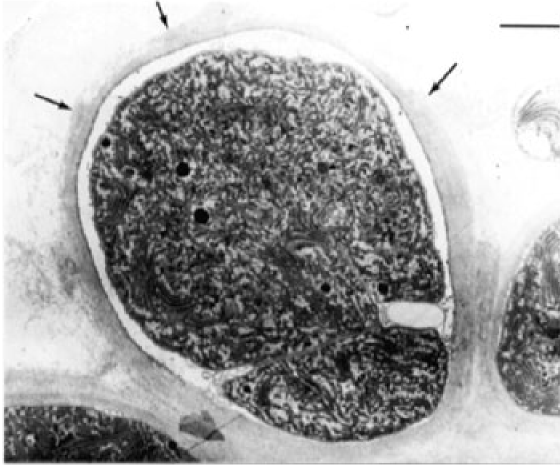


Fig. 52. Electron photomicrograph of a thin section of *Dermocarpella* at an early stage of fission. The cell has just completed binary fission, separating the apical cell (arrows) from the basal cell. Bar = 1 µm. (From Waterbury and Stanier, 1978.)

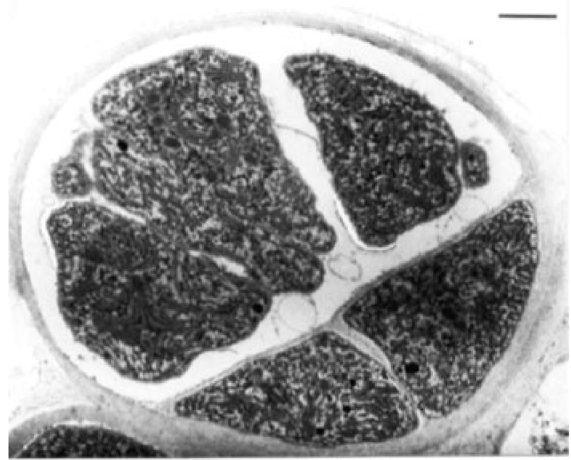


Fig. 53. Electron photomicrograph of a thin section of *Dermocarpella* at a later stage of fission. The basal cell has undergone a second binary fission, while the apical cell has begun its process of multiple fission. Note that the basal cells are beginning to be surrounded by the newly synthesized outer layer. Bar = 1 µm. (From Waterbury and Stanier, 1978.)

An interesting variation of this theme is manifested by the genus *Dermocarpella*. A mass culture of *Dermocarpella* is shown in Fig. 50. Figure 51 illustrates the sequence of events starting from a single baeocyte. The baeocyte enlarges, becomes ovoid and pyriform, and eventually releases its baeocytes; the structure that remains retains some of its cellular material. The series of electron photomicrographs in Figs. 52–54 illustrate the sequence of events as follows: The cell undergoes an asymmetric division, the larger daughter cell undergoing subsequent divisions to form the baeocytes and the smaller daughter becoming completely enclosed by the fibrous sheath. The larger portion of the cell ruptures, releasing the baeocytes, while the smaller portion presumably remains attached to its original site. This pattern is functionally similar to the life cycle of *Caulobacter*, with one of the asymmetric daughter cells remaining attached to its site and continuing to produce progeny, while the released, motile daughter cell(s) are free to seek a new site for attachment and growth. A schematic (Fig. 55) summarizes the life cycles of the pleurocapsalean cyanobacteria. This admirable work, begun in the laboratory of the late Roger Stanier, has been continued by John Waterbury at the Woods Hole Oceanographic Institute.

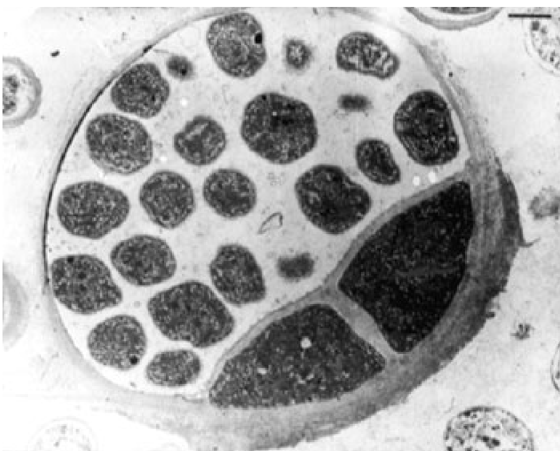


Fig. 54. Electron photomicrograph of a thin section of *Dermocarpella* at a terminal stage of fission. The cell is filled with baeocytes ready to be released. Bar = 1 µm. (From Waterbury and Stanier, 1978.)

Applications and Conclusion

The relationship between prokaryotic life cycles and the production of valuable secondary metabolites is well established, yet only a tiny fraction of these sorts of organisms have been explored for their ability to produce antibiotics. Furthermore, if we are truly serious in our attempts to determine the effects of our additions to or our manipulations of our environment, the effects on life cycles as well as on growth must be determined. Finally, and perhaps most importantly, one may justify an interest in prokaryotic life cycles solely on the ground that locked within them there is a vast array of undiscovered biological secrets. The developmental processes of differentiation and morphogenesis are far from being fully understood. And until we have a clearer picture of the nature and regulation of

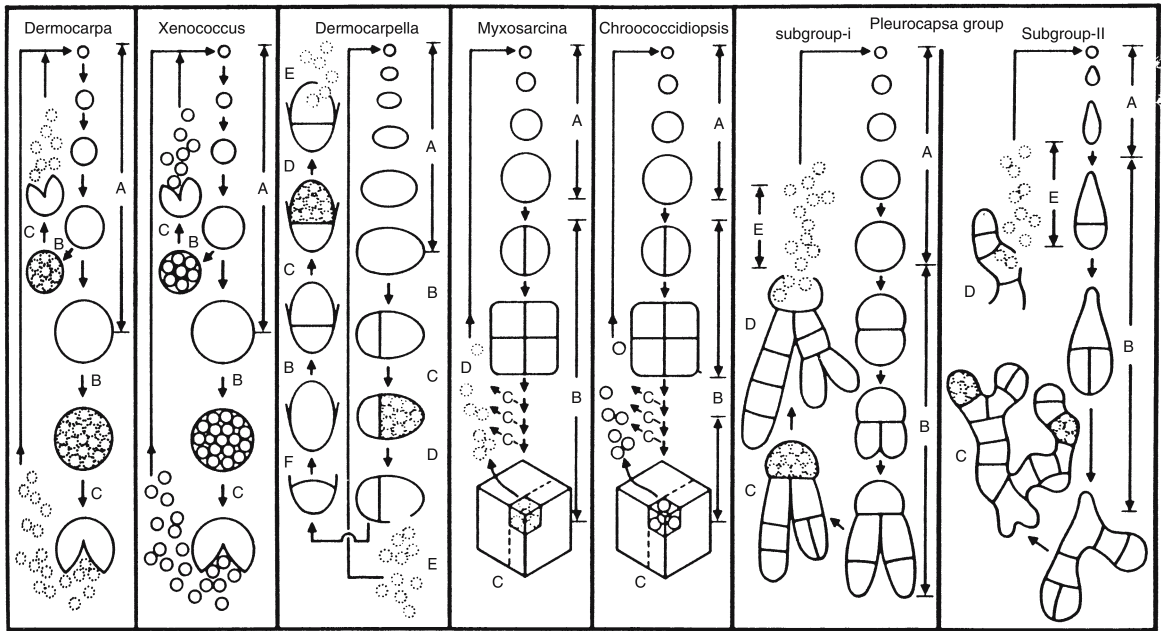


Fig. 55. Diagrammatic comparison of the pleurocapsalean life cycles. (From Waterbury and Stanier, 1978.)

life cycles, we will not fully understand the nature of the complex and delicate interactions between an organism and its environment.

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Prokaryotes (2006) 2:167–209
DOI: 10.1007/0-387-30742-7_7

CHAPTER 1.7

Life at High Temperatures

RAINER JAENICKE AND REINHARD STERNER

Introduction

In contrast to the simplistic definition of life as the quality that distinguishes a vital and functional being from a dead body, present-day biological sciences are mechanistically oriented, i.e., cells and their inventory are functionally determined by the nonvitalist principle that living matter is composed of chemical substances obeying the fundamental laws of physics. Any biological function, including ecological adaptation, differentiation and behavior, can be described in terms of the structures of those substances and the reactions that they undergo. However, one apparent difference between the life sciences on the one hand, and physics or chemistry on the other, deserves mentioning: Physics and chemistry study the unchanging properties of matter and energy, while the subject matter of biology (presently known organisms) is evolving, i.e., includes only a subset that has managed to produce descendents under the changing physical conditions of the biosphere.

Within the framework of biology and physical biochemistry, life refers to cellular organisms whose characteristics are 1) the capacity for metabolism (energy transformation), 2) growth, 3) response to stimuli, and 4) reproduction. Their constituent building blocks comprise a relatively small number of complex biomolecules (proteins, nucleic acids, carbohydrates and lipids), with the first two serving as substrates for the process of evolution. Evolution occurs because natural selection favors, among all the combinations available, those individuals whose characteristics increase their reproduction in a particular environment. At this point the physical or chemical conditions of the environment come into the play. As a consequence of the stochastic mechanism of "successful adaptation" to changing environmental conditions, all organisms are phylogenetically related to one another; in addition, they share most of the basic biochemical processes involved in replication, transcription, and translation, and in the basic reactions governing metabolic and energy-transfer pathways. Thus, fundamental biochemical and biophysical problems may be studied in whatever organism is practical or convenient. In the context of the present chapter, the specific properties of biomolecules from thermophilic microorganisms may provide us with a deeper understanding of general mechanisms underlying differences in the stability of proteins, nucleic acids and lipids, as well as in their metabolic turnover.

Dedicated to the memory of Professor John T. Edsall (3 November 1902–12 June 2002), one of the founders of physical biochemistry: scientist, teacher and mentor.

Adaptation to Extremes of Physical Conditions

Among the three alternative responses (avoidance, compensation or detoxification, and mutative adaptation) of microorganisms to extreme physical conditions, only mutative adaptation can cope with high temperature and high hydrostatic pressure, simply because cells in their natural aqueous environment are isothermic and isobaric (Jaenicke, 1981; Jaenicke, 1990). At this point, we include high pressure as a second variable because in many cases the term "environmental extremes" refers to a whole set of factors, e.g., high pressure and low or high temperature in deep sea hydrothermal vents (Somero, 1992; Kelley et al., 2001), or low pH and high temperature in acid solfataras (Brock, 1986; Stetter, 1996; Stetter, 1999). In addition, physicochemical parameters may have an indirect effect on an organism, e.g., temperature effects on the solubility of gases or on the viscosity and ionization of the aqueous medium. In such cases, in vitro experiments can easily compensate for such perturbations, this way eliminating indirect effects.

Considering mutative adaptation to extreme conditions, it is obvious that to grow and reproduce, the whole inventory of an extremophile needs to be adapted; fitness to survive in the

competitive situation of a given environment is defined by the least stable constituent of a species. In the context of thermophilic adaptation, the example of temperature-sensitive point mutants stresses this argument.

High temperature can be defined as the upper temperature range in which mesophilic organisms do not survive while specifically adapted hyperthermophilic organisms grow and multiply, not simply tolerating the high temperature but requiring it as their standard physiological condition. Commonly, species diversity in extreme environments is distinct from that in mesophilic environments, and therefore may be used as a criterion for extremophilism. In extreme environments (with low species diversity) often whole taxonomic groups are missing. For example, in saline and thermal lakes, as well as in hydrothermal vents, there are no vascular plants or vertebrates; in the most extreme high-temperature environments, only prokaryotic microorganisms have been discovered so far. Here, the low species diversity may sometimes be limited by extreme conditions to a few or even one species (Brock, 1978). In analyzing this phenomenon, we may ask how and at which level the extreme environment interferes with the normal growth of mesophilic organisms; in turn, to discover the essential characteristics of thermophiles, studying the adaptive mechanisms decelerating, or even inhibiting the growth of thermophiles at suboptimal temperature seems most promising. In the following, no strict distinction between thermophiles and hyperthermophiles will be made, because the limits are not well defined. Commonly, the temperature boundary of thermophiles is ca. 60°C, while hyperthermophiles show optimal growth temperatures of 80°C or above. Both estimates refer to growth, not survival. Needless to say, many bacteria, especially those capable of forming endospores, can tolerate temperatures much higher than those needed for optimal growth. However, it is the temperature range over which a microorganism is able to maintain growth and proliferation that is essential for evolution. The temperature range in the biosphere reaches from –80°C (in the Arctic [to approximately 65°N latitude] and in the Antarctic) to +350°C near white or black smokers in deep-sea vents. The upper temperature limit that still allows growth and proliferation of microorganisms is difficult to determine because of the extreme in situ turbulence in volcanic areas of the ocean. Evidently, "black smoker" bacteria alleged to grow at 250°C and 265 bar are in fact merely the subject of Jules Verne phantasy (Bernhardt et al., 1984).

Pyrolobus fumarii may serve as a well-established example for bacterial life at the upper temperature limit of viability. Its physiological characteristics are: growth between 90 and 113°C, with an optimum at 106°C (1 hr doubling time at $pH_{opt} = 5.5$ and $[NaCl]_{opt} = 1.7\%$), no growth at 85 and 115°C (Blöchl et al., 1997). The recent new world record, a Pyrodictium- and Pyrobaculum-related "Strain 121" with a temperature of maximal growth at 121°C and a survival half-life of ~6 min at 130°C draws a new line (Kashefi and Lovley, 2003). However, considering the rise of T_{max} during the last 40 years (Cowan, 2004), it is obvious that there is a sigmoidal time course that extrapolates to the expected upper temperature limit of viability around 140°C. The physical reasons are twofold: (i) crucial biomolecules undergo hydrothermal decomposition and (ii) the energetic costs of repair and resynthesis become unsustainable (Bernhardt et al., 1984; White, 1984; Stetter, 1998; Jaenicke and Böhm, 1998; Jaenicke, 2000a).

Since water in its liquid state is a necessary requirement for biological function in terms of the above criteria, the freezing temperature of homogeneous nucleation (–40°C) defines the lower limit at which life can exist. Chill conditions in the aqueous cytosol can persist down to this temperature and even below (Franks et al., 1990). Commonly, freezing damages cells irreversibly. In contrast, dehydration, e.g., in seeds and other dormant states of cells and tissues, allows various forms of cryptobiosis. Generally speaking, these limits are based on 1) the effect of low temperatures on the weak interactions between biomolecules (especially on the self-organization of proteins and lipids) and 2) metabolic and/or protective regulation mechanisms involving compatible solutes and other forms of stress response. For details regarding cold tolerance, resistance, acclimation, adaptation and cryptobiosis, see Crow and Clegg (1978); Finegold (1986); Laws and Franks (1990); Carpenter et al. (1993); Graumann and Marahiel (1996); Marshall (1997); Thieringer et al. (1998); Phadtare et al. (1999); Cavicchioli et al. (2000); Zachariassen and Kristiansen (2000), and Clegg (2001).

Over the whole biologically relevant temperature range, from psychrophiles up to hyperthermophiles, it is essentially impossible to predict how temperature changes may affect viability. Considering the complexity of metabolic pathways, the kinetics of each single step in any linear or cyclic reaction sequence may become rate limiting, either by its own high activation energy or by product inhibition. As evolution has produced efficient coupling mechanisms whereby products of reactions become the substrates for subsequent reactions, temperature perturbation will necessarily lead to a decrease in coupling efficiency. This holds because the relative reaction rates are determined not only by the specific dif-

ferences in the temperature coefficients of ligand binding and enzyme turnover, but also by the stability of the cellular microcompartmentation, e.g., in multienzyme complexes and by a wide variety of transport processes (Franks, 1985–1990; Jaenicke, 1990).

Water

General Properties

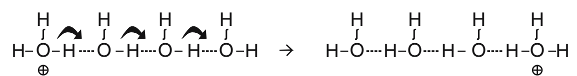
Because it is ubiquitous and the main component in the cell, water might be regarded as a mere space filler in living organisms. Actually, Thales of Miletus with more insight praised water as the basic element. As the various aspects of the physics, chemistry and biology of water have reached encyclopedic dimensions (Franks, 1975–1982; Franks, 1985–1990), this subject cannot be detailed to any great extent in this chapter. Instead, we will focus on liquid water and its significance in the context of the structure-function relation of biomolecules and their intrinsic and extrinsic stability.

Evidently, from a physico-chemical point of view, water is involved in biological processes as 1) medium in natural biotopes, 2) solvent within the cell, and 3) reactant or product in all biochemical and biophysical reactions. In the life cycle of the cell, this holds from the biosynthesis of proteins, nucleic acids, lipids, and carbohydrates to their degradation. Beyond this "housekeeping" part of life, water is of critical importance in the formation and maintenance of macromolecular and supermolecular structures; it determines not only the structure of the aforementioned biomolecules and their cellular compartmentation (e.g., in membranes), but also their function. For this reason, most cells have evolved mechanisms to control their water balance to avoid osmotic stress under extreme physical conditions (salt stress, desiccation, freezing, etc.; Hochachka and Somero, 1973).

When compared with other common solvents, liquid water exhibits unique properties (Table 1). Its anomalously high melting and boiling points, heat capacity, enthalpy of melting and vaporization, and its high surface tension, all indicate that the forces of attraction between molecules in the liquid state must be significant. As a consequence of this internal cohesion, the molecular mobility and fluidity of water remain unchanged, even if aqueous solutions are confined to subnanometer films or pores (Raviv et al., 2001). This property is attributable to the structure of the H_2O molecule itself (Fig. 1A): The O atom shares an electron pair with each of the two H atoms. Owing to the repulsion of the paired electrons by the unpaired ones, the H-O-H bond angle of 104.5° deviates from the tetrahedral arrangement (109.5°), which is a characteristic of ordinary hexagonal ice (Pauling and Hayward, 1964). As a consequence, the H_2O molecule possesses a high dipole moment (1.84 Debye) and a high dielectric constant, thus favoring dipole-dipole interactions involving hydrogen bonds (see below).

As shown in Fig. 1B and C, the water molecule has the ability to form four H-bonds, with two proton donor and two proton acceptor sites. The spatial disposition of these sites gives a clear visual conception of the geometry within each cluster.

To what extent does such ordered molecular arrangement persist in the liquid, bearing in mind that (compared to the covalent H-O-H bonds) the hydrogen bond is only a weak interaction? In this context, two observations are important: 1) As shown by the anomalously high conductivity of protons (= hydronium ions H_3O^+) in aqueous solution, the distinction between covalent and noncovalent bonds in a water cluster is blurred, since proton conductivity is "charge transfer without mass transport" according to



The reason is that in bulk water the transition of a bound proton to a neighboring free electron

Table 1. Anomalies of water in comparison with other solvents.

Substance	F_p (°C)	K_p (°C)	ΔH_{vap} (cal/g)	c_p (cal/g·degrees)	σ (erg·cm²)	ε	η (Poise)
H_2O	0	100	585	1.00	78	80	1.00
NH_3	–78	–34	296	1.125	18	15	0.27
CH_3CH_2OH	–114	78	204	0.58	22	24	1.20
CH_3COOH	17	118	96	0.47	28	7	1.22
CH_3COCH_3	–95	56	112	0.53	14	21	0.32
C_6H_6	5	80	104	0.41	29	2.3	0.65

Abbreviations: F_p, melting point; K_p, boiling point; ΔH_{vap}, specific enthalpy of vaporization; c_p, specific heat capacity; σ, surface tension at 25°C; ε, dielectric constant at 25°C; and η, viscosity. Data for σ, ε and η refer to 25°C, except for liquid NH_3, which was investigated at –34°C.

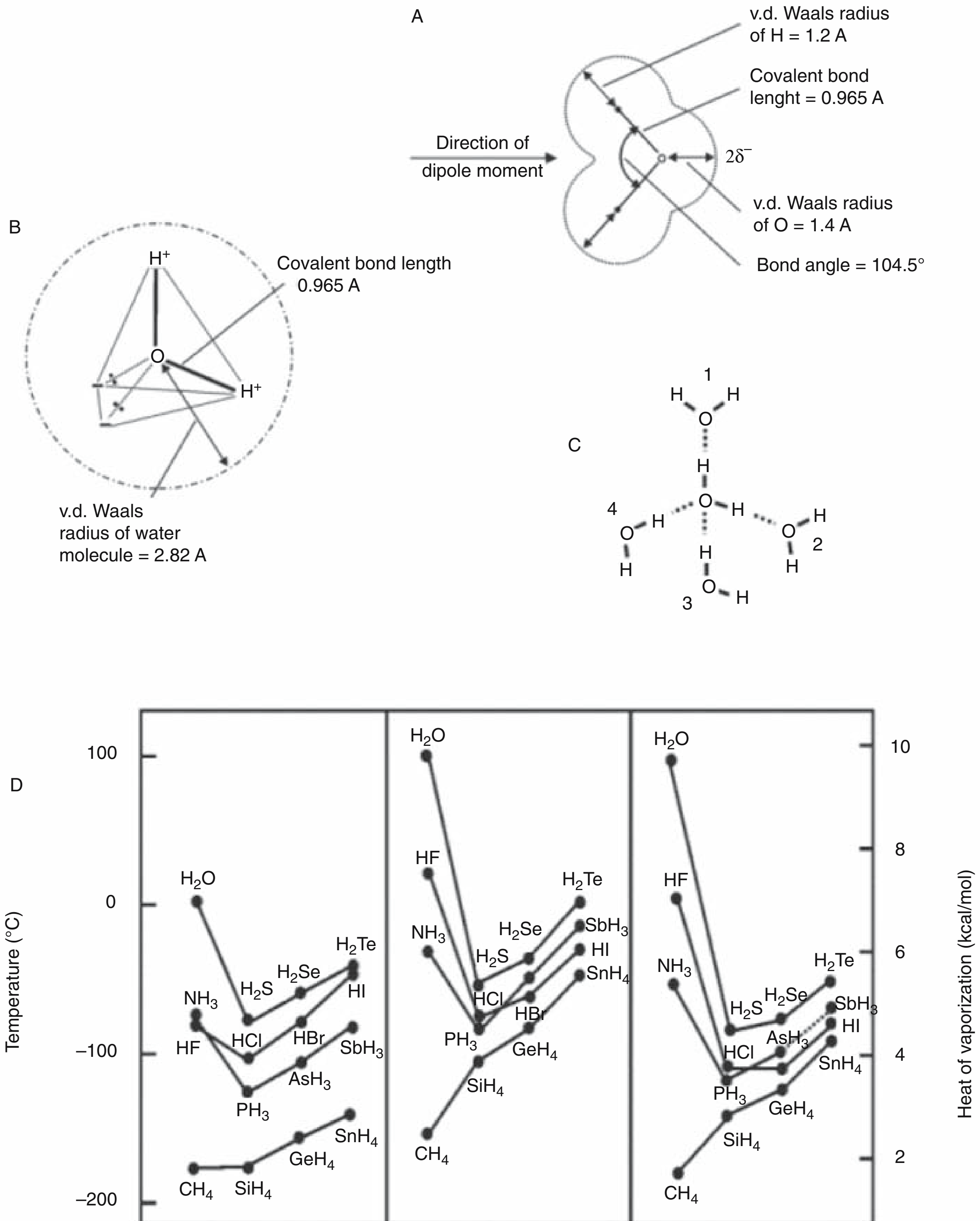


Fig. 1. The water molecule and its anomalies. A. Ball-and-stick crystallographic model giving the bond angles and lengths. B. Four-point-charge model with the oxygen atom placed in the center of a regular tetrahedron with vertices occupied by two positively charged hydrogen atoms and two negatively charged electron pairs. The distance of closest approach of two molecules (van der Waals radius) is 0.282 nm. C. Schematic view of the tetrahedral hydrogen bonding around a water molecule in ice; molecule (1) and (2) and the central H_2O molecule are in the plane of the paper, while (3) and (4) are above and behind it. D. Melting points (left), boiling points (middle) and heats of vaporization (right) of the isoelectronic sequences of hydrides in various rows of the periodic table. All three plots illustrate the effect of the anomalous interatomic forces between water molecules. Data from Pauling (1940), cf. Edsall and Wyman (1958).

pair in a hydrogen bond takes less than 10^{-12} second (<1 picosecond [ps]); thus, the lifetime of a given $(H_2O)_n$ arrangement is adequately described as a "flickering cluster." 2) The enthalpies of melting and vaporization of ice and water allow an estimate of H-bonding in liquid water to be given: close to the melting point, around 85% of the H-bonds in ice are still intact in the liquid state, whereas at the boiling point, single H_2O molecules prevail (Edsall and Wyman, 1958). Obviously, thermal energy opposes the structural forces so that physical parameters as well as solutes of various kinds are expected to easily perturb water structure. In turn, water is capable of modifying the intermolecular interactions between solute molecules as well as intramolecular interactions within each solute macromolecule. Thus, the biological significance of water stems from the intimate details of the compromise between water-water and water-solute interactions; the quantitative treatment of these interactions in terms of potential functions and activation profiles in the given multicomponent system is presently not feasible.

Temperature Dependence

As taken from its pressure/temperature (p/T) phase diagram over the whole biologically relevant range of hydrostatic pressure (<110 MPa, 1.1 kbar ~ 1100 atm), water either is in its liquid state or is hexagonal phase-I ice. Owing to its exceptionally low density, the latter shows a decrease of its freezing point with increasing pressure. Over the whole p/T range, there is no significant effect on the clustering of water molecules: evidently, pressure alone does not break H-bonds (Groß and Jaenicke, 1994). Focusing on isobaric conditions at atmospheric pressure, Table 2 summarizes the change of some important physical properties of water over the temperature range between –25 and 100°C, with +25°C representing the common seasonal temperature in the natural environment of mesophilic species. It is obvious that the temperature dependences are not linear but become more pronounced at low temperatures. In fact, many physical properties of water appear to diverge at –45°C (Franks, 1985; Franks et al., 1990).

Apart from the temperature effects on water structure in terms of cluster size and water-solute interactions, from the biochemical point of view, the most relevant change that takes place at varying temperature refers to the changed dissociation of water into H^+ and OH^-, described by the equilibrium constant K_w or its negative logarithm pK_w. In an aqueous environment, the solvent acts as conjugate acid or base, and any change in K_w or pK_w will produce changes in the respective dissociation constants K_d or pK-values of acids and bases that define ionization equilibria in solution. As both H^+ and OH^- are involved in most cellular processes (condensation, hydrolysis, reduction/oxidation, and membrane transport), it is likely that the large decrease in K_w with increasing temperature will affect equilibrium and kinetic processes. With increasing temperature, the dissociation of water increases, i.e., pK_w decreases. Most biopolymers are polyelectrolytes, but the pK values of their ionizable groups do not necessarily show the same temperature dependence observed for pK_w. Therefore, both their net charge and the state of ionization of crucial functional groups will change with temperature.

Table 2. Physical properties of liquid water at varying temperature.

	–25°C	+25°C	100°C
Density ($g{\cdot}cm^{-3}$)	0.987	0.996	0.958
Heat capacity C_p ($J{\cdot}[mol{\cdot}K]^{-1}$)	80	75	76
Isothermal compressibility ($10^6 MPa^{-1}$)	720	440	490
Hypersonic sound velocity ($m{\cdot}s^{-1}$)	1220	1480	1540
Dielectric constant	102	79	65
Self-diffusion coefficient ($10^5{\cdot}cm^2{\cdot}s^{-1}$)	0.32	2.2	8.4
Viscosity (mPa·s)	6.5	0.89	0.28
pKw[a]	17.3	14.0	12.3

[a]Refers to the changed dissociation of water into H^+ and OH^- at varying temperature.Data taken from the first volume of Franks (1975).

Thus, their conformational stabilities and biological activities may be affected in a complex way because any such influences would presumably be compounded by changes in the dielectric properties of the solvent, especially in structures with high charge density such as hyperthermophilic proteins, nucleotides and sulfated polysaccharides.

Hydration

The common knowledge that living cells and tissues contain around 70% water means that all cellular components interact with water; their native conformation results from the balance between intra- and intermolecular forces, on the one hand, and forces resulting from interactions with the aqueous solvent, on the other. Since all major biomolecules and water have the strongly polar hydroxyl group in common, it is obvious that what was called "clustering" is not restricted to H_2O, but also holds for proteins, nucleic acids, carbohydrates and fatty acids and their constituents. Here, from the energetic point of view, the stabilizing effect of H-bond formation within a biomolecule is expected to be marginal, because

most of the energy gained by forming the new "stabilizing bond" has to be paid by the breaking of a pre-existing solute-water bond. Evidently, multiple H-bonds in cooperative units such as α-helices or strands of nucleic acids, as well as additional contributions (e.g., from hydrophobic constituents) may accumulate to reach a high energy of stabilization, frequently referred to as "conformational energy" (Kauzmann, 1959; Franks, 1975–1982; Franks,1985–1990; Dill, 1990; Jaenicke, 1991b; Pace et al., 1996).

In the case of carbohydrates and fatty acids, the dominance of the polar hydroxyl and carboxyl groups is evident. They are responsible for the high solubility of sugars and other oligo- and polyhydroxy compounds as well as uronic acids, *N*-acetyl glucosamine, etc. (Suggett, 1975; Franks and Grigera, 1990). Conjugated with proteins, they allow the solubility and stability properties of their partner molecules to be modified (Kern et al., 1992; Kern et al., 1993). The net result of the aqueous environment for proteins is the protection of the nonpolar polypeptide core from the polar solvent. In nucleic acids, the situation is more complex: Here, secondary- and tertiary-structure formation is the result of an equilibrium between 1) electrostatic repulsion of the negatively charged phosphate groups along the linear polyelectrolyte, 2) stacking interactions and hydrogen bonding between the nucleotide bases, and 3) the conformational energy of the sugar-phosphate backbone. In its preferred conformation, the two polynucleotide strands in a duplex expose their deprotonated phosphates to the dielectric screening by the solvent, this way promoting the stacked arrangement of adjacent bases. As a result, a hydrophobic core is created in which H-bonds between the bases as well as additional sugar-base and sugar-sugar interactions are favored. The aqueous solvent contributes to the stability by 1) screening the charges of the phosphates, 2) hydrogen bonding to the polar exocyclic atoms of the bases, and 3) influencing the conformations of nucleotide constituents with methyl groups via nonpolar interactions (see below). Besides, because of the periodicity of the helical conformations, local binding sites of firmly bound structural water and linear arrangements of "bridges" of water molecules (involving nucleic acid polar atoms) can lead to favored structural arrangements with high conformational stability (Saenger, 1984; Westhof and Beveridge, 1990).

In spite of the well-established fact that the aqueous solvent is essential in accomplishing and maintaining the native state of biopolymers, so far theoretical treatments involving either distribution functions of the various intra- and intermolecular increments of stabilization, or energy functions aimed at structural parameters of hydration have been of limited success. On the other hand, a wealth of experimental data has been accumulated applying a wide variety of techniques: X-ray and neutron diffraction, hydrogen-deuterium exchange, Raman, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, sorption/desorption of water vapor, calorimetry (including heat capacity calorimetry), dilatometry, sedimentation analysis, viscometry and other hydrodynamic methods. The reason why all of them "gave a better view of the experimenters' interests than of water structure" (G.A. Jeffrey) is that there is no clear definition of "hydration" (or "solvation" in general); in addition, the problem of how to separate the contributions of the solute from the background of the solvent and from potential conformational effects in a highly dynamic system is still unresolved.

Considering the various experimental approaches, the most detailed information is expected from high-resolution X-ray data and solution NMR (Westhof and Beveridge, 1990; Otting et al., 1991; Frey, 1993; Goodfellow et al., 1994). Comparing the results, it is important to note that in the crystalline state, lattice forces may affect the number and position of spatially well-defined water molecules, so that in different crystal forms not all binding sites are conserved (Zhang and Matthews, 1994). NMR measurements allow two qualitatively different types of hydration sites to be distinguished: 1) a small number of interior water molecules (with residence times of 10^{-2}–10^{-8} s and X-ray coordinates in the crystal) and 2) surface hydration (with residence times $<10^{-9}$ s, not necessarily fixed in the crystal structure; Otting et al., 1991). How the latter type of hydration compares to the hydrodynamically relevant bound water is still unresolved. Regarding hydration/dehydration at elevated temperature, phase separation and neutron scattering measurements have been applied mainly to quantify macromolecular interactions, especially in protein mixtures. Available data are rudimentary and far from being understood in quantitative terms (Benedek, 1997; Tardieu et al., 1999; Jaenicke and Slingsby, 2001). In qualitative terms, at this point, theoretical approaches are promising in interpreting available experimental data: Using the RNA duplex $r(CpG)_{12}$ as a structurally well-defined model (Conte et al., 1996; Gyi et al., 1998), molecular dynamics simulations at $5 \rightarrow 40°C$ gave evidence for a significant decrease in the residence time of water molecules and potassium ions bound in the first coordination sphere of the duplexes, indicating decreased order in the solvent around the solute with increasing temperature (Auffinger and Westhof, 2002; E. Westhof, personal communication). In general, unfolding and/or aggregation of

the solute upon melting lead to a drastic further release of water (Jaenicke, 1971; Lauffer, 1975; Jaenicke and Seckler, 1997).

The present knowledge of the role of water in connection with the stability and activity of biomolecules may be summarized as follows:

1) *Dielectric constant (ε)*. The formation and maintenance of cellular components occur in the presence of excess water; thus, the weak interactions responsible for their various functions are governed by the energetics of solvated partners in a strong dielectric, dielectric (~80), not by interactions in vacuo (= 1) (Dill, 1990).

2) *Temperature effects on biomolecules and water*. High temperatures alter the energetic properties (e.g., vibrational modes) of biomolecules in their aqueous solvent, which itself shows a strong temperature-dependence in its interaction parameters (cf. Table 2); both levels are intertwined in a complex way.

3) *Hofmeister effects*. In the case of high charge densities on the surface of polyelectrolytes, as well as in the presence of high salt concentrations (e.g., in halophiles), electrostatics are complicated by the clustering of water around the charged groups and by the competition of (counter-)ions for their own water of hydration (Jaenicke, 1991b). At this point, the fundamental laws of electrochemistry have to consider the specific effects of the size, charge and hydration of each individual electrolyte, which taken together determine the solubility of polyelectrolytes (salting-in and salting-out effects) and many other physicochemical and biological phenomena. The immediate cause of the "Hofmeister series of cations and anions" is the differences in hydration attributable to the intensities of the electrostatic field around each specific ion (von Hippel and Schleich, 1969). The "Hofmeister series" was first reported by Hofmeister (1888) for the coagulation of lyophilic colloids, and later for many other physical, chemical and biological phenomena (Edsall and Wyman, 1958). Ordering cations and anions according to their capacity to promote the solubility of neutral and alkaline proteins, the following two series are observed: $Li^+ < Na^+ < K^+ < NH_4^+ < Mg^{2+}$ and $SO_4^{2-} < PO_4^{3-} < CH_3COO^- < Citrate^{3-} < Cl^- < ClO_4^- < Br^- < I^- < SCN^-$. The solubilizing or precipitating effects can be explained in terms of the competition for water between a polyelectrolyte (here the protein) and excess electrolyte in the solvent (Collins and Washabaugh, 1985; Baldwin, 1996; Jaenicke and Seckler, 1997). One important Hofmeister effect is that guanidine denaturation depends on the anion. While guanidinium sulfate ($[Gdm]_2SO_4$) has no denaturing effects, GdmCl is a strong denaturant and GdmSCN is the strongest chaotropic agent.

4) *Volume effects of solutes*. Considering the "structure-making effects" of biomolecules in water, peripheral charges and nonpolar groups exposed to the aqueous solvent show an anomalous increase in solvent density (decrease in volume), "electrostriction" in the case of ions and "iceberg formation" in the case of exposed nonpolar groups (Kauzmann, 1959; Jaenicke, 1981; Groß and Jaenicke, 1994).

5) *"Hydration numbers."* Attempts to quantify both the hydrophobicity and hydration of amino acid residues can be summarized by the following series (with hydration estimated by the number of moles of H_2O per mole of amino acid residue in parenthesis): Phe (0); Cys, Gly, Ile, Leu, Met, and Val (1); Ala (1.5); Arg^+, Pro, and Tyr (3); His^+, hydroxyproline (4); Lys^+ (4.5); Asp^- (6); Glu^-, Tyr^- (7.5) (Kuntz, 1971; Kuntz and Kauzmann, 1974; Kyte and Doolittle, 1982).

6) *Types of "bound water."* In comparing tabulated hydration data from X-ray crystallography, NMR (see above), and thermodynamic and hydrodynamic measurements, it becomes clear that different experimental approaches "see different types of bound water molecules." Most of the respective terms are self-explanatory; since space does not permit a detailed discussion, key references may suffice: Structural hydration (the number and positions are determined from X-ray and NMR coordinates), hydrodynamic hydration (the amount of "hydration shell" migrating with the solute is determined in sedimentation/diffusion experiments and viscometry), low-temperature hydration (estimated, e.g., as "non-freezable water"), and preferential hydration (measured by thermodynamic methods in the presence of low-molecular weight additives competing with the macromolecular component for its hydration) (Tanford, 1961; Kuntz and Kauzmann, 1974; Franks, 1975–1982; Franks, 1985–1990; Franks and Eagland, 1975; Eisenberg, 1976; Timasheff, 1995; Timasheff and Arakawa, 1997).

In the case of proteins, quantitative data vary between 0.25 and 0.40 g of H_2O per g of protein. A rough estimate of the bound water surrounding the protein whose properties differ from those of the bulk water, can be obtained as the sum of the hydration numbers of the constituent amino acids (see 5 above); this holds despite the fact that part of the amino acid residues are buried in the protein interior and not accessible to the solvent.

7) *Dehydration causes denaturation*. If the structure-function relation of lysozyme is taken as a model, it becomes clear that dehydration below the limiting value of 0.25 g of H_2O per g of protein causes reversible deactivation paralleled by drastic changes of all available physical characteristics (Careri et al., 1980).

8) *Residual hydration of proteins*. Complete dehydration (e.g., by freeze-drying or high-temperature dry-weight determination) cannot be accomplished. Even dry biopolymers still contain residual water of the order of 5–10%; before the dry state is reached, chemical modification (such as deamidation) occurs.

9) *X-ray structures yield biologically relevant information*. Biopolymer crystals investigated by X-ray crystallography contain ca. 50% aqueous mother liquor filling the space between the single molecules; thus in the crystal, there is sufficient water available to guarantee complete hydration, i.e., native-like conditions. From this we may conclude that three-dimensional (3D) structures based on crystallographic data are biologically relevant.

10) *Structure determination at high temperature*. Regarding the structural analysis of macromolecular components from thermophiles and hyperthermophiles at elevated temperature, only sparse high resolution data have been reported. It would be desirable to develop the necessary methods to bridge this gap in order to gain insight into the correlation of stability and molecular flexibility up to the temperature limit of viability.

Stability of Biomolecules

Intrinsic vs. Extrinsic Stability

The physical limits of life at high temperature are defined by the temperature dependence of the interatomic forces involved in the covalent and noncovalent stabilization of the molecular inventory of the cell. Except for membranes, the lipids of which often are anomalous phytanyl-ethers instead of fatty acid esters (see below), the inventory of thermophilic and hyperthermophilic cells consists of the same building blocks as those found in mesophilic cells. The occurrence of covalent modifications such as methylation and glycosylation of biopolymers from hyperthermophiles has been frequently reported; whether they are biologically relevant molecular strategies of stabilization is still unclear (Vieille and Zeikus, 2001; see sections on Adaptive Stabilization Mechanisms of Nucleic Acids and Adaptive Stabilization Mechanisms of Lipids and Membranes in this Chapter).

Mutational Adaptation

Given the conventional set of canonical nucleic acid bases and amino acids, the general response to evolutionary stress is the selection for beneficial mutations on the genome level. In the case of thermophilic adaptation, these mutations lead to an enhancement of the intrinsic stability of the protein inventory.

At high temperature, the integrity of nucleic acids is threatened by strand separation and chemical damage to the nucleotide chains. Mechanisms providing intrinsic stabilization comprise an increase in G+C content of tRNAs and rRNAs and posttranscriptional modification. Extrinsic stabilization may be provided by specific salts or histone-like proteins, and by efficient repair systems (Grogan, 1998; di Ruggiero et al., 1999; for details, see section on Adaptive Stabilization Mechanisms of Nucleic Acids in this Chapter).

Considering proteins, the thermal stabilities of the orthologous homologs are found to be positively correlated with the maximal environmental temperature (see also Alexandrov, 1969; Hochachka and Somero, 1984, and Dahlhoff and Somero, 1993; Fig. 2).

Thus, the balance between stabilizing and destabilizing forces is adjusted during evolution such that homologous proteins from different species retain similar conformational stabilities at their respective physiological temperatures (Jaenicke, 1991b; Somero, 1995; Somero, 2000). Generally, both thermophilic and hyperthermophilic proteins exhibit high intrinsic stabilities and long denaturation half-lives of the order of hours close to the boiling point of water (Jaenicke et al., 1996; Daniel, 2000; Jaenicke and Böhm, 2001). Basically, the natural amino acids would allow the formation of proteins with sta-

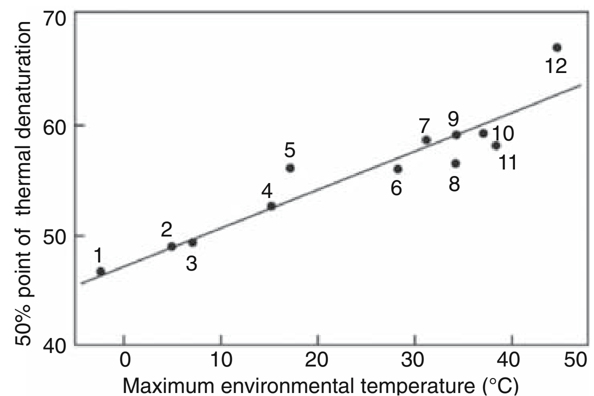


Fig. 2. The thermal stabilities of eye lens crystallins increase with the respective physiological temperatures of vertebrates. Coordinates refer to the 50% points of thermal denaturation and the adaptation temperatures of the following species: 1) *Pagothenia borchgrevinki* (Arctic fish); 2) *Coryphaenoides armatus* (deep-sea fish); 3) *Coryphaenoides rupestris* (deep-sea fish); 4) *Oncorhynchus mykiss* (rainbow trout); 5) *Cebidichthys violaceus* (tidepool fish); 6) *Rana muscosa* (frog); 7) *Alticus kirkii* (Red Sea fish); 8) *Rana erythraea* (frog); 9) *Gekko gekko* (lizard); 10) *Rattus norwegigus* (rat); 11) *Tropidurus hispidus* (reptile); and 12) *Dipssaurus dorsalis* (desert iguana). Data from McFall-Ngai and Horwith (1990).

bilities exceeding the magic upper temperature limit of ~115°C (de Grado, 1988; van den Burg et al., 1998). What constrains evolution for maximum thermal stability becomes obvious, keeping in mind that proteins are fundamentally multifunctional, combining the capacity to fold, serve a wide range of functions, and be degradable at the same time. As a consequence, protein evolution is a compromise between rigidity (stability) and flexibility (function, regulation, and turnover; Wetlaufer, 1980; Somero, 1995; Jaenicke, 2000a; Jaenicke, 2000b). At this point, it needs to be mentioned that the correlation between molecular flexibility and function (i.e., catalytic activity) is ambiguous and cannot be generalized because conformational stability is a global property, whereas the influence of flexibility on stability may be either global or local, as shown by kinetic unfolding experiments (Jaenicke, 1999; Bieri and Kiefhaber, 2000; Jaenicke and Lilie, 2000; Wright and Baldwin, 2000). The question of how local motions involved in the catalytic reaction are correlated with the fast anharmonic global dynamics, monitored spectroscopically or by X-ray analysis, is still open (Daniel et al., 1998, 1999). Comparing the Arrhenius activation energy and thermal stability of various enzymes, a new intrinsic thermal parameter, T_{eq}, was defined, that arises from the T-dependent equilibrium between the active and inactive enzyme at its true temperature optimum (Peterson et al., 2004). Beyond T_{opt}, the decrease in enzyme activity, induced by the T-dependent shift in the equilibrium, is up to two orders of magnitude greater than occurs through irreversible thermal denaturation. T_{eq} is central to the physiological adaptation of an enzyme to its environmental temperature, linking the molecular, physiological and environmental aspects of adaptation.

Disulfide Bonds

Disulfide bonds are known to be of utmost importance in stabilizing proteins such as hormones, plasma proteins or hydrolases and their inhibitors (Cecil and McPhie, 1959; Cecil, 1963; Friedman, 1973; Schulz and Schirmer, 1979; Fersht, 1998a; Branden and Tooze, 1999). Because the environment inside typical cells is reducing, cystine crosslinks are rarely found in intracellular proteins; in those exceptional cases where they are found, they usually exist transiently, playing roles in redox signaling or disulfide exchange, rather than serving to stabilize proteins, as they do outside the cell.

The suggestion that cystine might be essential in stabilizing cytosolic proteins in thermophiles was raised by a series of crystal structures of archaeal and bacterial proteins, e.g., elongation factor Ts from *Thermus thermophilus* (Jiang et al., 1996), TATA-box binding protein from Pyrococcus woesei (DeDecker et al., 1996), triosephosphate isomerase from *Thermotoga maritima* (Maes et al., 1999), adenylosuccinate lyase from *Pyrobaculum aerophilum* (Toth et al., 2000), ferric reductase from Archaeoglobus fulgidus (Chiu et al., 2001), and ferredoxin Fd1 from *Aquifex aeolicus* (Meyer et al., 2002). Based on this structural evidence, computational genomics and proteomics were applied to do a careful sequence-structure mapping study over the completely sequenced microbial genomes (Mallick et al., 2002). As a result it turned out that in the case of the intracellular proteins of certain hyperthermophilic archaea, especially the two crenarchaea *Pyrobaculum aerophilum* and *Aeropyrum pernix*, there is a clear preference for even numbers of cysteine residues that are mapped within disulfide bonding distance: More than 40% of the cysteine residues in *Pyrobaculum aerophilum* are predicted to be involved in disulfide bonds. Experimental findings support the computational results (T. O. Yeates, personal communication, 2002).

Stabilizing Additives and Molecular Chaperones

Apart from the intrinsic stabilization coming from contributions of intra- and intermolecular interactions within and between biomolecules, additional extrinsic stability increments may come from ligand binding, preferential solvation in the presence of high concentrations of compatible solutes, crowding and the action of molecular *chaperones*. Circumstantial evidence indicates that crowding effects within the living cell may extend the temperature range of stability significantly (Hochachka and Somero, 1973; Carpenter et al., 1993; Somero, 1995; Timasheff, 1995; Blöchl et al., 1997; Jaenicke, 2000a; Minton, 2000).

The discussion of intrinsic and extrinsic stability would be incomplete without mentioning molecular chaperones as accessory components involved in the stabilization of proteins at the borderline between self-organization and destruction. Functionally they are known to promote the long-term stability of proteins by regulating the kinetic partitioning of polypeptides between proper folding and association, on the one hand, and misfolding and subsequent aggregation or degradation, on the other (Jaenicke, 1987, 2004; Zettlmeissl et al., 1979; Goldberg et al., 1991; Kiefhaber et al., 1991; Jaenicke and Seckler, 1997). Using the primary meaning of the word, molecular chaperones avoid the "illegitimate interactions" between nascent or folding chains by keeping the level of aggregation-

competent polypeptide chains below a critical concentration, either by complex formation or by "iterative annealing," without becoming integral parts of the final native structure. The binding energy that drives the formation of the complex between the protein substrate and its chaperone may be used to rescue nascent or folding chains already on an off-pathway of proper folding (Beissinger and Buchner, 1998; Burston and Saibel, 1999; Jaenicke and Lilie, 2000; Leroux and Hartl, 2000; Walter and Buchner, 2002).

From their designation as "heat-shock proteins" (HSPs) (or "thermosomes" in the case of thermophilic archaea) one might expect that close to the limit of viability they would represent prototypes of proteins showing exorbitantly high intrinsic stability. However, as in the case of mesophiles, the term is a misnomer: heat-shock proteins are ubiquitous both in mesophiles and extremophiles, also under physiological conditions. Ubiquitous refers not only to the three phylogenetic domains, Eukarya, Bacteria and Archaea, but also to all the branches of the phylogenetic tree. In the case of (hyper-)thermophilic Archaea this means that all phyla, including the newly discovered nanoarchaeota (Huber et al., 2002) contain HSPs or thermosomes and other types of chaperones (Baross and Holden, 1996; Waters et al., 2003; Laksanalamai and Robb, 2004).

Considering the concise definition of molecular chaperones as "any protein that transiently interacts with and stabilizes an unstable conformer of another protein, facilitating its folding, assembly and interaction with other cellular components, as well as its intracellular transport or proteolytic degradation" (Leroux and Hartl, 2000), it is obvious that accessory proteins that assist protein folding, compartmentation and turnover, etc., must be of utmost importance in thermophiles. As a matter of fact, representatives of most chaperone families, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, GimC (prefoldin), and Hsp 16.5 (sHsp), have been isolated and studied in detail, in certain cases to the level of high-resolution 3D structures and systematic investigations of their structure-function relationship. From the physicochemical and functional point of view, they do not exhibit anomalous characteristics, except for differences in their quaternary structure and their ATP requirement. Space limits do not permit a detailed presentation of the rapidly growing field. Some aspects will be discussed in connection with the adaptive stabilization mechanisms of proteins. For reviews, see Fink and Goto (1997), Bukau (1999), Pain (2000), and Kiefhaber and Buchner (2004); and for special systems, Trent et al. (1991), Phipps et al. (1991), Trent (1996), Baross and Holden (1996), Trent et al. (1997), Andrä et al. (1998), Kim et al. (1998, 2003), Huber et al. (2002), and Laksanalamai and Robb (2004). At this point we focus our attention to only a few general observations that are connected with systematic, structural, cell biological and mechanistic aspects:

1. *Systematics*. The paradigm for chaperone-assisted protein folding has been the *group I* GroE system from *Escherichia coli*, which is generally found in bacteria and eukaryotic organelles of bacterial origin (Sigler et al., 1998; Hartl and Hayer-Hartl, 2002). The *group II* homologs in archaea (and the cytosol of eukaryotes) show a number of distinct features, as expected from the low sequence identity (<25%): Group I forms cages made up of seven-membered rings of Hsp60 subunits (as chaperone) and Hsp10 subunits (as co-chaperone), whereas *group II* consists of eight- or nine-membered hetero-oligomeric rings, with no general co-chaperone. The domain organization (equatorial ATPase domain, apical *recognition* domain and intermediate *connecting* domain) has been conserved between the two groups. However, in the "protrusion region" of the apical domain, significant differences have been discovered that allow the binding of the substrate protein without the help of the *group I* co-chaperone to be explained (Heller et al., 2004).

2. *Beyond systemaics: horizontal gene transfer*. As one would expect after the discovery of horizontal gene transfer, nature does not follow simple systematic rules. In the case of *group I* and *group II* chaperones, an archaeon has been found in which both types of HSPs, group I and group II, coexist. Inspecting the complete genomes of several species of the genus Methanocarcina, the first archaeal genomes were identified to contain both the GroEL/GroES (*group I*) and the thermosome/prefoldin (*group II*) genes. Both chaperones are coexpressed in the cytosol; under heat stress they are moderately induced. The GroE proteins show the structural features of their bacterial counterparts, whereas the thermosome contains three paralogous subunits (α, β and γ) which assemble at a molar ratio 2:1:1. As shown in vitro, the ATP- and ADP-dependent assembly reaction is regulated by the β subunit. The role of the two chaperones in one and the same cellular compartment with respect to substrate specificity and protein sorting from the ribosome to the proper chaperone machine is still enigmatic (Klunker et al., 2003; Figueiredo et al., 2004).

3. *Mechanism*. The functional significance of the structural flexibility ("plasticity") of proteins has been a well-established paradigm in the elucidation of enzyme mechanisms, self-assembly processes, molecular mechanics, etc. In the case of assisted protein folding, the molecular

machines nature has developed during evolution are absolutely unique: Binding a nascent or (re-)folding polypeptide chain via aggregation-competent hydrophobic core residues in a hydrophobic cage-like protein assembly, altering the hydrophobic surface of the cage into a hydrophilic one by closing the lid, and now allowing the secluded substrate polypeptide to find its energy minimum in the natural micro-environment sounds like a magician's trick, but that is what the thermosome manages to achieve (Gutsche et al., 1999; Bosch et al., 2000).

4. *Expression level*. The level of chaperone expression may vary over a wide range. In Pyrodictium occultum, a shift from 102 to 108°C has been reported to enhance the level of two bitoroidal hexadecameric ATPases of 56 and 59 kDa with optimal activity at 100°C to 80% of the total cytosolic protein concentration (Phipps et al., 1991; Baross and Holden, 1996). Similarly, electron micrographs of T-stressed *Sulfolobus shibatae* cells display a dense filamentous network of bitoroidal octodecamers, which suggests chaperone assemblies play a cytoscelet role in Archaea (Kagawa et al., 1995; Trent et al., 1997). On the other hand, the quantitative assessment of the role of the GroE system in protein folding in *Escherichia coli* suggested that there is sufficient GroEL to facilitate the folding of no more than 5% of all of cellular proteins within the cell (Lorimer, 1996); interestingly, overexpression of GroEL to high cellular levels in *Escherichia coli* does not inhibit cell growth (R. Rudolph, personal communication, 2004).

5. *Function of recombinant thermosomes*. The hyperthermophilic thermosomes from Pyrodictium occultum (*P.o.*) and Methanopyrus kandleri (M.k.) are members of the Hsp60 family. They form high-molecular-mass complexes, arranged in two rings of eight subunits each, stacked back to back, without Hsp10 as a co-chaperone. In *P.o.* two types of subunits (α and β) participate in the formation of the cage; they seem to alternate within each of the two rings. Overexpressing the two polypeptides separately and jointly in Escherichia coli yields authentic hexadecameric quaternary structures for all three-all-α, all-β and α+β. All three exhibit ATPase activity and bind denatured protein substrates, inhibiting their heat -aggregation. At temperatures up to 55°C, no release of renatured substrate was detectable. For technical reasons, experiments at physiological temperature were not feasible (Minuth et al., 1998). Switching to *M.k.* avoided the ambiguities caused by the hetero-polymeric quaternary structure of the *P.o.* thermosome. Strangely enough, its synthesis is not increased upon heat shock, and its ATPase activity depends on NH_4^+. The homo-hexadecameric recombinant protein is authentic and shows chaperone-like activity; again, no release of the substrate polypeptide chains is detectable at temperatures up to 60°C (Minuth et al., 1999).

6. *Structural studies at high temperature*. As taken from the thermosome example, collecting structural and functional data at the temperature limits of hyperthermophiles would be highly desirable. In this context, novel approaches have been developed. For example, in the case of the above mechanism of *group II* archaeal chaperones (see point 1) the functional details were deduced from differences between X-ray and solution-NMR data at varying temperature (Heller et al., 2004). Unfortunately, for both experimental approaches, presently data collection at temperatures close to or beyond the boiling point of water is impeded by crystallization problems and line broadening as well as signal overlap. In the case of NMR, using dipolar couplings allowed the loss of NOE information at high temperature to be compensated; on the other hand, assigning and separating single resonances of aromatic amino acids was facilitated by selective ^{19}F-labeling of Trp residues. Using the cold-shock protein (Csp) from *Thermotoga maritima* as a model, both methods were applied to extend structural studies into the physiological temperature regime. Taking the room-temperature structure as a reference, most significant alterations at high temperature occur in regions of the molecule that have been modeled as binding sites for single-stranded DNA, in agreement with the idea that *Tm*Csp plays a central role in the regulation of gene expression under cold-shock conditions (Jung et al., 2004). ^{19}F tryptophan labeling was used to study the folding of *Tm*Csp over a wide temperature range. In combination with stopped-flow experiments at lower temperatures, global line-shape analysis showed that the folding rate of *Tm*Csp closely resembles data collected for mesophilic Csps. However, the unfolding rate constant of *Tm*Csp is two orders of magnitude lower over the entire temperature range. Thus, stability differences are solely due to differences in the unfolding rates of the mesophilic and thermophilic proteins (Sterner and Liebl, 2001). A thermodynamic analysis points to an important role for entropic factors in the stabilization of *Tm*Csp relative to its mesophilic conterparts (Schuler et al., 2002).

7. *Small heat-shock proteins (sHSPs)*. sHSPs from thermophilic and hyperthermophilic organisms form multimeric complexes with (occasionally heterodisperse) molecular masses ranging from 200 kDa to more than 1 MDa. Although they show high diversity, the majority of acid sHSPs share amino acid sequence similarity with the vertebrate eye-lens α-crystallins; both groups are molecular chaperones (Jacob et al., 1993;

Jaenicke and Creighton, 1993). Presently available sparse structural data suggest that their monomeric structures share a common building-block structure (van Montfort et al., 1992). The mechanism of action seems to be defined by the individual quaternary structure; evidently, dissociation/association reactions play a role in the regulation of chaperone activity (Laksanalamai and Robb, 2004). In contrast to the acidic chaperones (IP~4.6), basic sHSPs (IP~9) are involved in nucleotide binding (Korber et al., 2000).

Measuring Thermodynamic Stability

The conventional introduction to the subject, with its emphasis on heat engines, is almost certain to convince the student that thermodynamics is sheer sophistry and unrelated to the real business of biochemistry. But an understanding of some of the ideas of thermodynamics is important to discover how molecules make organisms work (van Holde, 1985).

The thermodynamic stability, e.g., of nucleic acids or proteins, can be quantified by measuring the temperature- or denaturant-induced unfolding, excluding irreversible side-reactions such as chemical modifications or aggregation (Tanford, 1968; Tanford, 1970; Privalov, 1979; Jaenicke and Seckler, 1997). To illustrate the procedure, we assume an N U equilibrium transition of a monomeric globular protein from its native (N) to the denatured state (U). The free energy of conformational stability is the difference between the free energies of the unfolded and the folded states

$$\Delta G_{stab} = G_{unfolded} - G_{native} \quad (1)$$

as well as the Gibbs-Helmholtz equation

$$\Delta G_{stab} = \Delta H_{stab} - T\Delta S_{stab} \quad (2)$$

where T is the absolute temperature, and ΔH_{stab} and ΔS_{stab} are the enthalpy and entropy differences between the unfolded and the folded states

$$\Delta H_{stab} = H_{unfolded} - H_{native} \quad (3)$$

can be determined either directly from calorimetric experiments (ΔH_{cal}) or from a "van't Hoff plot," i.e., from the temperature dependence of the apparent equilibrium constant K of the transition

$$N \overset{K}{\Leftrightarrow} U \quad (4)$$

according to

$$\Delta H_{van't\ Hoff} = RT^2\, d(\ln K)/dT \quad (5)$$

where R is the gas constant. The "two-state assumption" underlying Eq. (4) can be tested by comparing the results of both approaches. If only N and U and no intermediates are populated in the N→U transition, ΔH_{cal} equals $\Delta H_{van't\ Hoff}$, otherwise $\Delta H_{cal}/\Delta H_{van't\ Hoff}$ is >1 (Privalov, 1979; Jaenicke, 1991b).

A further qualitative standard criterion to test the two-state assumption is to compare the denaturant-induced equilibrium transitions monitored by different spectral properties of a protein. If the unfolding profiles do not coincide, intermediates are present in significant amounts, and Eq (4) does not adequately describe the denaturation process (Pace and Scholtz, 1997). If Eq (4) is sufficient to describe the reaction, the thermodynamic stability of the protein can be calculated from the apparent equilibrium constant K according to

$$\Delta G_{stab} = -RT \ln K \quad (6)$$

Under physiological conditions, i.e., in water at constant pH, pressure and temperature (pH 7, 1 bar, 25°C), the change in Gibbs free energy as 1 mole of substrate is converted to 1 mole of product ([substrate] = [product] = 1 mole/liter) represents the standard Gibbs free energy change $\Delta G°'$. Given the high molecular mass of proteins and their relatively high partial volume, molar concentrations are experimentally inaccessible. In the case of simple first-order reactions such as Eq (4), this is irrelevant; in more complex reactions, to compare the stabilities of proteins, their free energies have to be normalized, e.g., to millimolar or micromolar concentrations (Dams and Jaenicke, 1999).

It is obvious that the above equilibrium constant K depends on the denaturation conditions, because in a given experiment different variables may be superimposed in the destabilization of a protein. For example, sometimes neither low pH nor chaotropic agents (urea or guanidinium chloride) alone are able to denature a protein. This holds especially for ultrastable proteins from hyperthermophiles, where most proteins only unfold beyond the boiling point of water; thus to shift the melting temperature (T_m) at which half of the protein is thermally unfolded down to a manageable range, low pH values or the addition of chaotropic agents are required. The measured ΔG_{stab} values at these nonphysiological solvent conditions have to be extrapolated to obtain ΔG_{stab} data at zero denaturant concentration; this allows the thermodynamic stabilities of different proteins, e.g., from mesophiles and hyperthermophiles, to be compared (Privalov, 1979; Pace, 1986; Pace and Scholtz, 1997; Pfeil, 1998).

Because of the significant temperature dependence of ΔH_{stab} and ΔS_{stab}, ΔG_{stab} versus T profiles observed for the thermal unfolding of proteins exhibit parabolic characteristics (Privalov, 1979; Schellman, 1997; Fig. 3A); their maxima cluster in a narrow range between 30 and 80 kJ/mol (7–20 kcal/mol; Fig. 3B); other modes of denaturation such as guanidinium chloride, urea

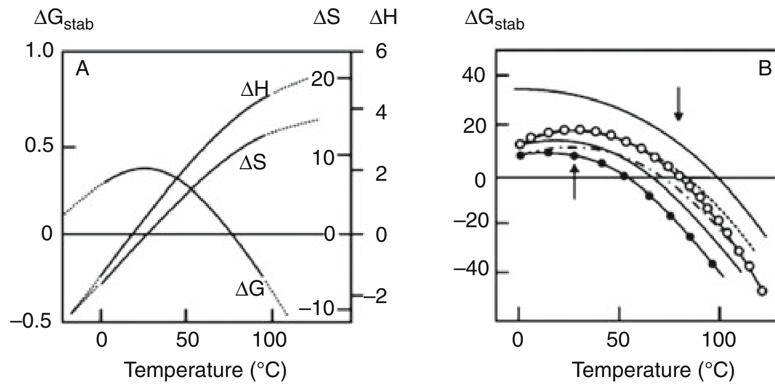


Fig. 3. Temperature dependence of ΔH, ΔS and ΔG_{stab} of proteins. A. Temperature-dependence of the enthalpy, entropy and free energy of stabilization (ΔG_{stab}) of sperm whale myoglobin, calculated per mole of amino acid residues (Privalov, 1979; Privalov and Gill, 1988). B. ΔG_{stab} versus T profiles of structurally related proteins from mesophiles and hyperthermophiles. The various profiles illustrate that enhanced thermal stability may be accomplished either by flattening the parabola, or by an upward shift (to a higher overall free energy), or by a shift to higher temperature; they belong to the β-barrel DNA-binding protein Sso7d from *Sulfolobus solfataricus* (—), all-β tyrosine kinases BtkSH3 (- - -) and Tec-SH3 (• – • –), α-spectrin (--), CspA from *Bacillus subtilis* (•) and Csp from *Thermotoga maritima* (o). The arrows at ~30 and 80°C refer to the physiological optimum temperatures of *B. subt*, and *T. maritima*, respectively. Taken from Jaenicke (2000a).

or pH give the same result, provided they are corrected to standard conditions (Makhatadze and Privalov, 1995; Pfeil, 1998). Thus, in spite of the large number of noncovalent contacts maintaining the native structure of proteins, ΔG_{stab} is only marginal, no more than the equivalent of a few weak intermolecular interactions, even in the case of extremophilic proteins. Representing the minute difference between the large contributions of attractive and repulsive forces, ΔG_{stab} determines the close packing of the polypeptide chain and the minimization of the hydrophobic surface area that are typical for globular proteins in aqueous solution. The balance of attraction and repulsion corresponds exactly to the abovementioned compromise between rigidity and flexibility.

In contrast to the two-state assumption, protein denaturation is rarely fully reversible, so that determining the thermodynamic stability in terms of ΔG_{stab} may be difficult or even impossible. In these cases, operational definitions of stability are used to characterize proteins, especially if homologs from different species or wildtype and mutant proteins are compared. To this end, most commonly apparent (nonequilibrium) T_m values or the denaturant concentration at which half of the protein is unfolded ($c_{1/2,urea}$ or $c_{1/2,GdmCl}$) are used. For multidomain and multisubunit proteins this approach has often been the only means to obtain at least a qualitative estimate of stability.

Thermodynamics vs Kinetics

Thermodynamics and kinetics are commonly considered as separate domains; the first, with emphasis on energy and mass action, ignores time as a physical parameter, while the second is focused on reaction coordinates and rates. However, describing chemical equilibrium as the situation defined by the balance of the formation and decay of the reaction product.

$$K = \frac{\vec{k}}{\overleftarrow{k}} \tag{7}$$

with K as the equilibrium constant, and and as rate constants of the forward and backward reactions, kinetics and thermodynamics are obviously connected. Combining equations (6) and (7), evidently, kinetics can be used to define and measure stability: Higher ΔG_{stab} values, e.g., of thermophilic proteins compared to homologous mesophilic proteins, could be due to an increase in the rate of folding or a decrease in the rate of unfolding, or a combination of the two. The rate of the unfolding reaction is limited by the highest activation barrier upon the N→U transition. Thus, protein stability may be governed, in terms of kinetic stabilization, by a high free energy of activation ($\Delta G_{N\rightarrow U}$) that separates N from the transition state. In a number of cases the rate of unfolding of hyperthermophilic proteins has been shown to be drastically decelerated compared to their mesophilic counterparts, supporting the view that the increase in $\Delta G_{N\rightarrow U}$ is responsible for the enhanced ΔG_{stab} (Sterner and Liebl, 2001). To give an example, the cold-shock proteins from *Bacillus subtilis* (equilibrium unfolding at $T_m = 52°C$), *Bacillus caldolyticus* ($T_m = 72°C$) and *Thermotoga maritima* ($T_m = 90°C$) show a dramatic decrease in their unfolding rates with increasing T_m, whereas folding occurs at

closely similar high rates ($\tau = 1.0 \pm 0.2$ ms; Perl et al., 1998; Perl et al., 2000; Perl and Schmid, 2001). Evidently, from the ecological point of view, a high $\Delta G_{N\rightarrow U}$ may provide a significant advantage for the hyperthermophilic organism, because, owing to the turbulence in hydrothermal vents, exposure times to a lethal temperature range may be short compared to the half-time of the N→U unfolding transition; on the other hand, the fast folding reaction allows the rapid formation of the compact native structure, this way protecting the nascent protein during the folding process against irreversible damage by covalent modification or aggregation (Hensel et al., 1992; Jaenicke and Böhm, 1998; Plaza del Pino et al., 2000).

Forces and General Mechanisms in Protein Stabilization

The observation that the Gibbs free energy of stabilization represents a minute difference between strong attractive and repulsive potentials means that proteins exist close to the borderline of denaturation. Comparing tabulated ΔG_{stab} values with the bond energies of the relevant weak interactions (Bernal, 1939; Bernal, 1958; Kauzmann, 1959; Stillinger, 1977; Pfeil, 1998), it becomes clear that a few hydrogen bonds, or a hydrophobic patch, or just one ion pair may suffice to shift the optimum stability of proteins from the mesophilic to the thermophilic temperature regime (Fig. 3B). Evidently, evolution can choose between an astronomical number of ways to adapt to extreme conditions; therefore, no general rules of protein stabilization are to be expected. The following brief survey of the interatomic forces that contribute to macromolecular stability may provide some understanding of the many reasons why certain amino acid substitutions are essential for protein stability, while others are neutral.

Basically, the spatial structure of proteins is determined by electrostatic forces between polar and ionized groups and by hydrophobic interactions involving nonpolar residues (Dill, 1990; Jaenicke, 1991a; Jaenicke and Böhm, 2001a; Petsko, 2001; Spyracopoulos and Sykes, 2001). The electrostatic forces include ion pairs, hydrogen bonds and van der Waals forces.

Single surface-exposed ion pairs have often been considered of secondary importance because they are normally present in small numbers and not highly conserved in proteins; thermodynamic data and evidence from protonation/deprotonation experiments seemed to confirm this assumption (Kauzmann, 1959; Dill, 1990). The relatively small contribution to the thermal stability of no more than ~4 kJ/mol could be easily explained because the gain in the free energy is practically compensated by the entropic cost of dehydration plus the reduction of the conformational freedom when the protein goes from the denatured to the native state (Jaenicke, 1991b; Matthews, 1996; Fersht, 1998b). One would predict that at high temperature this effect becomes even more important. However, again referring to the above cold-shock proteins, about half of the difference in $\Delta G_{stab,70°C}$ between the mesophilic *B. caldolyticus* and the thermophilic *B. stearothermophilus* protein is due to electrostatic interactions between two exposed amino acid residues (Pace, 2000; Perl et al., 2000; Mueller et al., 2000; Perl and Schmid, 2001). Along these lines, an increase in the number of electrostatic interactions, which are often organized in clusters of ion pairs between charged amino acid side chains, has been frequently found in hyperthermophilic proteins compared to their mesophilic counterparts (Jaenicke and Böhm, 1998; Szilágyi and Závodszky, 2000; Sterner and Liebl, 2001). These networks can increase protein stability by a number of mechanisms: 1) Each extra ion pair added to the network requires the desolvation and localization of only a single residue; 2) networks of charged groups are often located in cavities and at interfaces where their conformational freedom is restricted; as a consequence, part of the entropic cost has already been provided during the folding of the polypeptide chain; 3) hydration effects play a minor role at high temperature (Elcock, 1998); and 4) the dielectric constant (in the denominator of the Coulomb equation) decreases with temperature, resulting in an increase in electrostatic energy upon ion-pair formation. Perutz's early hypothesis on the significance of ion pairs for the stability of thermophilic proteins may be rationalized on the basis of given arguments (Perutz and Raidt, 1975).

In summarizing our present understanding of the contribution to ΔG_{stab} attributable to electrostatic potentials between charged groups, one important conclusion is that the key stabilizing feature is not so much the type and the number of groups, but their structural context and optimum placement. The ion-pair network in the glutamate dehydrogenase family illustrates this conclusion: The comparison of the crystal structures of the mesophilic, moderately thermophilic and hyperthermophilic enzymes and homology modeling clearly indicate that the decrease in T_m correlates with the reduction ("fragmentation") of ion-pair networks (Yip et al., 1995; Yip et al., 1998).

The significance of hydrogen bonds as the dominant stabilizing force in protein folding and stability has been controversial for more than 60 years (Mirsky and Pauling, 1936; Bernal, 1939;

Bernal, 1958; Kauzmann, 1959; Fersht, 1987; Dill, 1990; Jaenicke, 1991a; Jaenicke, 1991b; Makhatadze and Privalov, 1995; Pace, 1996; Schellman, 1997; Petsko, 2001). Attempts to quantify their bond energy resulted in a range between 12 and 38 kJ/mol (3–9 kcal/mol), including 21 kJ/mol (5 kcal/mol) for the amide-amide NH···O bond (Fersht, 1998b). Mutant studies focusing on the energy increment inherent to an intrachain H-bond relative to the H-bond with the aqueous solvent yielded 1.2 ± 0.6 kcal/mol (Fersht, 1987; Matthews, 1995; Matthews, 1996; Pace et al., 1996; Jaenicke, 1999). Using standard proteins such as ribonuclease T1 or phage T4 lysozyme, a large amount of experimental data corroborated this estimate. For the contribution of the H-bond to the stability of nucleic acids, also 1 kcal/mol was suggested by Crothers and Zimm (1965); their prediction was confirmed using polynucleotides as models (Freier et al., 1986).

Commonly, forming an H-bond, e.g., in a helix or a β-strand, requires the transfer of polar groups from the periphery into the interior of the protein. This unfavorable process has been hypothesized to reduce the above H-bond energy to a value close to the thermal energy (kT); however, given the large number of H-bonds involved in secondary structure formation, it was assumed that even a marginal increase in the free energy of stabilization ΔG_{stab} value will accumulate to a significant net stabilization (Pace et al., 1996). Recent studies, comparing Asn→Ala, Leu→Ala and Ile→Val mutants, have shown that the burial of an amide group contributes more to protein stability than the burial of an equivalent volume of $-CH_2-$ groups. Obviously, this is in contrast to the above hypothesis because it clearly indicates that the desolvation penalty for peptide groups is much smaller than assumed so far; at the same time, it allows the conclusion that the hydrogen bonding and van der Waals interactions of peptide groups in the tightly packed interior of the folded protein are more favorable than similar interactions with water in the unfolded polypeptide chain (Pace, 2001).

In summarizing the present state of theory and experiment regarding the role of hydrogen bonds in the stabilization of globular proteins and other biopolymers in aqueous solution, it is now well-established that H-bonds exhibit a significant stabilizing effect over the whole biologically relevant temperature range.

The hydrophobic effect is a way of describing the tendency of nonpolar compounds such as hydrocarbons to transfer from water to an organic solvent. Its physical nature was previously considered to be entropic, attributable to the reorganization of the normal hydrogen-bonding network in water (called "iceberg formation") by the presence of a hydrophobic compound or nonpolar groups. In forming a hydrophobic interaction, the release of the icebergs from nonpolar groups or surfaces into the bulk phase of the aqueous solvent leads to an increase in entropy which exceeds the free energy terms due to losses of rotational or translational degrees of freedom (Kauzmann, 1959; Tanford, 1962; Tanford, 1980; Lauffer, 1975; Privalov, 1979; Dill, 1990; Privalov and Gill, 1988). According to Eq (2), the entropic nature of hydrophobic interactions would suggest that their contribution to ΔG_{stab} increases with temperature. As a logical consequence, it has been claimed that the increased stability of proteins from thermophiles compared to mesophiles is attributable to an increase in hydrophobicity. A critical analysis proved the differences to be statistically insignificant (Böhm and Jaenicke, 1994); this result has been recently confirmed for the much larger data base gained from the complete genomes of mesophilic, thermophilic and hyperthermophilic bacteria and archaea (Jaenicke and Böhm, 1998; Sterner and Liebl, 2001). The results do not allow the conclusion that hydrophobic interactions do not contribute to ΔG_{stab}, for various reasons: 1) The result of determining the probabilities with which polar and nonpolar amino acids are localized in the core or in the exterior confirm the relative solubilities of all amino acids in water or other less polar solvents (Nozaki and Tanford, 1971). However, detailed normalized distributions of the solvent accessibilities of all 20 amino acids, calculated from known 3D structures clearly show that in the folding process, there are roughly equivalent decreases in the accessibility of both the polar and nonpolar groups (Richards, 1977; Rose et al., 1985). Obviously, the relevant forces and the final structure of proteins require more careful definition than is implied by the common assumption that inside equals nonpolar and outside equals polar. 2) Using the relative solubilities, i.e., the transfer of nonpolar substances into water, as a model reaction to quantify hydrophobic interactions, the temperature dependence is characterized by parabolic profiles (Fig. 4). At the minimum, the hydrophobic effect shows its maximum, $\Delta H_{transfer}$ equals zero, and $\Delta G_{transfer}$ is fully determined by $T\Delta S_{transfer}$ (Privalov, 1988a; Pace, 1992).

At higher temperatures, the hydrophobic effect decreases and finally vanishes at ~120–140°C (Sturtevant, 1977; Baldwin, 1986; Privalov and Gill, 1988; Dill, 1990; Makhatadze and Privalov, 1995). Evidently, the hydrophobic effect is a highly complex phenomenon. 3) Recent thermodynamic data have shown that there is a significant enthalpic contribution to the hydrophobic effect, which can be attributed

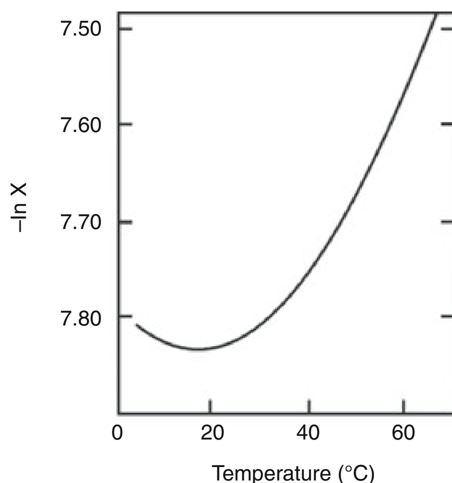


Fig. 4. The temperature dependence of the solubility (x) of benzene in water reflects hydrophobic interactions. Data taken from Franks et al. (1963). The corresponding thermodynamic data for the transfer of benzene and ethylbenzene from the pure liquid phase to water at 25°C are:

	Surface area (Å^2)	Solubility (mole fraction)	$\Delta H_{transfer}$ (kJ/mol)	$\Delta S_{transfer}$ (J/K·mol)	$\Delta G_{transfer}$ (kJ/mol)	ΔC_p (J/K·mol)
Benzene	240	4.01×10^{-4}	2.08	−58	19.4	225
Ethylbenzene	302	0.258×10^{-4}	2.02	−81	26.2	318

ΔC_p, the change in heat capacity, may be gained from a ΔH versus T plot according to the Kirchhoff equation $\Delta C_p = \partial(\Delta H)/\partial T$ (cf. Privalov and Gill, 1988; Dill, 1990; and textbooks of physical chemistry).

to van der Waals interactions (P.L. Privalov, personal communication, 1998). A sound thermodynamic treatment of the correlation of temperature, stability and the hydrophobic interaction has been put forward by Schellman (1997).

In summarizing the forces and general mechanisms involved in the stabilization of proteins, it is important to note that, as a state function, the free energy of stabilization ΔG_{stab} is an additive quantity. According to the Gibbs-Helmholtz equation (Eq [2]), its increments are either enthalpic or entropic. Three major contributions are dominated by enthalpy: attractive forces between ion pairs, hydrogen bonds, and van der Waals interactions. Hydrophobic interactions have enthalpic and entropic increments. Apart from its significance in the hydrophobic effect, entropy clearly dominates three characteristics of thermophilic proteins: 1) the increase in proline or the decrease in glycine residues, 2) the reduced lengths of loops, and 3) anomalously high states of association. In the case of 1) and 2), stabilization comes from the destabilization of the denatured state, attributable to the decrease in the degrees of freedom of the unfolded polypeptide chain (Matthews et al., 1987; Suzuki et al., 1991; Watanabe et al., 1991; Watanabe et al., 1994; Watanabe et al., 1996; Watanabe et al., 1997). In the case of 3), both the burial of nonpolar sites in the inner core of the protein and the release of water (accompanying ion-pair formation), lead to an increase in entropy and a gain in free energy (Lauffer, 1975; Jaenicke, 1987; Jaenicke and Seckler, 1997; Schellman, 1997; Thompson and Eisenberg, 1999).

Temperature Effects on Ligand Binding and Enzyme Function

Concerning thermal effects on enzymatic catalysis and regulation, ligand binding is of crucial importance. At this point, binding-site geometry, active-site fitting of the ligands, and conformational changes upon binding, transformation and release of substrates, products and effectors are expected to be temperature dependent. However, binding of substrates and/or coenzymes often exhibits entropy-enthalpy compensation (Lumry and Rajender, 1970), so that the overall free energy of ligand binding seems to be more or less indifferent to temperature changes (Fig. 5A).

Michaelis-Menten constants (K_m) for homologous enzymes from mesophiles and thermophiles often cluster in a narrow range, when compared at optimum physiological temperatures (Fig. 5B). At a fixed temperature, enzymes from mesophiles show higher catalytic turnover numbers (k_{cat}) than their homologs from thermophiles; however, owing to the general enhancement of reaction rates with temperature, at

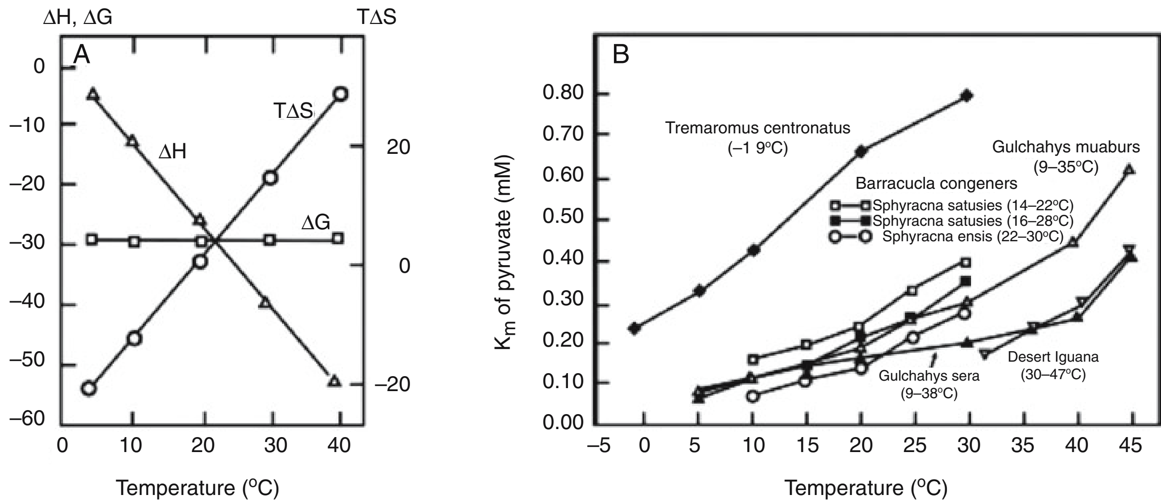


Fig. 5. Thermal adaptation and protein function. A. Entropy-enthalpy compensation upon binary complex formation of NADH and lactate dehydrogenase from pig muscle, monitored by calorimetric analysis; ΔH, ΔS and ΔG refer to ligand binding in kJ/mol. Data from Hinz and Schmid (1977). B. Temperature effect on the Michaelis-Menten constant (K_m) of pyruvate for the H4 isoenzyme of lactic dehydrogenase (LDH-H_4) of organisms adapted to different temperatures; physiological temperatures given in parentheses. Data taken from Somero (1995).

physiological temperature k_{cat} also is conserved in accordance with the frequently observed unity in diversity observed in comparative physiology (Somero, 2000).

Biochemical Limitations at High Temperature

The high-temperature regime of life is limited not only by the above-mentioned temperature coefficients of the relevant interatomic interactions in biomolecules, but also by their hydrothermal decomposition (Dill, 1990; Jaenicke, 1991a; Jaenicke, 1991b; Blokzijl and Engberts, 1993; Elcock, 1998; Jaenicke and Böhm, 1998; Sterner and Liebl, 2001). In the cytosol's multicomponent system, amino acids, nucleic acid bases, carbohydrates and lipids are prone to a wide variety of covalent chemical modifications, especially under various extreme physical conditions. In the case of proteins at elevated temperature, these modifications comprise deamidation of the amide side chains of Asn and Gln residues, succinimide formation at Glu and Asp, β-elimination, oxidation of His, Met, Cys, Trp and Tyr, disulfide interchange, lanthionin formation, Maillard reactions, hydrolysis, ring cleavage, etc. (Zale and Klibanov, 1986; Ledl and Schleicher, 1990; Creighton, 1994; Table 3).

Since these reactions have high activation energies, they become increasingly important at high temperatures. On the other hand, their reaction rates have been shown to be higher for small peptides with high flexibility than for proteins, when comparing the same amino acid sequence (Wearne and Creighton, 1989). Similarly, enhanced rates were observed for unfolded proteins compared to the same proteins in their native state (Hensel et al., 1992). Thus, the typical high packing density of thermophilic proteins will have a protecting effect, to the extent that degradative reactions may be assumed to play no significant role as long as the native conformation of a given protein is intact (Daniel et al., 1996). For this reason, hyperthermophiles and thermophiles must either inhibit unfolding and subsequent degradation of proteins or compensate for both processes. Possible strategies are the accumulation of compatible solutes, or enhanced protein synthesis and repair. Little is known about the chemistry of thermal degradation or specific protection of proteins, and even less about repair. An exception is the L-*iso*aspartyl methyltransferase from *Thermotoga maritima*, a highly active repair enzyme that catalyzes the transfer of the methyl group from S-adenosylmethionine (SAM) to the α-carboxyl group of L-*iso*aspartyl residues, resulting from the deamidation of Asn and the isomerization of Asp (Ichikawa and Clarke, 1998). Interestingly, the k_{cat} of the enzyme at 80°C is ~20-fold higher than that of mesophilic homologs at 37°C, supporting the view that it is specifically adapted to the high need of protein repair close to the boiling point of water.

The thermal stability of the different canonical natural amino acids at neutral pH decreases in the following series: (Val, Leu) > Ile > Tyr > Lys > His > Met > Thr > Ser > Trp > (Asp, Glu, Arg).

Table 3. Degradative chemical reactions and isomerization reactions important to irreversible protein denaturation, especially at elevated temperature.

Reaction	Amino acids involved	Comments
Deamidation	Asn, Gln (especially in Asn-Gly and Asn-Ser sequences)	Independent of pH, product: *iso*-Asp as substrate of methyl transferase, leading to repair or clearance.
Racemization	Asp	
Isomerization	Pro (*cis-trans* isomerization)	Catalyzed by peptidyl prolyl-*cis-trans* isomerases.[a]
Glycation	Lys and other amino acids reacting with reducing sugars	Cross-linking by Maillard reactions, involved in in vivo degradation.[b]
Oxidation	Cys ⇔ sulfenic → cysteic acid (oxidation or SH/SS exchange via mixed disulfides) Met ⇔ sulfoxide → sulfone	Thiolate mechanism catalyzed by Cu^{2+} or Fe^{2+} or protein disulfide isomerases (PDI, DsbA/DsbB, etc.). Significant both in vivo and in vitro in the presence of oxygen radicals.
Proteolysis	Polypeptides → amino acids	Caused either by proteases or autolysis, or by H^+-catalyzed peptide cleavage; nonenzymatic, between Asp and Pro and Asp and (C-terminal) Asn.
Photodegradation	Trp → kynurenin → *N*-formyl kynurenin Tyr → DOPA, dityrosine Cystine → 2 Cys	Caused by nonionizing or ionizing radiation, depending on the local microenvironment of the amino acids.

Abbreviations: Asn, asparagine; Gln, glutamine; Ser, serine; Asp, aspartate; Lys, lysine; Pro, proline; Cys, cysteine; Met, methionine; PDI, protein disulfide isomerase; DsbA/DsbB, disulfide-bond forming proteins; and DOPA, dihydroxyphenylalanine.

[a]Schiene-Fischer and Fischer (2000).

[b]cf. Barrett (1985).

For further references and details, cf. Greenstein and Winitz (1961); Meister (1965); Cecil (1963); Gottschalk (1972); Freedman (1973); Barrett (1985); Stadtman (1990); Stadtman and Oliver (1991); Volkin et al. (1995); Berlett and Stadtman (1997); Jaenicke and Seckler (1997); Daniel and Cowan (2000); Jaenicke and Lilie (2000); Schiene-Fischer and Yu (2001); and Vieille and Zeikus (2001).

Cysteine exhibits low stability: Depending on temperature and pH, it undergoes either oxidation (to form cystine), or elimination of sulfur (lanthionin formation). The lower limit at which degradation in aqueous buffer solutions was detectable was ca. 110°C (Bernhardt et al., 1984). One may assume that up to this temperature range, biosynthesis can still balance the thermal decomposition. In the temperature regime of hydrothermal vents, e.g., at 250°C (265 bar), the half-lives of the amino acids, peptides and proteins undergoing degradation were found to be too short to be offset by biosynthesis of these molecules (White, 1984).

ATP and ADP hydrolysis become significant between 110 and 140°C (Leibrock et al., 1995). This upper temperature limit coincides with the temperature range at which the hydrophobic hydration of nonpolar residues in aqueous solution vanishes (Sturtevant, 1977; Privalov, 1979; Baldwin, 1986; Jaenicke, 1991b; Jaenicke, 2000a).

In summarizing the biochemical limitations of viability from the point of view of water-soluble proteins, temperatures beyond ca. 130–140°C are not tolerable, for two reasons: 1) natural amino acids are hydrothermally decomposed and 2) the solvent properties of water are altered, blurring the difference between polar and nonpolar residues, thus interfering with the "hydrophobic collapse" (as the initial step of protein folding) and the formation of the densely packed hydrophobic core (as the prerequisite of protein stability). For nucleoproteins and lipoproteins or membranes, the same holds true because of the temperature limits of the intermolecular interactions between the polar and nonpolar components in the respective complexes. For both classes of proteins, extrinsic factors and compatible solutes may enhance the stability as well as the limits of growth (see below). The given upper temperature limit of viability has been confirmed for cells of the most extreme hyperthermophiles *Pyrolobus fumarii*, and strain 121 with its temperature of maximal growth at 121°C (Blöchl et al., 1997; Kashefi and Lovley, 2003). Whether the protective action of compatible solutes and/or crowding induced by high levels of molecular chaperones contribute to this extreme thermotolerance, needs further investigation (cf. Carpenter et al., 1993; Zimmerman and Minton, 1993; Somero, 1995; Trent et al., 1997; Minton, 2000).

Clearly, the biochemical limit of viability depends not only on the intact organization of the cell's standard high-molecular weight components, but also on the low-molecular weight compounds such as coenzymes and metabolites. Again, in general, extremophiles make use of the common repertoire of compounds known from the metabolism of mesophiles. Keeping in mind the high catalytic rate of most enzymes under physiological conditions, the majority of meta-

bolites do not limit viability at temperatures close to 100°C. The reported half-lives of ATP and ADP range from ~1–6 hours at 100°C, depending on the pH and the presence of metal ions (Ramirez et al., 1980; Leibrock et al., 1995; Daniel et al., 1996). However, the oxidized nicotinamide adenine dinucleotide (NAD^+) has a half-life at 100°C of no more than 10 min. To cope with this instability, nature can make use of at least four strategies: 1) high catalytic turnover, or 2) channeling of labile intermediates, 3) local stabilization in enzyme-ligand complexes, and 4) usage of an alternate metabolic pathway or a different, more stable compound. In the case of 3), the high affinity of ligands for their respective enzymes has frequently been shown to cause mutual stabilization (Danson, 1988; Jaenicke et al., 1996; Dams and Jaenicke, 1999).

Adaptive Stabilization Mechanisms of Nucleic Acids

The integrity of nucleic acids is threatened at high temperatures, which can induce either strand separation and chemical damage of the nucleotide constituents or, at the extreme, breakage of backbone phosphodiester bonds (Grogan, 1998; Daniel and Cowan, 2000).

Mechanisms to Avoid Strand Separation

An increased G+C content is known to increase the temperature T_m at which melting, i.e., strand separation of DNA and RNA occurs. Thus, a possible adaptation mechanism of nucleic acids to thermophilic and even more to hyperthermophilic conditions would be an increase in G+C. Indeed, a systematic study revealed a strong positive correlation between the G+C content of tRNAs and rRNAs with the optimum growth temperatures of prokaryotes (Galtier and Lobry, 1997; Fig. 6A). The same study showed, however, that the G+C content of genomic DNA is not correlated with the growth temperature (Fig. 6B).

Quite the contrary, the DNA of some of the most hyperthermophilic archaea has a strikingly low G+C content, with values as low as 31 mol%, e.g., for *Acidianus fervidus* and *Methanococcus igneus* (T_{max} >90°C), and an average of ca. 45 mol% for all presently known hyperthermophilic archaea and bacteria (Stetter, 1996; Grogan, 1998). These data clearly suggest that in these organisms, the DNA double helix must be stabilized either by extrinsic factors such as ions and small metabolites or by proteins. It has been known for a long time that the addition of salts or polyamines leads to an increase in T_m. Actually some, but not all, hyperthermophiles accumulate high concentrations of putative ionic thermoprotectants such as potassium di-inositol-1′,1′-phosphate and tripotassium cyclic-2,3-diphosphoglycerate (Hensel and König, 1988; Scholz et al., 1992). However, there is no clear correlation between the level of polycationic polyamines and growth temperature (Kneifel et al., 1986). In a number of archaeal hyperthermophiles, two unrelated groups of highly basic proteins were identified, which bind to DNA without marked sequence preference. Both the members of the HMf histone family, which are homologs of the eukaryal core histones, and the histone-like proteins from *Sulfolobus* species, for which no eukaryal homologues are known, increase the T_m of the DNA double helix significantly (McAfee et al., 1996; Soares et al., 1998). Thus, there is clear evidence that hyperthermophiles make use of different strategies to prevent DNA strand separation at their extreme growth temperatures. Certainly, the physiological interpretation of in vitro T_m data gained from topologically open molecules has to be taken with a grain of salt because cellular DNA is in a topologically closed conformation, and denaturation will not result in two independent single-stranded molecules, but in a random-coil structure with intertwined strands (Marguet and Forterre, 2001). As a result, topologically closed DNA is undoubtedly more resistant to denaturation than open DNA. It was postulated that the introduction of positive supercoils into closed DNA, which is catalyzed by reverse gyrases from hyperthermophiles, specifically stabilizes the double helix and keeps it in a functional state at high temperature (Forterre et al., 1996; Lopez-Garcia and Forterre, 1997, 2000). However, the hyperthermophile *Thermotoga maritima* contains both "normal" and reverse gyrases and propagates negatively supercoiled plasmid DNA (Guipaud et al., 1997).

tRNA molecules are not permanently integrated into larger macromolecular complexes. Therefore, in adapting to high temperatures, they must have developed mechanisms for intrinsic stabilization. Part of the stabilization energy may originate from an increased G+C content. However, unfractionated tRNA from the hyperthermophiles *Pyrococcus furiosus* and *Pyrodictium occultum* showed T_m values around 100°C, too high to be attributable to the measured G+C content (Kowalak et al., 1994). An early investigation identified a broad variety of covalent posttranscriptional modifications in nucleosides from tRNA preparations of thermophiles and hyperthermophiles, six of which were structurally novel in showing alterations of their bases as well as methylation of their ribose moiety (Edmonds et al., 1991). Altogether, 23 modified nucleosides were identified in *Pyrococcus furiosus*; three of them (Fig. 7) not only

A

n=165 genera

16S rRNA stems G+C content %

n=51 genera

tRNA G+C content %

n=38 genera

23S rRNA stems G+C content %

n=71 genera

5S RNA G+C content %

Optimal growth temperature °C

B

n=224 genera

Genomic G+C content %

Optimal growth temperature °C

Fig. 6. G+C contents of A) various RNAs and B) genomic DNAs plotted against optimal growth temperatures. Data taken from Galtier and Lobry (1997).

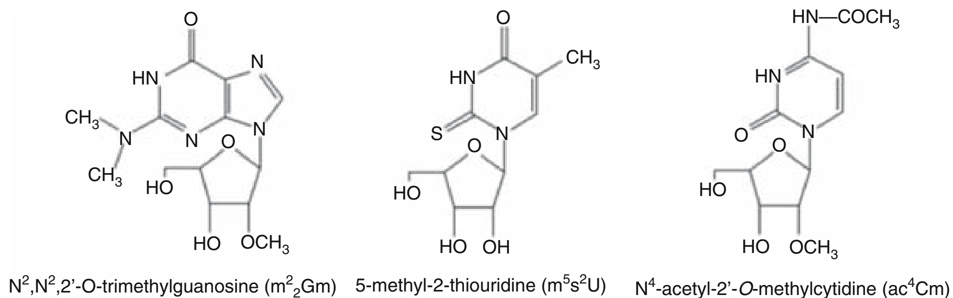


Fig. 7. Modified nucleosides implicated in the stabilization of hyperthermophile tRNA. From Daniel and Cowan (2000).

exhibited enhanced relative abundance with increasing growth temperature, but also higher stability, which they effected by 1) restricting the conformational flexibility of the ribose ring, 2) favoring the A-type helix, and 3) preventing phosphodiester-bond hydrolysis (Inoue et al., 1987; Kawai et al., 1992; Kowalak et al., 1994; Cummins et al., 1995).

Apparently, the protecting effect of posttranscriptional tRNA modification is not restricted to the archaea: both the level of 5-methyl-2-thiouridine and the T_m value of tRNA from the bacterium *Thermus thermophilus* show a significant increase with increasing growth temperature (Watanabe et al., 1976). The effect becomes even more compelling if tRNAs from psychrophiles are included in the comparison. While the abundance and the variety of posttranscriptional tRNA modifications are more pronounced in thermophiles and hyperthermophiles than in mesophiles, significantly less modifications are found in tRNAs from psychrophiles (Dalluge et al., 1997). The most abundant one is dihydrouridine, whose nonplanar base resists stacking, this way decreasing stability. In addition, dihydrouridine favors the C-2′-endo sugar conformation, which is less rigid than the C-3′-endo conformer (Yokoyama et al., 1981). Obviously, enhanced flexibility is essential for optimal functioning at low temperature, whereas high intrinsic stability has lower priority.

In the case of rRNAs, significant stabilization is provided by their conjugation with proteins within the ribosomal complex. In accordance with this argument, the levels of posttranscriptional modifications of rRNAs are much lower than in tRNAs, both in mesophiles and in thermophiles. Still, rRNA modifications are much more abundant in *Sulfolobus solfataricus* than in *Escherichia coli*, and the level of stabilizing ribose O-2′ methylations significantly increases with the culture temperature of the hyperthermophile (Noon et al., 1998).

Mechanisms to Avoid and Repair Chemical Damage of Nucleotides

Chemical damage of nucleic acids by hydrolytic attack close to the boiling point of water is an enormous potential threat for hyperthermophiles. The most common damages to DNA are 1) base deamination, 2) loss of bases from one strand with apurinic or apyrimidinic sites as final products, and 3) hydrolytic cleavage of phosphodiester bonds. It was suggested that, above 100°C, DNA would be subject to a ca. 3000-fold increase in the levels of deamination and depurination compared with DNA at 37°C (Lindahl, 1993). Furthermore, it was estimated from in vitro stability data that under the physiological conditions of *S. solfataricus* (intracellular pH 6, 80°C) two apurinic sites per gene per cell division would accumulate (Grogan, 1998). The most severe damage of nucleic acids is the hydrolytic cleavage of the backbone phosphodiester bond. For this reaction, it was postulated that the preceding depurination at an adjacent site is the rate-limiting step (Marguet and Forterre, 1998; Marguet and Forterre, 2001). In contrast, for RNA, hydrolytic strand breakage is not coupled to depurination; instead it occurs via the direct attack of the phosphodiester bond by the ribose 2′-OH oxygen. In vitro, at around 100°C, single-strand breaks occur at a high rate (Marguet and Forterre, 1994; Grogan, 1998). The corresponding lesions could lead to lethal double-strand breaks, if not prevented or repaired in vivo. Therefore, it was suggested that hyperthermophiles must have evolved highly efficient mechanisms to protect and/or repair their DNA (Grogan, 1998). In support of this hypothesis, when *Pyrococcus furiosus* cells are exposed to 100°C, their DNA is about 20 times more resistant to breakage than DNA from *Escherichia. coli* at the same temperature (Peak et al., 1995). Furthermore, passive protection of DNA might be provided by similar mechanisms as used to increase the T_m of the DNA double helix, i.e., high salt concentrations, and binding to proteins (see above). Indeed, it has been shown that the presence of Mg^{2+} and K^+ protect double-stranded DNA from depurination, probably by directly stabilizing the N-glycosidic bond between the deoxyribose and the base (Marguet and Forterre, 1998). With respect to the formation of nucleoprotein complexes, archaeal histones are known to protect plasmid DNA against radiation (Isabelle et al., 1993).

In spite of these well-established protection mechanisms, DNA in hyperthermophiles will almost certainly be damaged to a larger extent than DNA in mesophiles. A model organism for comparative research in this context is the radiation-resistant bacterium *Deinococcus radiodurans*. Both, γ-irradiation and heat have been shown to induce double-strand breakage of DNA, which can be repaired efficiently by *D. radiodurans*. This capacity derives from multiple copies of its chromosome providing intact copies for repair by a DNA recombinase (Minton and Daly, 1995). In analogy, the chromosome of the archaeon *Pyrococcus furiosus*, after irradiation-induced fragmentation, was reassembled by the cells upon incubation at 95°C (di Ruggiero et al., 1997). Open reading frames encoding homologues of RecA proteins involved in recombination repair in bacteria and eukarya were found in the archaeal genomes sequenced so far. Strong experimental evidence suggests that at least one of these homologs, FEN-1 from *P. furiosus*, is involved in double-strand breakage repair

(di Ruggiero et al., 1999). Other than double-strand breakage-repair, activities have been demonstrated in vitro for several other archaea. For example, *Methanobacterium thermoautotrophicum* is able to remove ultraviolet light-induced photoproducts, supposedly with a photolyase as catalyst (Kiener et al., 1989; Ögrünc et al., 1998). Furthermore, a T/G-selective DNA thymine N-glycosylase takes care of the mutagenic effect of hydrolytic 5-methylcytosine deamination (Horst and Fritz, 1996), while uracil-DNA glycosylases seem to be involved in the repair of cytosine deamination (Koulis et al., 1996); in addition, O_6-alkylguanine-DNA transferase activities were also found in hyperthermophiles (Skorvaga et al., 1998). On the other hand, MutL and MutS, which are used in all bacterial and eukaryal mismatch-repair systems, have not been found in any of the archaeal genomes so far.

In summary, the present knowledge of the specific mechanisms by which hyperthermophilic microorganisms preserve the integrity of their genetic material is still incomplete. More information is needed about the intracellular salt concentrations and the DNA-binding and DNA-protecting proteins, to establish in vitro test systems that come as close as possible to the in vivo situation. Moreover, homologs of known bacterial and eukaryal repair enzymes from hyperthermophiles need to be characterized to identify their catalytic properties under physiological conditions. The ongoing genome-sequencing projects will help identify the most promising candidates for this approach.

Adaptive Stabilization Mechanisms of Lipids and Membranes

Living cells have a cytoplasmic membrane serving as a barrier between the cytoplasm and the environment. It consists of lipid layers with embedded proteins that generate specific and vital solute concentration gradients across the membrane. Penetration of small solutes through the lipid component of the membrane is caused either by active transport or passive diffusion. Being directly proportional to the thermal energy (kT), passive diffusion is accelerated with increasing temperature (Einstein, 1905; Einstein, 1906; van de Vossenberg et al., 1998). In hyperthermophiles, extreme temperature may lead to the breakdown of solute gradients. Therefore, their membranes need to be extremely thermostable, but they also require specific adaptive mechanisms to limit the permeability of ions. This holds especially for protons because of the essential role of proton gradients in energy-requiring processes such as ATP synthesis, active transport of specific solutes across the membrane, flagellar rotation, and maintenance of the intracellular pH and turgor (Albers et al., 2000).

Chemical Composition of Membrane Lipids

At physiological temperatures, membrane lipids are in a liquid-crystalline state (Melchior, 1982), forming a suitable matrix for the attachment or integration of membrane proteins. The overall structure of the lipid membrane is conserved between eukarya, bacteria and archaea. The inner and outer hydrophilic surfaces, which are composed of polar headgroups, enclose the hydrophobic interior consisting of long hydrophobic hydrocarbon chains. At this point, the chemical composition of archaeal membranes has been found to be significantly different from the chemical composition of bacterial and eukaryal membranes. Both bacterial and eukaryal lipids have esters between glycerol and fatty acid chains (glycerol fatty acyl diesters), whereas the lipids of archaeal membranes are formed by ethers between glycerol (or another alcohol such as nonitol) and branched C_{20}-hydrocarbon side chains (Langworthy and Pond, 1986). The side chains consist of repeated saturated isoprenoid units containing a methyl side group at every fourth carbon atom in the backbone. These methyl side groups restrict the mobility of the chains, thereby stabilizing them and restricting ion permeability (see below). The two hydrocarbon chains can be ether-linked to either one glycerol unit (forming a C_{20}, C_{20}-isopranyl glycerol diether = diphytanylglycerol diether = archaeol), or two glycerol units (forming a dibiphytanylglycerol tetraether = caldarchaeol; Fig. 8A and B). The archaeols are found in all archaea, whereas the caldarchaeols (and nonitol-caldarchaeols) are only found in thermophilic archaea. The caldarchaeols can be further modified by cyclopentane rings in the biphytanyl side chains (Fig. 8C–E).

The caldarchaeols of thermophilic archaea are typically glycosylated at C_3 and C_6 of the glycerol and nonitol backbones, respectively. Probably, hydrogen bonds between the glycosyl headgroups stabilize the membrane structure by reducing lateral lipid mobility (van de Vossenberg et al., 1998; Daniel and Cowan, 2000).

An unsaturated diether lipid was found in the archaeon *Methanopyrus kandleri* (Hafenbradl et al., 1993). This lipid, 2,3-di-*O*-geranylgeranyl-*sn*-glycerol, resembles terpenoids, but the consequences for membrane function are still unknown. Another type of unsaturated lipid was discovered in the psychrophilic archaeon *Methanococcoides burtonii* (Nichols and Franzmann, 1992). This lipid contains a double bond that can distort the short-range order of the membrane, thus allowing the necessary fluidity of the mem-

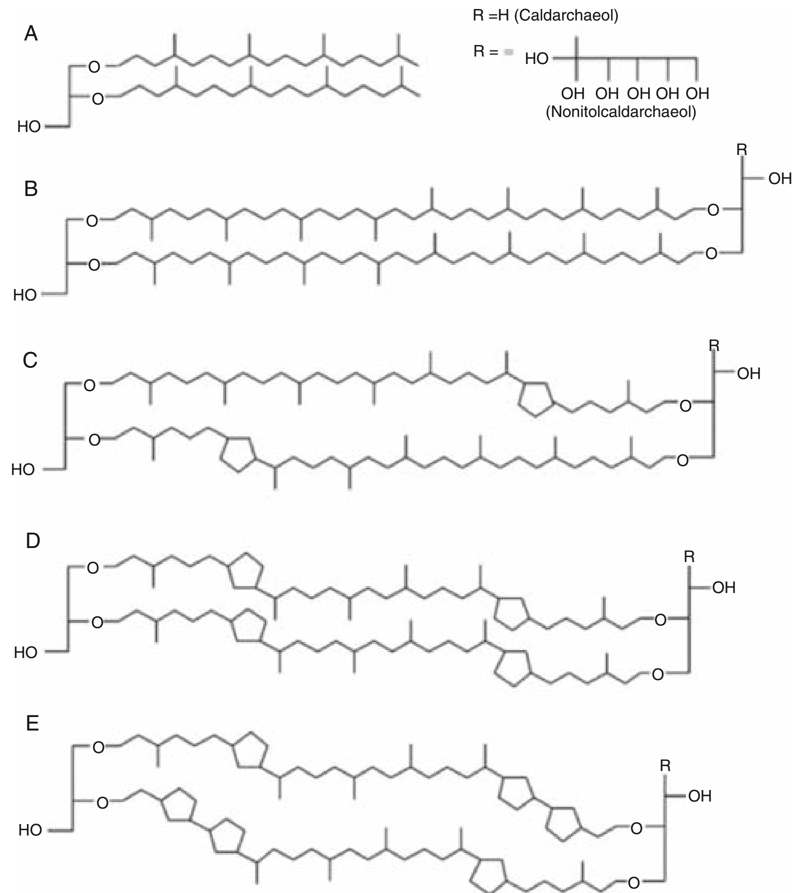


Fig. 8. Archaeal lipid architecture. (A) Diphytanyl glycerol diethers, (B) dibiphytanyl diglycerol tetraethers, and (C–E) internal cyclization in dibiphytanyl diglycerol tetraethers. From Daniel and Cowan (2000).

brane to be adapted to the physiological low temperature (Suutari and Laakso, 1992).

Topology, Stability and Permeability of Membranes

The glycerol-diester lipids of bacteria and eukarya form bilayer membranes. The same holds for the archaeol lipids of halobacteria and most other archaea growing under moderate conditions (Kates et al., 1993; Upasani et al., 1994; Kates, 1995). In contrast, the caldarchaeol lipids of the thermophilic and acidophilic archaea form monolayers spanning the entire membrane (de Rosa et al., 1991; Relini et al., 1996). In monolayers, two glycerol units are covalently linked by the phytanyl side chains, whereas in bilayers the glycerol units are noncovalently linked by hydrophobic interactions between the fatty acid side chains. As a consequence, monolayers have a diameter between 2.5 and 3.0 nm (Gliozzi et al., 1983), somewhat thinner than typical C_{18} glycerol-diester bilayers, but much more stable: Vesicles generated from *Thermoplasma acidophilum* ether lipids are more resistant to high temperature and surface-active agents than vesicles of bacterial dipalmitoyl phosphatidyl-choline (Ring et al., 1986). Moreover, liposomes prepared from tetraether lipids from a number of archaea were shown to be extremely stable toward high temperature, alkaline pH and enzymatic degradation by phospholipases (Chang, 1994; Choquet et al., 1994).

As has been mentioned, to guarantee energy production, membranes of all microorganisms, no matter whether they are psychro-, meso-, thermo- or hyperthermophilic, must provide an efficient barrier against the flux of protons. Liposomes prepared from lipids derived from a variety of organisms with different growth temperatures were compared for their proton permeabilities (van de Vossenberg et al., 1995). This study showed that, at the respective growth temperature, proton permeability was closely similar for the various liposomes (Fig. 9).

This “homeoproton permeability adaptation” is reminiscent of the “corresponding states” observed for homologous pairs of enzymes from mesophiles and thermophiles, most of which were shown to exhibit comparable stabilities, flexibilities and activities at their respective physiological temperatures (Jaenicke, 1991b; Somero, 1995; Jaenicke and Böhm, 1998). As a logical consequence, at a given fixed temperature, the proton permeability of membranes is decreased with increasing temperature of adaptation, fol-

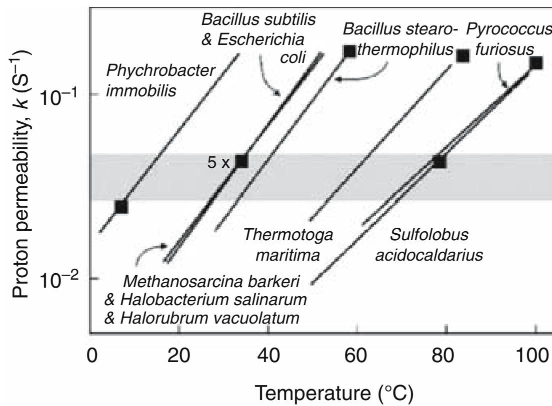


Fig. 9. The proton permeability of archaea and bacteria that live at different temperatures. At the respective growth temperatures, the proton permeability falls within a narrow range (gray bar). *Thermotoga maritima* and *Bacillus stearothermophilus* have higher permeabilities than those of other organisms. Both organisms overcome this problem differently. From Albers et al. (2000).

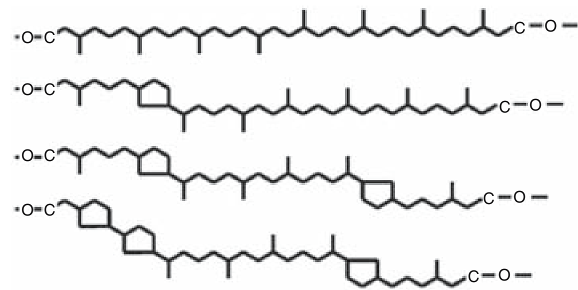


Fig. 10. Cyclization of the phytanyl chains of the *S. solfataricus* tetraether lipids. Only one of the phytanyl side chains is shown. The degree of cyclization increases from top to bottom. From Albers et al. (2000).

lowing the order: psychrophiles > mesophiles > thermophiles > hyperthermophiles. Various archaeol and caldarchaeol lipids were 6–120-fold less permeable to water, solutes, protons and ammonia than bacterial diphytanyl-phosphatidylcholine liposomes (Mathai et al., 2001). It was shown that the crucial factor ensuring low permeability are cyclopentane rings in the phytanyl side chains, which limit the mobility in the midplane hydrocarbon region. The substutitution of ether- for ester-bonds provides an additional barrier that specifically impairs the flux of protons.

Bacterial thermophiles have membrane lipids rich in saturated fatty acids, which make the membranes more rigid and stable at high temperatures because stronger hydrophobic interactions are formed between saturated fatty acids compared with unsaturated ones (Brock, 2000). Other differences between membranes from mesophilic and thermophilic bacteria include alterations in acyl chain length, branching, and/or cyclization (Tolner et al., 1998). Interestingly, the extremely thermophilic *Thermodesulfobacterium* contains lipids combining bacterial and archaeal properties; here, glycerol is ether-linked to a unique C_{17} hydrocarbon side chain along with some fatty acids instead of phytanyl side chains (Brock, 2000).

Adaptation of Membrane Structure and Function to Temperature Fluctuations

Bacteria and archaea can grow over a wide range of temperatures. When facing environmental temperature shifts, most of them adapt the structure of their membranes to ensure constant stability and permeability. In archaea, as well as in mesophilic and psychrophilic bacteria, this adaptation is achieved by adjusting the chemical composition of the lipids. Archaea adapt to low temperatures by decreasing the degree of saturation of their hydrocarbon side chains (Nichols and Franzmann, 1992), whereas they respond to high temperature by the cyclization of the side chains and by replacing diether to with tetraether lipids (de Rosa and Giambacorta, 1988; de Rosa et al., 1991; Yamauchi and Kinoshita, 1995): For *Sulfolobus solfataricus* and *Thermoplasma*, it was shown that the number of cyclopentane rings incorporated into the lipid diphytanyl side chains increase with growth temperature, this way rigidifying the membrane and limiting passive diffusion of small molecules (Mathai et al., 2001; Fig. 10).

In *Methanococcus jannaschii*, a different mechanism is observed: here, increasing temperatures induce the change from diether lipids to the more thermostable tetraether lipids (Sprott et al., 1991).

To investigate adaptive changes of membranes from bacteria, *Bacillus subtilis* was grown at the boundaries of its growth temperature (van de Vossenberg et al., 1999). The average lengths of lipid acyl side chains, the degree of saturation, and the ratio of *iso*- and *anteiso*-branched fatty acids increased with temperature. In accordance with the concept of homeoproton permeability adaptation, these modifications kept the proton permeability of the cytoplasmic membrane at a rather constant level. Likewise, in psychrophiles, the proton permeability is maintained at a constant level when the growth temperature is varied (van de Vossenberg et al., 1995). In contrast, in thermophilic bacteria such as *Bacillus stearothermophilus* and *Thermotoga maritima*, homeoproton permeability cannot be maintained, as their membranes become porous at high temperatures. Some moderately thermophilic bacteria can compensate for the high proton leakage by drastically increasing the respiration rate, and together with that, the rate of proton pumping (de Vrij et al.,

1988). A different strategy is found in the moderate thermophile *Caloramator fervidus*, which, instead of the proton, uses the less permeable sodium ion as the main coupling component for energy transduction (Speelmans et al., 1993a; Speelmans et al., 1993b).

In summary, a number of different mechanisms have been identified that keep membranes stable and functional at high temperatures. Archaea contain lipids with ether linkages between various alcohols and hydrocarbon side chains, in which cyclopentane rings are incorporated in a growth-temperature dependent manner. Thermophilic bacteria, which contain less stable ester lipids prone to proton leakage, evolved alternative strategies to maintain vital chemiosmotic gradients under physiological conditions. As the number of novel lipid structures constantly grows, more variations on these themes are to be expected.

Adaptive Stabilization Mechanisms of Proteins

To fulfil their diverse functions, proteins from hyperthermophiles need to be in their native, folded state at temperatures around 100°C. In contrast, most proteins from mesophiles are unfolded at ~50°C (Fig. 2), often followed by irreversible aggregation and/or chemical damage (Jaenicke and Seckler, 1997). What are the structural determinants that render proteins from hyperthermophiles much more thermostable than their homologs from mesophiles? As mentioned in the section on "Stability of Biomolecules," few additional favorable electrostatic or hydrophobic interactions suffice to shift ΔG_{stab} of a protein from the mesophilic to the thermophilic temperature regime (Jaenicke and Böhm, 2001; Fig. 3B). In addition, proteins from hyperthermophiles are not only stabilized intrinsically, but also by extrinsic factors such as compatible solutes or molecular chaperones. What follows briefly summarizes our current knowledge of the intrinsic and extrinsic stabilization of hyperthermophilic proteins. For further details see (Jaenicke and Böhm, 2001; Petsko, 2001; Sterner and Liebl, 2001; Vieille and Zeikus, 2001).

Intrinsic Stabilization: There Are No General Rules

In the section on "Stability of Biomolecules," the electrostatic and hydrophobic interactions that stabilize proteins were discussed. Moreover, the contributions of enthalpy and entropy to the free energy gain caused by these interactions was pointed out. Pairwise comparisons of amino acid sequences and X-ray structures of homologous proteins from mesophiles, thermophiles and hyperthermophiles showed that one or more of these stabilizing interactions were more frequent or more pronounced in the thermophilic and hyperthermophilic variants. These additional stabilizing interactions can in principle occur at all levels, from primary to the quaternary structure (Jaenicke and Böhm, 1998; Daniel and Cowan, 2000; Vieille and Zeikus, 2001; Sterner and Liebl, 2001; Yano and Poulos, 2003).

A large number of mutational studies have been performed to identify stabilizing interactions, which were frequently detected in hyperthermophilic proteins. To this end, selected amino acid residues were substituted by site-directed mutagenesis, and the resulting changes in stability were measured. Instructive examples are the enzymes phosphoribosylanthranilate isomerase (PRAI) and indoleglycerol phosphate synthase (IGPS), which catalyze two successive reactions within tryptophan biosynthesis and adopt the frequently encountered $(\beta\alpha)_8$-barrel fold (Höcker et al., 2001; Wierenga, 2001). PRAI is monomeric in most mesophiles but dimeric in *Thermotoga maritima* (Sterner et al., 1996). The two identical monomers of *Thermotoga maritima* PRAI are associated via intimate hydrophobic contacts at the N-terminal faces of their central β-barrels (Hennig et al., 1997). By replacing a Phe residue at the monomer-monomer interface of *T. maritima* PRAI by a Glu residue, the hydrophobic interactions are weakened. As a consequence, the enzyme becomes monomeric and thermolabile, without losing its catalytic activity (Thoma et al., 2000; Fig. 11).

The importance of increased association states for increased thermostability was also shown for ornithine carbamoyltransferase, which consists of four trimers in *Pyrococcus furiosus*, but only one in mesophiles. Gradual dissociation of dodecameric ornithine carbamoyltransferase from *Pyrococcus furiosus* into trimers, as induced by site-directed mutagenesis at subunit interfaces, led to a gradual decrease in thermal stability (Clantin et al., 2001). Indoleglycerol phosphate synthase is monomeric both in mesophiles and hyperthermophiles. However, IGPS from *Sulfolobus solfataricus* and *T. maritima* contain twice the number of potentially stabilizing ion pairs compared with *E. coli* (Hennig et al., 1995; Merz et al., 1999). Two *T. maritima* IGPS variants, which had one of these ion pairs disrupted by site-directed mutagenesis, showed significantly decreased thermostabilities (Merz et al., 1999). The stabilizing role of ion pairs was also proven by site-directed mutagenesis experiments performed with glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) from *T. maritima*, glutamate dehydrogenases from both *Thermococcus litoralis* and *P. furiosus*,

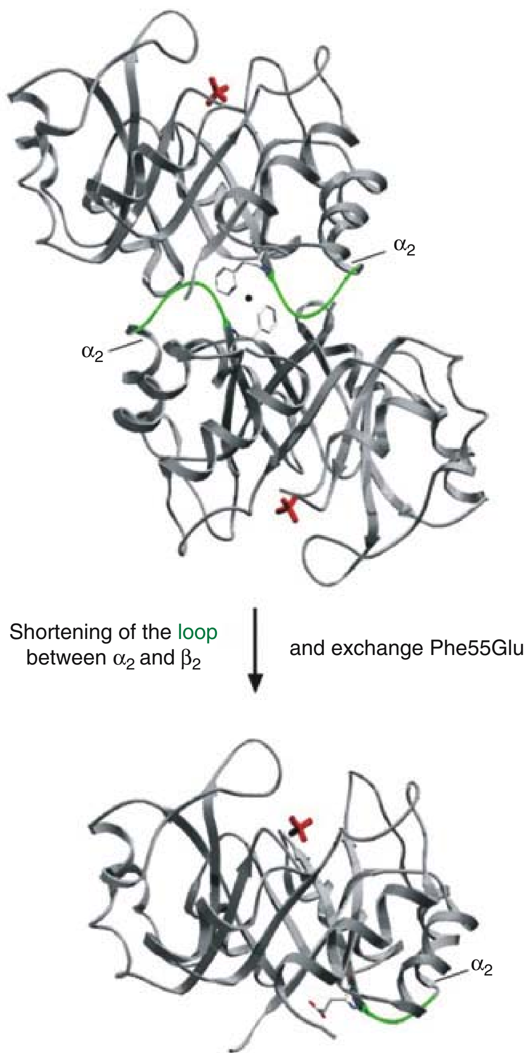


Fig. 11. Monomerization of the native homodimer of PRAI from *Thermotoga maritima* by rational design. Monomers were generated by shortening of the loops connecting helices α2 with strands β3 (in green), and by replacing the two Phe55 residues located close to the twofold symmetry axis (shown as a black dot) with glutamates (shown in stick format). The bound phosphate ions (red tetrahedrons) identify the active sites. The monomeric variants are catalytically as active as the dimer, but far more thermolabile. From Höcker et al. (2001), with permission.

3-isopropyl-malate dehydrogenase from *Thermus thermophilus*, rubredoxin from *P. furiosus*, and the archaeal histone from *Methanothermus fervidus*, and citrate synthase from psychrophiles to hyperthermophiles. The latter represents a good example for the whole spectrum of adaptive changes, including intersubunit ionic networks and varying states of association (Wrba et al., 1990; Tomschy et al., 1994; Pappenberger et al., 1997; Vetriani et al., 1998; Li et al., 2000; Nemeth et al., 2000; Strop and Mayo, 2000; Nordberg Karlsson et al., 2002, 2003; Bell et al., 2002, and references therein). The increased $\Delta G_{stab,70°C}$ of the hyperthermophilic cold shock protein from *T. maritima* compared to its mesophilic counterpart from *B. subtilis* was shown to be largely due to Arg3, whose positive charge improves the global electrostatic potential of the protein (Perl and Schmid, 2001; cf. the section on "Stability of Biomolecules"). This result suggests that the optimum placement of charged groups on the surface of a protein is crucial for its thermostability (Xiao and Honig, 1999), a hypothesis that is strengthened by a number of other experimental studies (Grimsley et al., 1999; Loladze et al., 1999; Spector et al., 2000).

In spite of these examples, in many cases the predicted stabilizing interactions (as deduced from pairwise mesophile-thermophile comparisons of sequences and structures) could not be verified experimentally. Therefore, it is still not possible to deduce general mechanisms that would lead to high protein thermostability. The reason for this shortcoming is the large number of neutral changes of amino acid residues and 3D structures that have accumulated during evolution without affecting protein stability (Böhm and Jaenicke, 1994; Arnold et al., 2001b). Based on this argument, large-scale structural comparisons of amino acid sequences and 3D structures, which reduce the large "phylogenetic noise," are likely to provide more significant results. Such systematic comparisons are now possible owing to the growing number of complete genome sequences from mesophiles and hyperthermophiles, and the fast rate with which new X-ray structures become available.

The amino acid compositions of a number of mesophiles and thermophiles were deduced from their genome sequences and compared in several systematic studies (Table 4).

These comparisons allow the following conclusions. Hyperthermophilic proteins 1) contain a decreased content of uncharged polar amino acids, this way avoiding deamidation of Gln and Asn catalyzed by Thr and Ser (Wright, 1991; Haney et al., 1999; cf. the section on "Biochemical Limitations at High Temperature"), 2) show an increased content of the charged amino acids Glu and Asp, a significant fraction of which may be involved in stabilizing ion pairs at the protein surface (see above; Haney et al., 1999; Cambillau and Claverie, 2000), and 3) are on avarage significantly smaller than their mesophilic homologs (Chakravarty and Varadarajan, 2000), presumably owing to shorter solvent-exposed surface loops (Thompson and Eisenberg, 1999) or extensions at the N- and/or C-terminal ends (Fig. 12).

Upon unfolding, small proteins show a smaller heat capacity change (ΔC_p) than large proteins (Murphy and Freire, 1992; Myers et al., 1995); a decrease in ΔC_p flattens the ΔG_{stab} versus T profile and leads to an increase in T_m (Fig. 3B).

The three-dimensional structures of proteins from mesophiles and thermophiles were compared in a number of comprehensive studies. From a non-redundant dataset of high-quality X-ray structures of protein subunits from mesophiles, thermophiles and hyperthermophiles, it revealed that the increase in intrinsic stability was paralleled by more ion pairs (apart from slight differences with respect to cavities), hydrogen bonds, secondary structure content and polarity of surfaces (Szilagyi and Závodszky, 2000; Table 5).

A similar study suggested that ion pairs and side chain-side chain hydrogen bonds are more frequent in thermophilic than in mesophilic proteins (Kumar et al., 2000a; 2000b). There was no evidence for significant differences with respect to compactness, hydrophobicity, polar and nonpolar surface area, protein size, and number of Pro residues in loops; however, thermophilic proteins appeared to have a higher fraction of residues in α-helices.

Two further investigations confirmed that the α-helices of thermophilic proteins show increased stability, mainly due to the higher intrinsic helical propensities of the amino acids involved (Petukhov et al., 1997; Facchiano et al., 1998). Two systematic comparisons of lactate dehydrogenases (LDH) and triosephosphate isomerases (TIM) from psychrophiles, mesophiles and hyperthermophiles revealed positive correlations between thermostability and the number of intra-subunit (LDH) and inter-subunit ion pairs (TIM), respectively (Auerbach et al., 1998; Maes et al., 1999).

Table 4. Change in amino acid composition going from proteins of mesophiles to proteins of thermophiles.

Amino acid	Gains	Losses	Ratio	Net change	Change, %
Ile	842	658	1.28	184	9.5
Glu	739	562	1.31	177	9.1
Arg	383	214	1.79	169	16.5
Lys	789	620	1.27	169	8.3
Pro	167	96	1.74	71	7.0
Tyr	224	177	1.27	47	5.8
Ala	504	458	1.10	46	2.8
Trp	23	11	2.09	12	8.3
Leu	560	548	1.02	12	0.6
Cys	72	69	1.04	3	0.9
Phe	200	202	0.99	–2	–0.3
Asp	429	432	0.99	–3	–0.2
Val	666	670	0.99	–4	–0.2
His	80	92	0.87	–12	–2.8
Gly	201	264	0.76	–63	–3.4
Met	174	248	0.70	–74	–11.3
Gln	158	234	0.68	–76	–13.1
Thr	336	431	0.78	–95	–8.4
Asn	313	481	0.65	–168	–15.9
Ser	271	664	0.41	–393	–31.7

Abbreviations: Ile, isoleucine; Glu, glutamic acid; Arg, arginine; Lys, lysine; Pro, proline; Tyr, tyrosine; Ala, alanine; Trp, tryptophan; Leu, leucine; Cys, cysteine; Phe, phenylalanine; Asp, aspartic acid; Val, valine; His, histidine; Gly, glycine; Met, methionine; Gln, glutamine; Asn, asparagine; Ser, serine.
Data from Haney et al. (1999).

The results of the cited mutational studies, and those of the systematic and comprehensive comparisons between the amino acid sequences and 3D structures of psychrophilic, mesophilic and thermophilic proteins can be summarized as follows: Owing to the small differences between ΔG_{stab} of hyperthermophilic and mesophilic proteins (Matthews, 1993; Matthews, 1996; Jaenicke and Böhm, 1998), attempts to find a unifying set of rules of stabilization must fail. The structural features that characterize some of the known hyperthermophilic proteins are increased numbers of hydrogen bonds, higher packing densities and α-helical contents, improved hydrophobic interactions, optimized surface areas, decreased volumes, fewer cavities, and a shortening of the polypeptide chains. Attempts to define the relative significance of these many different factors by counting their frequency in comprehensive comparative studies led to four major contributions: 1) stabilized α-helices, 2) decreased entropy of the unfolded state by increased numbers of Pro and β-branched amino acid residues, 3) decreased content of chemically labile polar amino acid residues, and in particular, 4) increase in the number of optimized ionic interactions (Sanchez-Ruiz and Makhatadze, 2001). The latter finding is in accordance with theoretical work suggesting ion pairs are more stabilizing at high than at low temperatures and might therefore be crucial for the stability of hyperthermophilic proteins (Elcock and McCammon, 1997; Elcock, 1998; De Bakker et al., 1999). It is important to note that the stabilizing effect of a given ion pair depends on its structural context. Ion pairs that connect N- and C-termini in IGPS and GAPDH from *T. maritima* contribute significantly to thermostability, probably by preventing the fraying of the N- and C-termini, which might initiate thermal denaturation (Pappenberger et al., 1997; Merz et al., 1999). Also, for entropic reasons, clusters of ion pairs are likely to be more stabilizing than individual ion pairs (Yip et al., 1995; Yip et al., 1998).

Although our knowledge of the structural basis of high intrinsic protein thermostability is still incomplete, considerable operational progress has been achieved in the last years, especially in the first successful examples of rational or semi-empirical improvements of protein thermostability (Malakauskas and Mayo, 1998; van den Burg et al., 1998). An alternative approach to improve protein thermostability is "directed molecular evolution" (Wintrode and Arnold, 2000; Arnold et al., 2001b). It mimics the

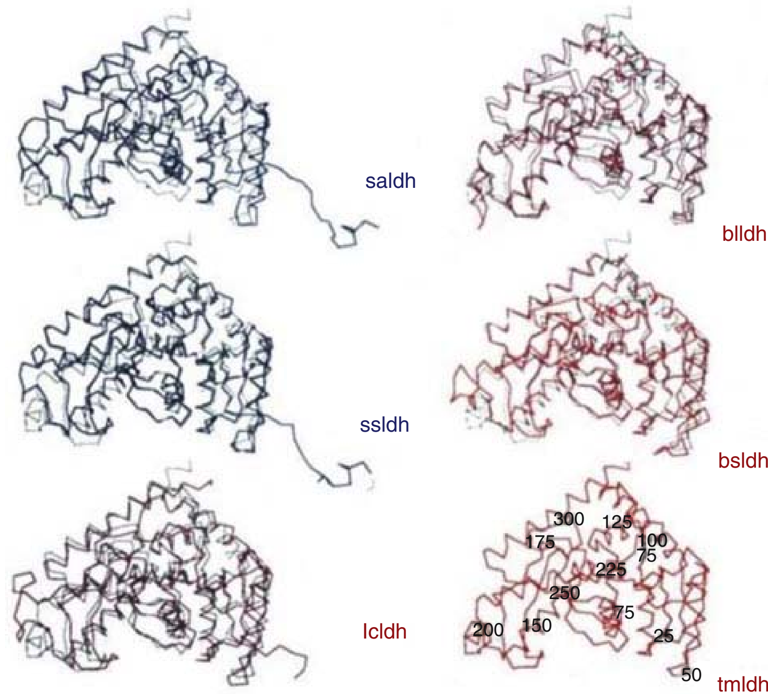


Fig. 12. The three-dimensional structures of lactate dehydrogenases (ldh) from hyperthermophiles, on the one hand, and mesophiles as well as a cold-blooded fish, on the other, are practically isomorphous, with root-mean-square (r.m.s.) differences below 2.4 Å. Comparisons of the 2–3 Å resolution crystal structures of the various homologs with the enzyme from *Thermotoga maritima* (as the reference; in gray) show that the increase in thermostability is parallelled 1) by a reduction in the length of the C-terminal extension, 2) by an increase in compactness of the tetrameric assembly, and 3) by the presence of an additional "thermohelix" (αT) in each of the subunits of the hyperthermophile enzyme. The shift from blue to red in the figure characterizes the temperature range of the organisms from which the various enzymes were isolated The corresponding abbreviations and physiological T_{opt}-values refer to: sa, *Squalus acanthias* (dogfish, ~10°C); ss, *Sus scrofa* (pig, 37°C); lc, *Lactobacillus casei* (~30°C); bl, *Bifidobacterium longum* (~40°C); bs, *Bacillus stearothermophilus* (~65°C) and tm, *Thermotoga maritima* (~80°C). For details, see Auerbach et al. (1998).

natural evolution process by applying iterative rounds of random mutagenesis and selection (or screening) of stabilized protein variants. Given an appropriate selection or screening system, this approach is generally applicable because it does not require specific knowledge of the structure of the protein to be stabilized. Moreover, directed evolution is instructive because it is unbiased and may provide stabilizing amino acid exchanges at positions in the protein that were

Table 5. Systematic comparison of the structures of proteins from mesophiles, thermophiles and extreme thermophiles.

Property		Correlation with temperature	Change in proteins from moderate thermophiles	Change in proteins from extreme thermophiles
Cavities	Number	↓↓	0	↓↓↓
	Volume	↓	↑	↓
	Area	↓	↑	↓↓
Hydrogen bonds	Number	0	0	0
	Unsatisfied	↓	↓	↓
Ion pairs	<4.0 Å	↑↑	↑	↑↑↑
	<6.0 Å	↑↑	↑↑	↑↑↑
	<8.0 Å	↑↑↑	↑↑↑	↑↑↑
Secondary structure	α	0	↑	0
	β	↑	0	↑↑
	Irregular	↓	↓	↓
Polarity of surfaces		↓↓	↑↑↑	0
	Exposed			
	Buried	0	↑	↑

The number of arrows (1, 2 or 3) shows whether the represented correlation or change is considered insignificant, moderately significant of highly significant.
From Szilagyi and Závodszky (2000).

not anticipated. Moreover, because wildtype and stabilized proteins differ only in few amino acids, the analysis of the observed effects is considerably simplified, compared with the analysis of the much more diverse homologous mesophilic-thermophilic protein pairs. Recent successful examples of stabilizing proteins by directed evolution were summarized in Arnold (2001a) and Sterner and Liebl (2001).

Extrinsic Stabilization by Accessory Compounds

It has been known for some time that the intrinsic stability of some proteins from hyperthermophiles is too low to allow their function in vivo (Thomm et al., 1986; Fabry and Hensel, 1987). These observations suggested that these proteins are stabilized by extrinsic factors such as metabolites or proteins. Many organisms accumulate high concentrations of organic solutes in response to various stress conditions. These solutes are called "compatible solutes" because they do not compromise cellular functions (Carpenter et al., 1993; da Costa et al., 1998). Low-molecular mass solutes in the aqueous environment of proteins can have various effects on protein solubility and stability. At low concentrations, salts can increase protein solubility (i.e., have a salting-in effect), whereas at high concentrations they can lead to protein precipitation (i.e., a salting-out of protein; cf. the section on "Water," subsection "Hydration"). Also, some solutes (e.g., urea or guanidinium chloride) destabilize proteins, whereas others (e.g., glycerol) have a stabilizing effect (Timasheff, 1995; Timasheff and Arakawa, 1997). Two conclusions follow from the fact that in all these cases high concentrations (usually >1 M) of the additives are required: 1) the intermolecular interactions involved must be nonspecific and weak, and 2) water (i.e., hydration) must play an important role, since the effect of a particular compound depends on the differential affinities of the protein and the additive for water (Timasheff, 1995). The precipitating and stabilizing compounds are preferentially excluded from the surface of the protein, that is, the protein has a higher affinity for water than for these agents. As a consequence, proteins are preferentially hydrated, which favors the native state and makes unfolding more unfavorable. In contrast, destabilizing agents bind more strongly than water to proteins. Stabilizing compounds include sugars and polyols (sucrose, trehalose, glycerol, mannitol and sorbitol), amino acids (proline and glycine), methyl amines (sarcosine, trimethylamine-*N*-oxide, and glycine betaine), tetrahydropyrimidine derivatives (ectoins), and some salting-out salts (Timasheff, 1995; Knapp et al., 1999).

In recent years, a number of compatible solutes have been found specifically in thermophiles and hyperthermophiles (Fig. 13). Some of them are likely to be adaptations to life at high temperatures: they are overproduced upon up-shifting the growth temperature of a given microorganism, and significantly increase the stability of a number of its proteins in in vitro measurements. For example, cyclic 2,3-diphosphoglycerate (cDPG) was discovered in hyperthermophilic methanogens (Hensel and König, 1988; Martins et al., 1997), and increasing growth temperature was found to increase its concentration in *Methanothermus fervidus*. The addition of cDPG drastically increased the in vitro stability of GAPDH from *M. fervidus*, but not that of the homologous enzyme from rabbit (Hensel and König, 1988). Various derivatives of *myo*-inositol phosphate were found in hyperthermophilic archaea and bacteria; a correlation between solute accumulation and growth temperature was detected in some cases (Ciulla et al., 1994; Martins and Santos, 1995; Martins et al., 1996; Martins et al., 1997; Ramakrishnan et al., 1997; Lamosa et al., 1998). Di-*myo*-inositol phosphate was found to stabilize GAPDH from *Pyrococcus woesei*, but sodium citrate had a similar effect (Scholz et al., 1992). Clearly, more information is required about the stabilization of proteins by *myo*-inositol phosphate derivatives. It is remarkable that a number of hyperthermophiles with a low salt requirement, e.g., *Thermotoga thermarum*, *Fervidobacterium islandicum*, *Pyrobaculum islandicum* or *Thermococcus zilligii* AN1, do not produce significant amounts of compatible solutes, either at their optimum growth temperatures or after a temperature up-shift (Martins et al., 1996; Lamosa et al., 1998). It was therefore postulated that some compatible solutes, which are produced by slightly halophilic thermophiles, might act when osmotic and temperature stress occur simultaneously (Lamosa et al., 1998).

MOLECULAR CHAPERONES As mentioned earlier, molecular chaperones are ubiquitous in all living cells (cf. to the section "Stability of Biomolecules"). As heat-shock proteins (HSPs) they regulate the kinetic partitioning between folding→association and misfolding→aggregation of polypeptide chains at elevated temperature (Bukau, 1999; Jaenicke and Lilie, 2000). "Thermosomes" in thermophilic and hyperthermophilic Archaea are double-ring cages with eight- to ninefold symmetry consisting of HSP60-subunits (Archibald et al., 1999). As shown for *Pyrodictium occultum*, at the upper temperature limit of viability they may be expressed to protein levels up to 80% of the total cellular protein, indicating that the chaperone is essential for survival under stress conditions (Phipps et al., 1991).

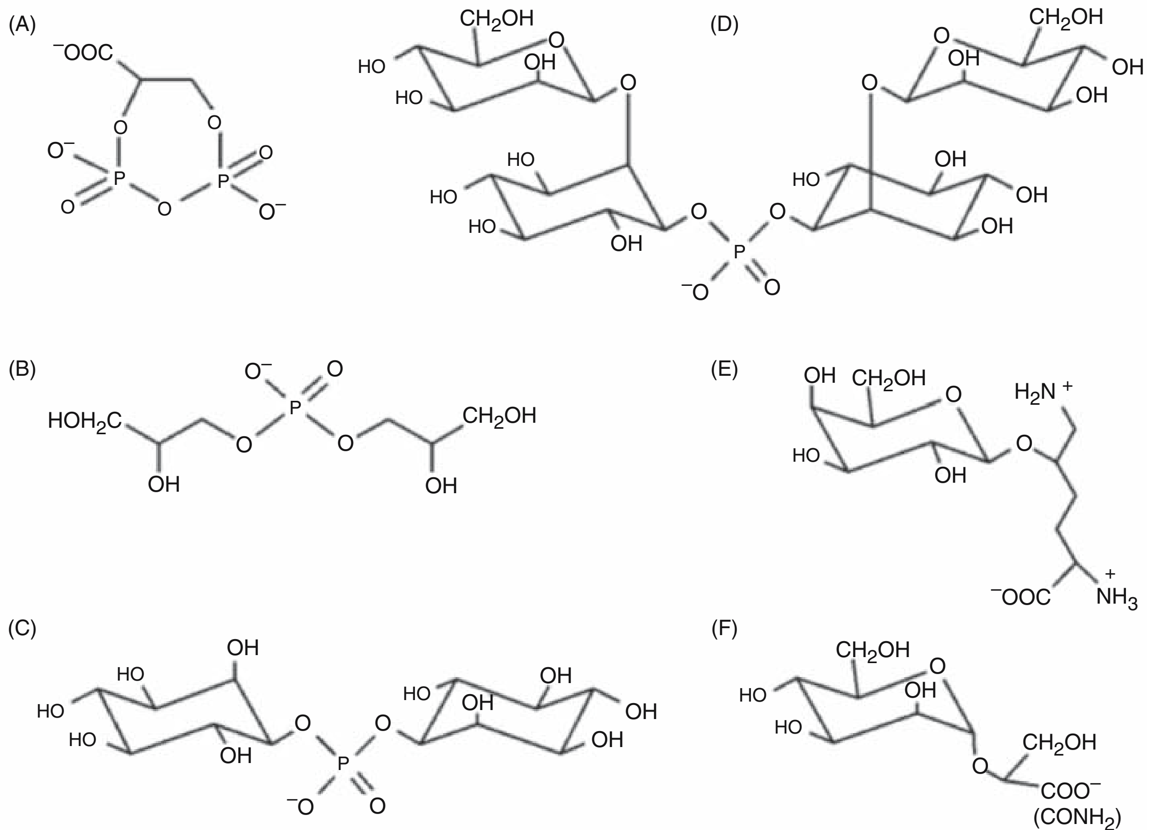


Fig. 13. Compatible solutes from hyperthermophiles. A) Cyclic 2,3-diphosphoglycerate, B) diglycerol phosphate, C) di-*myo*-inositol-1,1′-phosphate, D) di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′-phosphate, E) β-galactopyranosyl-5-hydroxylysine, and F) α-mannosylglycerate and α-mannosylglyceramide.

Similarly, the hyperthermophiles *Sulfolobus shibatae* and *Archaeoglobus fulgidus* display heat-induced synthesis of high chaperone levels (Kagawa et al., 1995; Emmerhoff et al., 1998). Apart from the correlation of heat stress and HSP expression, relatively little is known about the specific functions of thermosomes. In vitro experiments with the recombinant proteins clearly showed, that the thermosomes from *Pyrodictium occultum* and *Methanopyrus kandleri* display chaperone-like activities, but only dead-end complexes with non-native substrates bound to the reconstituted thermosomes were observed (Minuth et al., 1998, 1999). Because of the complexity of the systems, these experiments were performed with mesophilic substrate proteins below the optimum growth temperatures of the hyperthermophiles. To elucidate the in vivo function, further experiments under more physiological conditions are required.

Hsp70 (or DnaK) proteins have a multitude of functions; they are coupled to nucleotide binding and hydrolysis, and modulated by the co-chaperones Hsp40 (DnaJ) and GrpE (Bukau and Horwich, 1998). Some moderately thermophilic archaea such as *Methanobacterium thermoautotrophicum* possess Hsp70, while the most thermophilic ones have no *hsp*70-homolog encoding genes in their genomes (Gribaldo et al., 1999; Macario et al., 1999); it was therefore speculated that a structurally unrelated chaperone may take over its role in these archaea (Leroux et al., 1999; Siegert et al., 2000). In contrast, the hyperthermophilic bacteria belonging to the genera *Thermotoga* and *Aquifex* possess Hsp70 homologs (Macario et al., 1999).

An important function of chaperones, in addition to the inhibition of aggregation, is the unfolding of proteins, which either feeds misfolded proteins into the cellular degradation system, or offers aggregated protein molecules another chance for proper folding by "iterative annealing" (Horwich et al., 1999; Shtilerman et al., 1999; Weber-Ban et al., 1999; Chen et al., 2001; Grantcharova et al., 2001). Members of the Hsp100/Clp family display significant unfoldase activity of misfolded proteins in yeast and *E. coli*, cooperating with Hsp70 (DnaK) in the subsequent refolding process (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Mogk et al., 1999; Weber-Ban et al., 1999). Protein aggregates are first bound by ClpB; an ATP-triggered structural change leads then to the presentation of hydrophobic regions of aggregated proteins, which are

subsequently solubilized by DnaK. The cooperation of ClpB and DnaK was also demonstrated for ClpB and DnaK from the extreme thermophile *Thermus thermophilus*. In vitro, the DnaK system (DnaK, DnaJ, GrpE and ATP) suppressed heat-induced aggregation of substrate proteins, and ClpB-induced efficient refolding (Motohashi et al., 1999). It is not clear whether similar DnaK-ClpB systems exist in other hyperthermophiles. While the genomes of the hyperthermophilic bacteria *T. maritima* and *Aquifex aeolicus* contain *dnaK* and *clpB* gene homologs, no such homologs have been detected in the archaea so far.

In summarizing, the extrinsic stabilization of proteins by compatible solutes and molecular chaperones appears to be crucial for many hyperthermophiles, especially when they grow close to their upper temperature limits. Further experiments under these extreme conditions promise insights into the cellular mechanisms that set the upper temperature limit at which life is possible.

Conclusions

This survey is based on the assumption that the upper temperature limit of life is dictated by molecular instability at the level of both cytosolic low-molecular weight compounds and biopolymers. As indicated by in vitro experiments, a number of metabolites, especially coenzymes, are unstable under optimum growth conditions of thermophiles and hyperthermophiles. Mechanisms to overcome this instability include 1) rapid catalytic turnover, 2) metabolic channelling and 3) local stabilization by weak intermolecular interactions. In the case of biopolymers, the canonical building blocks are sufficiently stable to allow structural and functional integrity at temperatures close to or even beyond the boiling point of water. Depending on the structural context, significantly higher stabilities can be accomplished, e.g., by evolutionary protein design or chemical modification. However, evolution in the biosphere optimizes for (multi-)functionality rather than stability. Basically, the free energy of stabilization is accumulated from small increments involving electrostatic and hydrophobic interactions. Covalent modifications or extrinsic factors such as salts, specific ligands and compatible solutes may significantly improve thermostability.

In the case of nucleic acids, chemical modification (e.g., RNA methylation) and binding of extrinsically stabilizing components (salts and histone-like basic proteins) are essential in maintaining replication, transcription and translation at temperatures close to or even beyond the melting temperature of DNA and RNA. Only for RNAs, enhanced base-pairing has been observed, whereas thermophile and hyperthermophile DNA shows unexpectedly low G+C contents, even for archaea with optimal growth temperatures above 100°C.

Considering the lipid constituents of mesophilic and thermophilic membranes, characteristic differences have been discovered: Thermophilic archaea contain highly caldarchaeol ether-lipids, which form stable monolayers that span the entire membrane (Fig. 8), whereas the ester-lipids of bacterial thermophiles are stabilized by a high content of saturated fatty acids. The necessary fluidity is regulated either by differences in the degree of saturation, or by adjusting the chemical composition of the fatty-acid hydrocarbon side chains.

In the case of proteins, enhanced intrinsic stability in thermophiles compared to their mesophilic counterparts is the cumulative effect of minute improvements of local interactions at the secondary-, tertiary- and quaternary structural level, e.g., higher packing efficiency, networks of ion pairs and/or hydrogen bonds, and reduction of conformational strain. Taken together, these increments suggest that thermostability corresponds to increased rigidity at low temperature and shifts to normal flexibility at physiological temperature; evidently, evolutionary adaptation to a physical parameter tends to maintain "corresponding states" with regard to conformational flexibility. At this point, it seems appropriate to stress that any generalization in considering the structure-function relationship of biopolymers, on the one hand, and their stability on the other, needs careful controls. In the present case, this may be illustrated by a number of contradicting observations: 1) There are hyperthermophilic enzymes with high intrinsic thermostability that are more active than their mesophilic counterparts, even at room temperature, thus combining high catalytic efficiency with high overall rigidity (Sterner et al., 1996; Ichikawa et al., 1998; Merz et al., 1999). 2) In stressing overall rigidity, it is important to notice that there is not necessarily a single measure of flexibility: a given 3D structure of a protein molecule may provide a rigid scaffold, e.g., a $(\beta\alpha)_8$-barrel, at the same time showing high catalytic efficiency due to the local flexibility of its active center (Shoichet et al., 1995). Along these lines, attempts have been reported to distinguish the "macro-" and "microstability" of proteins (Závodszky et al., 1998). 3) There is no fundamental reason for stability and rigidity to be strictly correlated because flexibility implies high conformational entropy of the folded state, which is favorable to thermodynamic stability. In addition, rigidity and flexibility may depend on the methods applied; one and the same protein

may be rigid on a nanosecond time scale, but flexible on a millisecond time scale (Lazarides et al., 1997; Daniel and Cowan, 2000). 4) Making use of the amide hydrogen-exchange rates in rubredoxin from *Pyrococcus furiosus* (the most thermostable protein presently known), it was shown that conformational opening processes occur within milliseconds for all amide positions along the polypeptide chain; the corresponding distribution of amide protection factors is indistinguishable from data reported for typical mesophilic homologs (Hernández et al., 2000; Jaenicke, 2000b). Obviously, these data are in contrast to the above generalization that enhanced conformational rigidity in the folded native state determines the increased thermal stability of thermophilic and hyperthermophilic proteins. At present, there is no way to resolve the apparent discrepancies; more experiments need to be done to combine the data to a new general view of protein stabilization.

Another open question refers to the phylogeny of microorganisms and their genes (cf. Doolittle, 1998; Koonin et al., 1998). Considering the protein repertoire of mesophiles and thermophiles, a wealth of experimental data proved that the average stabilities of thermophilic proteins exceeds the stabilities of the corresponding mesophilic proteins (Fig. 2). Roughly speaking, the mutative adaptation of a mesophile to a high-temperature environment requires the adaptation of the complete proteome to the higher temperature. For the reverse shift, a single temperature-sensitive mutation is sufficient. In spite of this simple argument, it is still unclear which of the two alternatives describes the direction of natural selection in the early evolution of the biosphere. The accumulation of extreme thermophiles close to the root of the (16S rRNA) phylogenetic tree (Fig. 14) favored the hypothesis that the prebiotic soup was hot, suggesting that ther-

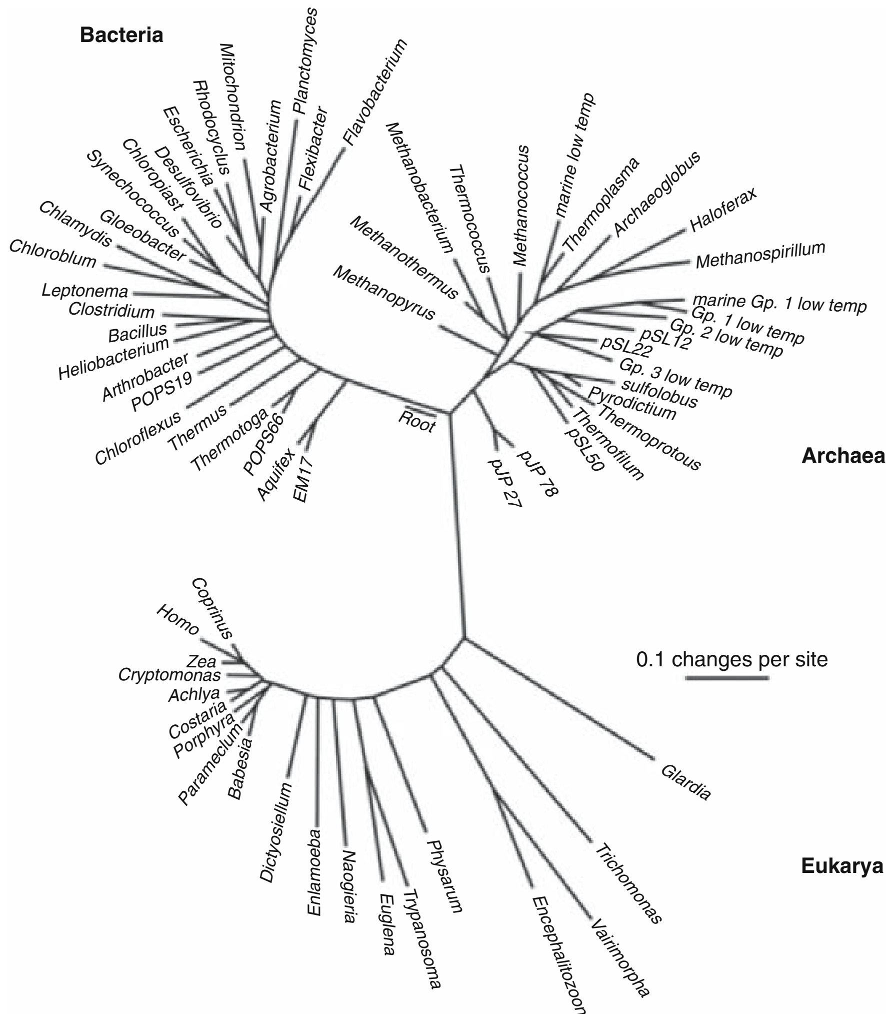


Fig. 14. Universal phylogenetic tree based on rRNA sequences. The scale bar corresponds to 0.1 changes per nucleotide. From Pace (1997), with permission. Regarding the positioning of the Nanoarchaeota, see Huber et al. (2003).

mophiles preceded mesophiles in the early history of life (Woese et al., 1990).

However, the advent of complete genome sequences made it clear that the phylogenetic tree has more complex roots than expected from a single genetic marker molecule inasmuch as different genes may or may not agree with the tRNA tree. Even more perplexing, genomes may contain a mix of DNAs, some related to archaea, while others are close to bacteria, so that a given microorganism, depending on the marker gene, ends up at different phylogenetic placements. It looks as if each gene has its own history, possibly due to mechanisms such as horizontal gene transfer or "gene swapping" (Nelson et al., 1999; Ochman et al., 2000). Although the mechanism of gene swapping is still unknown, there seems to be no better explanation for the observation that 17 out of 34 families of eukaryotic proteins that date back to early cell evolution look as if they come from bacteria, while only 8 families show a greater similarity to archaea, the supposed ancestor of eukarya. In spite of these inconsistencies, presently available genome sequences still fit the three-kingdom hypothesis (Miller and Lazcano, 1995; Woese, 1998; Deckert et al., 1998; Doolittle, 1998; Daniel and Cowan, 2000). No thermophilic eukarya have been discovered so far (R. Rachel, personal communication, 2001); possible reasons for this observation have been mere speculation.

Concerning the geological time when hyperthermophilic microorganisms might have evolved, there are claims for the occurrence of a variety of early archaea around 4 billion years ago. Photosynthetic life (both anoxygenic and oxygenic) has been established as early as 3.5–3.7 billion years ago (Rosing, 1999), molecular fossil evidence allowed the existence of cyanobacteria to be traced back 2.7 billion years (Brocks et al., 1999), and in addition, chemotrophic archaea have been spotted in 3.2 billion year-old volcanogenic massive sulfide rocks (Rasmussen, 2000). The latter finding extends the realms of thermophilic archaeal life into the extreme of deep-sea hot springs, in addition to the open ocean, mid-ocean ridges, lake communities, costal sediments and coastal hydrothermal systems. It does not show that abyssal hydrothermal life came before photosynthesis; however, it may be taken to support the idea that steps in the early history of life took place around hydrothermal systems (Nisbet, 2000).

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Prokaryotes (2006) 2:210–262
DOI: 10.1007/0-387-30742-7_8

CHAPTER 1.8

Life at Low Temperatures

SIEGFRIED SCHERER AND KLAUS NEUHAUS

Introduction

Most habitats on our planet are permanently cold. By volume, 90% of the world's oceans have a temperature of 5°C or less, supporting both psychrophilic and psychrotolerant microorganisms. When terrestrial habitats are included, over 80% of the earth's biosphere is permanently cold (Russell, 1990a). Archaea contribute significantly to biomass in cold environments, although few have been isolated (Goodchild et al., 2004).

Microorganisms that are able to grow at low temperatures are termed "psychrophilic" (sometimes also "obligate psychrophiles") and "psychrotolerant" (or "facultative psychrophiles" or "psychrotrophs") or even "psychroactive" (Nozhevnikova et al., 2001b). We recommend here the use of only the designations "psychrophilic" and "psychrotolerant." (Morita, 1975) has defined psychrophiles "as organisms having an optimal temperature for growth at about 15°C or lower, a maximal temperature for growth at about 20°C, and a minimal temperature for growth at about 0°C or lower." This author summarized the confusing discussion started about 100 years ago concerning which definitions and terms are appropriate. The above-mentioned terms have no generally accepted definitions. However, this is not surprising since microorganisms are adapted to low temperature to very different degrees, and a continuum of evolutionary transitions exists in all genera that harbor cold-adapted strains. For instance, strains of typical psychrophiles or psychrotolerants within the same species may have quite different doubling times at the same low temperature. The terms "mesophile," "psychrotolerant," and "psychrophile" are therefore impossible to define exactly.

Table 1 suggests the use of three temperatures for defining principal types of cold adaptation. Because of the evolutionary variability of bacteria, many transitions between thermal types commonly exist; therefore, any such temperature ranges should be considered approximate guidelines only.

The first reported growth of a bacterium at low temperature was probably by (Forster (1887)) and about luminescent bacteria from salt water: ". . . they grow almost as well in the ice box as at the usual room temperature." For a review, see Morita (1975). However, within the last five years, the number of papers dealing with cold adaptation has increased sharply. This is true both for the analysis of the cold shock response (see the section on The Cold Shock Response and Cold Adaptation in this Chapter), as well as the description of new psychrophilic and psychrotolerant species, especially from permanently cold habitats. This sharp increase in interest (as reflected in the increased number of reviews written about bacterial life at low temperature in recent years) is certainly fueled by many emerging biotechnological uses of cold tolerant organisms (Margesin and Schinner, 1999).

Table 2 lists selected psychrotolerant and psychrophilic species described in the literature between 2000 and 2003. Generally, cold-adapted species are found throughout the entire taxonomic range of bacteria and archea as well as in many climates.

Some general reviews on the physiological adaptations of cold-adapted microorganisms have been published within the last decade by Russell (1998), (1990b); Gounot (1991), Margesin and Schinner (1999), and D'Amico et al., (2002a). The reader may consult these reviews for further references. Reviews on specific subjects within the area of cold adaptation are mentioned below (see Table 9). This article will mainly focus on data published within the last few years.

The Cold Shock Response and Cold Adaptation

Organisms respond to a cold shock by a specific pattern of transient gene expression (Yamanaka, 1999a); reviewed in Weber and Marahiel (2003). However, note that the "specific pattern" described in literature is based nearly exclusively on observations made in cultures growing exponentially in a liquid broth. Comparison of cold shocked gel-entrapped *Escherichia coli* cells to

Table 1. Definitions for typical cold-adapted and mesophilic microorganisms.

	Growth temperature[a]		
	Minimal	Optimal	Maximum
		(°C)	
Typical psychrophile (obligately psychrophilic)	<0	<15	<20
Typical psychrotolerant (facultatively psychrophilic, psychrotrophic)	<7	>20	>25
Typical mesophile	>10	>25	>35

[a]The temperatures given for a psychrophile are taken from Morita (1975). Taking into account the temperature variations for the growth of microorganisms in refrigerated food reported in the literature, the lower growth limit of psychrotolerants in Table 1 has been set to 7°C.

Table 2. Selection of psychrotolerant and psychrophilic species described between 2001 and beginning of 2005.

Species	TT	Isolated from	Reference(s)
Algoriphagus antarcticus	pp	microbial mats in Antartic lake	Van Trappen et al., 2004c
Alkalibacterium olivoapovliticus	pt	Olive wash-waters	Ntougias and Russell, 2001
Alkalibacterium psychrotolerans	pt	fermented polygonum indigo	Yumoto et al., 2004
Alteromonas stellipolaris	pt	Antartic sea water	Van Trappen et al., 2004a
Arthrobacter psychrophenolicus	pp	alpine cave	Margesin et al., 2004
Bacillus psychrodurans	pt	Soil, Egypt	Abd El-Rahman et al., 2002
Bacillus psychrotolerans	pt	Soil, Germany	Abd El-Rahman et al., 2002
Carnobacterium pleistocenium	pt	permafrost, Alaska	Pikuta et al., 2005
Chromohalobacter sarecensis	pt	saline Andean region	Quillaguaman et al., 2004a
Clostridium sp. PXYL1	pp	Cattle manure digester, India	Akila and Chandra, 2003
Dietzia psychralcaliphila	pp	Fish-processing plant, Japan	Yumoto et al., 2002
Flavobacterium frigidarium	pp	Antarctica	Humphry et al., 2001
Flavobacterium frigoris	pp	microbial mats in Antartic lake	Van Trappen et al., 2004d
Geopsychrobacter electrodiphilus	pt	marine sediment fuel cell	Holmes et al., 2004
Gillisia limnaea	pp	microbial mats in Antartic lake	Van Trappen et al., 2004e
Glaciecola polaris	pt	Artic ocean	Van Trappen et al., 2004b
Halomonas boliviensis	pp	Bolivian hypersaline lake	Quillaguaman et al., 2004b
Hyphomonas aff. jannaschiana	pp	Deep sea	Edwards et al., 2003
Lactovum miscens	pt	acidic forest soil	Matthies et al., 2004
Marinilactibacillus piezotolerans	pt	deep sub-seafloor sediment	Toffin et al., 2005
Marinobacter aff. aquaeolei	pp	Deep sea	Edwards et al., 2003
Marinomonas ushuaiensis	pp	coastel sea water, Argentina	Prabagaran et al., 2005
Methanogenium marinum	pt	Marine sediment, Alaska	Chong et al., 2002
Methanosarcina mazei	pt	Tundra	Simankova et al., 2003
Mycobacterium psychrotolerans	pt	pond water near uranium mine	Trujillo et al., 2004
Paenibacillus antarticus	pt	Antartic sediment	Montes et al., 2004
Pseudomonas alcaliphila	pp	Seawater, Hokkaido, Japan	Yumoto et al., 2001b
Pseudomonas antartica	pp	microbial mats from Antartica	Reddy et al., 2004
Pseudomonas psychrophila	pp	Food storage room, Japan	Yumoto et al., 2001a
Pseudomonas psychrotolerans	pt	vetinary hospital, Vienna	Hauser et al., 2004
Psychrobacter marincola	pp	Sea water	Romanenko et al., 2002
Psychrobacter maritimus	pt	coastal sea ice, sea of Japan	Romanenko et al., 2004
Psychrobacter nivimaris	pt	Southern Ocean	Heuchert et al., 2004
Psychrobacter proteolyticus	pt	Antarctic krill	Denner et al., 2001
Psychrobacter salsus	pt	fast ice, Adelie Land, Antartica	Shivaji et al., 2004
Psychrobacter sumarinus	pp	Sea water	Romanenko et al., 2002
Psychromonas profunda	pp	Deep Atlantic sediment	Xu et al., 2003c
Rhodoferax ferrireductans	pt	Marine sediment, United States	Finneran et al., 2003
Sejongia antartica	pt	terrestrial samples, Antartica	Yi et al., 2005

Abbreviations: TT, thermal type; pt, psychrotolerant; and pp, psychrophilic.

free floating cells revealed significant differences in the protein response (Perrot et al., 2001). What one would define as a specific cold shock stimulon is therefore dependent on the experimental procedures. To our knowledge, virtually no report deals with the cold shock response of stationary phase cells, though many bacteria spend most of their lifetime in stationary phase (Kjelleberg, 1993).

Although low temperature induces an adaptation of many cellular components, e.g., the membrane composition, the supercoiling of the DNA,

and the transcriptome (see the section Cold Shock and the Degradation in this Chapter), the most severe problem seems to be initiation and translation of bulk mRNA at low temperatures (Broeze et al., 1978; Jones and Inouye, 1994). Shifting a culture of *Escherichia coli* from 37°C to 8°C or below resulted in polysomal run-off and accumulation of free ribosomes (Broeze et al., 1978; Xia et al., 2002).

Other stresses, e.g., some antibiotics (VanBogelen and Neidhardt, 1990), dilution of a culture with fresh medium (Brandi et al., 1999a; Brandi et al., 1999b), an upshift in the concentration of nutrients (Yamanaka and Inouye, 2001a), diauxic lag (Novotna et al., 2003), oxidative stress (Smirnova et al., 2001b), hydrostatic pressure (Wemekamp-Kamphuis et al., 2002), or exposure to colicin E9 (Walker et al., 2004) can mimic a cold shock response or at least induce the cold shock stimulon to some extent (Wick and Egli, 2004). Therefore it could be hypothesized that every event stopping or stalling the ribosomes leads to an induction of the cold shock response (Walker et al., 2004). This is most obvious for the cold shock response itself (Gualerzi et al., 2003), the use of certain antibiotics affecting the translational speed (VanBogelen and Neidhardt, 1990), or after nutrient upshift (Brandi et al., 1999a) in which many new mRNAs are synthesized to adapt to the new nutrition provided.

Chilling of bacterial cells affects their viability. In *Bacillus subtilis* it was shown that death after cold shock is not only due to a passive event (a reaction velocity decrease and outrun of energy), but also to translocation of a DNase (YokF) from the periplasm into the cytoplasm attacking the DNA of the cell. This partly resembles apoptosis in eukaryotic cells (Sakamoto et al., 2001).

Most work on cold shock was done with the mesophiles *E. coli* and *B. subtilis*. This article, therefore, reports mainly on these two organisms. The reader may consult the detailed review by Weber and Marahiel (2003) about bacterial cold shock responses. Besides cold shock proteins, some cold adaptation proteins have been described. However, much less is known about the permanent response of bacteria towards low temperature. This work is summarized in the section Cold Adaptation.

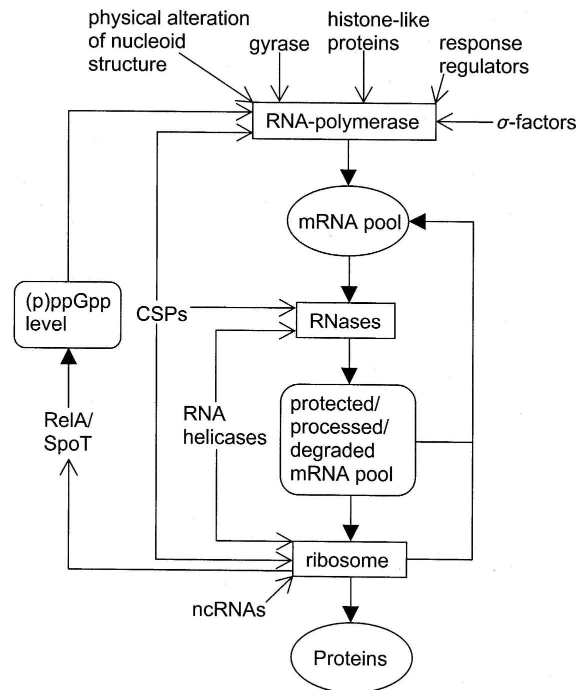


Fig. 1. Regulation of bacterial cold shock responses as a multiple filter model. The arrows indicate the flow of genetic information from DNA (via RNA polymerase, top) to protein (bottom), in turn affecting via a feedback circuit response (eventually conducted by effector molecules) this genetic flow. The T-arrows have to be read as "modulates activity of." The filter systems are boxed in square boxes. These filters are integration systems reacting directly or indirectly to temperature changes. Adapted from Weber and Marahiel (2003).

Major Cold Shock Proteins: CspA–CspI

In most free-living bacteria, a cold shock protein (Csp) family has been identified (Francis, 1997) and displays homology to CspA, first discovered in *E. coli* (Jones et al., 1987); reviewed in Ermolenko and Makhatadze (2002). Since CspA has the highest induction level, these proteins are often termed "major cold shock protein(s)" (MCSPs; Goldstein et al., 1990; Etchegaray and Inouye, 1999b; Lopez and Makhatadze, 2000). However, note that many parasitic or pathogenic bacteria do not contain such a protein family (e.g., *Chlamydia trachomatis*, *Helicobacter pylori*, *Mycoplasma* sp. and others (Yamanaka, 1999a) and some psychrotrophic bacteria, as *Aeromonas hydrophila*, may not respond with a "typical" cold shock response (Imbert and Gancel, 2004). After a downshift from 37°C to 10°C, the major cold shock proteins of *E. coli* (CspA, B, G and I) are induced. The Csps reach 13% of the total protein synthesis, and synthesis of CspA is increased 30-fold under certain circumstances (Jones et al., 1987; Goldstein et al., 1990; Lee et al., 1994; Thieringer et al., 1998; Etchegaray and Inouye, 1999b; Wang et al., 1999; Gualerzi et al., 2003). In the psychrotolerant *Yersinia enterocolitica*, a CspA tandem has been discovered which may lead to a higher rate of CspA synthesis (Neuhaus et al., 1999). A first hypothesis formulated on the basis of CspA's abundance after a cold shock was that this protein may function as an antifreeze protein.

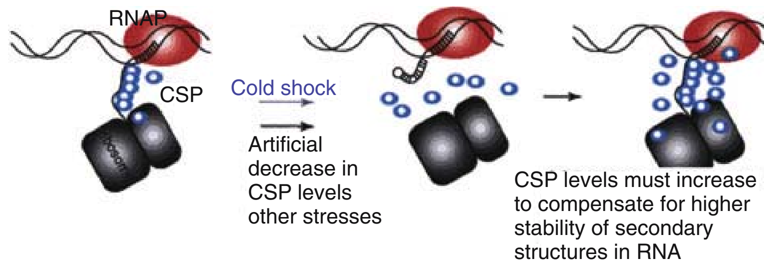


Fig. 2. Model for the function of cold-shock proteins (CSPs) as RNA-chaperones that couple transcription to translation of mRNA. During growth at 37°C, CSPs bind to mRNA as it protrudes from the RNA-polymerase complex (RNAP) and maintain the RNA in a linear form. The ribosome then displaces CSPs, which have only low affinity for RNA, and initiates translation. Accordingly, an artificial decrease in the CSP concentration would lead to the formation of secondary structure in RNA and prevent translation. After cold shock or other stresses (e.g., carbon starvation), an increase in the CSP concentration is needed to counterbalance the increased stability of RNA secondary structure. Redrawn after Graumann and Marahiel (1998).

Although a cold adaptation period preceding freezing enhances freezing tolerance (Thammavongs et al., 1996); Kim and Dunn, 1997; Kim et al., 1998), the role of MCSPs in that protection has not been demonstrated unequivocally (Wouters et al., 1999).

Apparently, CspA is-at least in part-an mRNA chaperone, opening the secondary structures of mRNAs at low temperature, an alteration which helps the ribosomes to function after a cold shock (Jiang et al., 1997); Fig. 2). Besides CspA, *E. coli* contains a family of highly similar Csps, containing eight other members: CspB consists of 71 amino acids (aa) and is 79% identical to CspA. Similarly, CspC contains 69 aa and has 70% identity to CspA; CspD (74 aa) has 45% identity; CspE (69 aa) has 70% identity; CspF (70 aa) has 44% identity; CspG (70 aa) has 73% identity; CspH (70 aa) has 47% identity; and CspI (70 aa) has 70% identity (Lee et al., 1994; Yamanaka et al., 1994; Yamanaka et al., 1998; Nakashima et al., 1996; Wang et al., 1999). Also, Bacillus cereus contains a family of MCSPs (Mayr et al., 1996). All these different CspA homologs are believed to be stress adaptation proteins for different tasks, but the cold-inducible Csps can replace each other to some extent (Graumann et al., 1997; Yamanaka et al., 1998; Gualerzi et al., 2003). CspA is induced after a cold shock from 30°C down to 10°C, CspB and CspG occur between 20°C and 10°C, and CspI occurs below 15°C (Wang et al., 1999). A quadruple deletion mutant missing CspA, CspB, CspE and CspG was cold sensitive and formed filamentous cells at 15°C. This phenotype was suppressed by overexpression of each member of the cold-shock protein family except CspD, which causes lethality (Phadtare and Inouye, 2004b; Xia et al., 2001b). A different function of CspA and CspD was supported by another line of evidence. Green fluorescent protein (GFP) fusions were found in the nucleoid in the case of CspD, and in a polar position of the cell in the case of CspA (Giangrossi et al., 2001a), however, in Pseudomonas Csp seems to be distributed evenly in the cytosol of the cell (Khan et al., 2003).

Most cold inducible MCSPs have an unusually long mRNA leader region of 156–256 bp upstream of the translational start. An exception is the mRNA leader region of the *cspH* gene from *Salmonella enterica*, which is only 55 bp long (Kim et al., 2001). Another exception was reported recently, the cold-inducible CspA-homolog CspV from *Vibrio cholerae*, which exhibits a leader of only 12 bp (Datta and Bhadra, 2003).

The CspA molecule is small (7.4 kDa and 70 aa), acidic (pI 5.92), and very hydrophilic (Goldstein et al., 1990). A remarkable feature is the high sequence similarity of the bacterial major cold shock proteins to eukaryotic Y-box factors, including human YB-1 (which is 44% identical with CspA) and frog FRG Y1/2 (Didier et al., 1988; Tafuri and Wolffe, 1990; Lee et al., 1994). These are domains of DNA or RNA-binding motifs which bind to a specific regulatory sequence called the “Y-box motif,” ATTGG/CCAAT (Wolffe, 1994), and are therefore designated “cold-shock domains” (CSDs; Karlson and Imai, 2003). Interestingly, a protein with a similar fold, initiation factor 1, can complement in a *B. subtilis cspB cspC* double mutant (Weber et al., 2001a). Figure 3 shows the three-dimensional (3D) structure of CspB from *B. subtilis* (Schindelin et al., 1992; Schindelin et al., 1993; Schindelin et al., 1994; Schnuchel et al., 1993), which is similar to that CspA from *E. coli* (Schindelin et al., 1994; Feng et al., 1998). It consists of five antiparallel β-sheets, which form a barrel. The RNP-1 motif KGFGFI (Landsman, 1992; Lee et al., 1994) and RNP-2 motif VHVHF

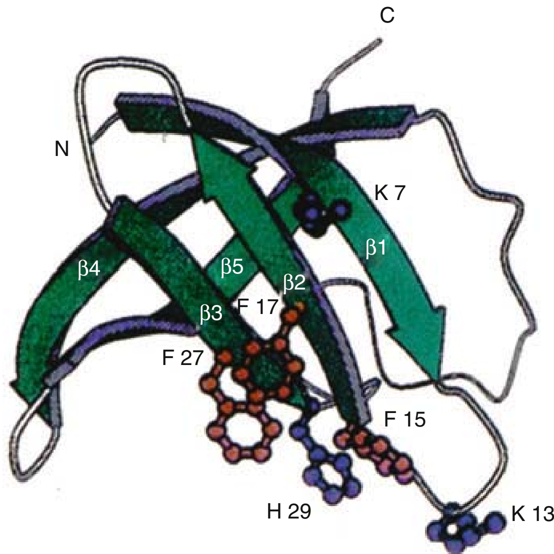


Fig. 3. Three-dimensional structure of CspB from *B. subtilis* features five antiparallel β-sheets, which form a barrel. From Graumann and Marahiel (1996a).

(Landsman, 1992; Schnuchel et al., 1993) contain most of the aromatic residues. They are exposed to the water phase and interact with single-stranded (ss) DNA (Schröder et al., 1993; Newkirk et al., 1994). If the aromatic residues in the RNP-1 or RNP-2 are replaced as a result of mutations, binding of ssDNA containing the Y-box motif is abolished (Schröder et al., 1995).

CspB is very similar to CspA, the time frame of its induction follows closely that of CspA (Etchegaray et al., 1996). The promoter regions of *cspA*, *cspB* and *cspG* display similar features with similar promoter sequences and the same unusually long leader region of about 156–256 bases (Nakashima et al., 1996; Datta and Bhadra, 2003). The function of CspH needs to be elucidated (Yamanaka et al., 1998); however, in *Salmonella enterica*, *cspH* was described as cold inducible (Kim et al., 2001).

Among the non-cold-inducible MCSPs, CspC is rather highly expressed at normal growth temperature (37°C) and its level remains unchanged after a cold shock (Lee et al., 1994). Also, CspC was found to be a multicopy repressor of the *mukB106* mutant (Yamanaka et al., 1994). This mutant has a defect in chromosomal partitioning (Niki et al., 1991). CspD is induced at the onset of the stationary phase and inversely dependent on growth rate or glucose starvation. Guanosine-3′-diphosphate-5′-(tri)diphosphate, collectively abbreviated (p)ppGpp, is a positive regulator for CspD (Yamanaka and Inouye, 1997; Yamanaka et al., 1998). Because overproduction of CspD is lethal, the presence of overproducing cells is indicated by a typical morphology, which is due to impaired DNA replication. CspD inhibits effectively both the initiation and the elongation of minichromosome replication in vitro (Yamanaka et al., 2001c), and it is switched off after cold shock (Lee et al., 1994). CspD has been found associated with the nucleoid in *E. coli* (Giangrossi et al., 2001a). The function of CspF is unknown and needs to be elucidated (Yamanaka et al., 1998).

CspE is abundantly produced at 37°C, but a *cspA* deletion mutant also has higher levels of CspE in the cold. Originally, CspE was found as a multicopy repressor of the *mukB106* mutant gene that codes for a protein, which, together with CspC, plays a role in chromosomal partitioning (Yamanaka et al., 1994). Later, CspE was found to interact with nascent RNA in transcription complexes, causing antitermination. The latter function is coupled to the nucleic acid melting abilities of this protein (Phadtare et al., 2002a; Phadtare et al., 2002b; Phadtare et al., 2004c). Furthermore, it binds to the Y-box motif and functions as a repressor for *cspA* at 37°C through an interaction with the transcription elongation complex (Bae et al., 1999). Recently, it was discovered that CspE binds to poly(A) tails of mRNAs (which is a decay signal) and subsequently impedes the 3′ to 5′ exonucleolytic decay by polynucleotide phosphorylase (PNPase). CspE also inhibits both internal cleavage and poly(A) tail removal by RNase E, thus stabilizing mRNA (Feng et al., 2001). CspE was also found to be important in radiation resistance of *E. coli* (Chattopadhyay, 2002; Mangoli et al., 2001). All evidences taken together imply that CspE is a regulator also important for translational fidelity of DNA in cold environments, and for DNA condensation and partitioning during growth (Mangoli et al., 2001; Sand et al., 2003).

The most prominent Gram-positive bacterium examined with respect to cold shock is the mesophile *B. subtilis* (Weber and Marahiel, 2002) extensively reviewed the cold shock response of this organism. Briefly, after a cold shock from 37°C to 15°C, protein synthesis resumed 2 h later. During the adaptation, CspB (which is a homolog to CspA of *E. coli*) is induced and remains at higher than pre-cold shock levels (Willimsky et al., 1992; Kunklova, 1995; Graumann et al., 1996b). A *cspB*::*lacZ* fusion showed a sevenfold induction after cold shock from 37°C to 10°C. In addition to CspB, CspC and CspD of *B. subtilis* are homologs; CspC is also cold inducible (Graumann et al., 1997) but differs slightly from CspB since the CspC increases more rapidly. Interesting genome-wide transcriptional profilings of the *B. subtilis* cold shock response were conducted by Kaan et al. (2002) and Beckering et al. (2002), the former

study describing genes not only induced but also repressed after cold shock in this organism.

In *B. subtilis*, CspB and CspC not only participate in the cold shock response, but also act as major stationary-phase induced proteins. This illustrates the broad functionality of these Csps in cellular physiology (Graumann and Marahiel, 1999a).

In Anabaena sp., no MCSP could be detected. However, an RNA helicase CrhC was found to be induced after cold shock (Chamot et al., 1999; Chamot and Owttrim, 2000). This helicase is completely membrane bound and mainly polar localized in this organism (El-Fahmawi and Owttrim, 2003). Interestingly, *E. coli* CspA has also been found in a polar position, but it remains in the cytoplasm (Giangrossi et al., 2001a). Even though the function of CrhC is not completely clear, the RNA unfolding abilities of both proteins, CrhC and CspA, seems to be needed in polar positions. In Synechococcus sp., a heat shock protein (Hsp)90 homolog, HtpG, was found to be heat and cold shock inducible (Hossain and Nakamoto, 2003).

Regulation of the Major Cold Shock Proteins

The regulation of CspA induction in *E. coli* after cold shock is rather complex and not yet fully understood. Transcriptional and posttranscriptional regulation of cold shock genes, including the MCSPs, was reviewed in detail by Gualerzi et al. (2003), and an overview of some aspects is given in Fig. 4. The cspA gene exhibits an unusually long leader sequence. The major transcription start +1 is located 159 bp upstream from the translational starting point. The promoter seems to be σ-70 dependent, since the –35 region (TTGCAT) and the –10 region (CTTAAT) are found to be similar to a σ-70 consensus sequence (TTGACA for the –35 and TATAAT for the –10 (Qoronfleh et al., 1992; Tanabe et al., 1992). Other regulatory elements in the gene sequence of cspA include the cold box (Fang et al., 1998; Jiang et al., 1996b), the upstream (UP) element, the downstream box (Mitta et al., 1997), the upstream box (Yamanaka et al., 1999c), and others (Yamanaka, 1999a). An overview of the features of the cspA gene sequence is given in Fig. 5.

The 5′ end of the *cspA* mRNA contains a regulatory sequence (cold box), which stabilizes the mRNA at low temperature, enabling cold shock induction (Xia et al., 2002). The consensus cold box sequence (5′ UGACGUACAGA) is found in *cspA*, *cspB* and *csdA* (Jiang et al., 1996b). However, if the 5′ end of *cspA* containing this cold box is overproduced, the expression of cold shock genes is no longer transient, and the synthesis of bulk proteins is impaired (Jiang et al., 1996b; Xia et al., 2002). Also, the cessation of regrowth after cold shock is prolonged. This fits

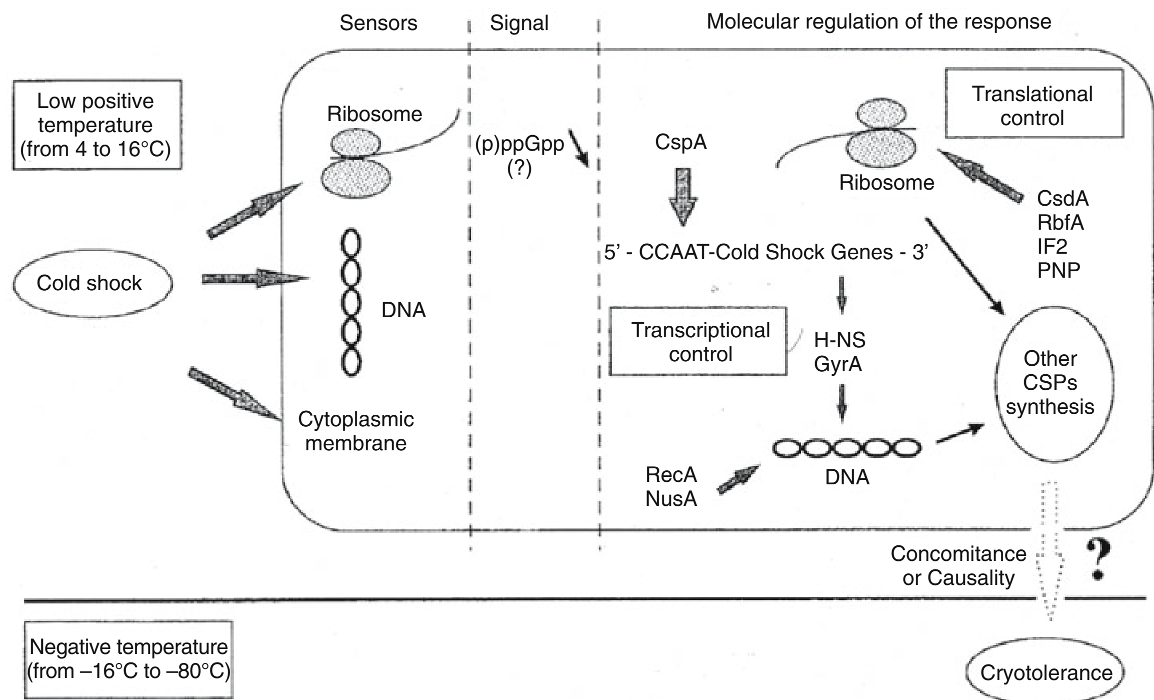


Fig. 4. Some aspects of the cold shock response. From Panoff et al. (1998).

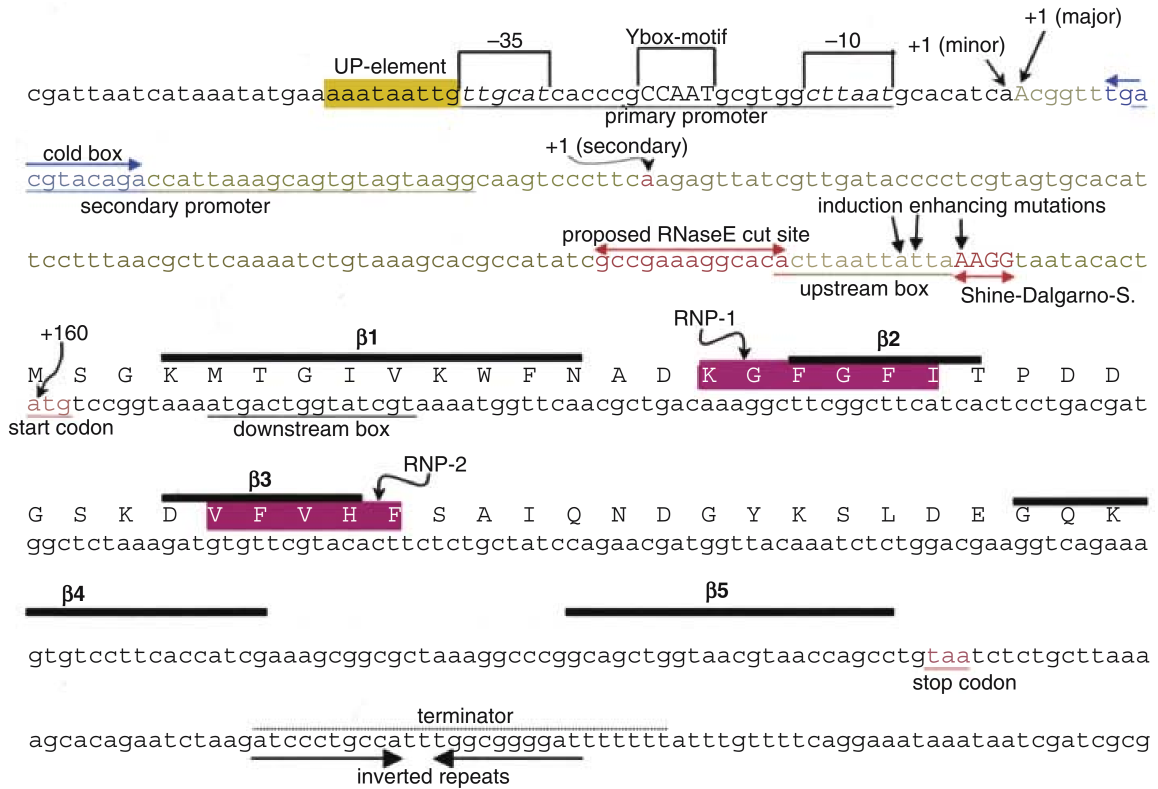


Fig. 5. Overview of the features of the *cspA* gene and the CspA protein.

nicely with the observation that *cspA* mRNA in excess is poisonous to the cell. The robust translatability of *cspA* mRNA depends on initiation, and the ribosome appears to be preadapted to translate *cspA* mRNA (Etchegaray and Inouye, 1999b; Xia et al., 2001a). Since overproduction of CspA together with the overproduction of the 5′-end restores the normal cold shock response, CspA itself probably interacts with the cold box (Jiang et al., 1996a). Furthermore, Giuliodori et al. (2004) could repeat the preferential translation of cold-shock mRNAs after cold shock in vitro. Apparently, cold-shock 70S ribosomes display some translational selectivity for MCSP mRNAs. The *trans*-acting factors involved are 1) CspA itself (increasing translatability of mRNA in the cold) and 2) the cold shock-induced stoichiometric imbalance between the initiation factors IF1, IF2 and IF3, on the one hand, and the ribosomes, on the other. Possible *cis*-acting elements discussed are the secondary or tertiary structures of the unusual long 5′ leader sequences of MCSP mRNAs.

Furthermore, in addition to CspA-mediated autoregulation, a repressor for *cspA* was found, which turned out to be CspE. CspE is abundantly produced at 37°C, and in a *cspE* mutant, *cspA* is derepressed (Fang et al., 1998). In vitro, CspE and CspA cause transcriptional pausing just behind the cold box of *cspA*, and CspA production is inhibited by addition of CspE to the translating ribosomes (Bae et al., 1999; Phadtare and Inouye, 1999).

Mutational analysis of the 5′-untranslated leader of *cspA* showed another element to be involved in regulation of CspA. A deletion of a few bases upstream from the Shine-Dalgarno sequence (SDS) decreased the CspA amount more than 10-fold. It turned out that a 13-bp sequence located 11 bp upstream of the SDS is conserved in the cold-inducible genes *cspA*, *cspB*, *cspG* and *cspI*. This element was designated the "upstream box," and it is speculated that this region may form different secondary structures at different temperatures, leading to an efficient translation at low temperatures or nearly zero translation at higher temperatures (Yamanaka et al., 1999c).

Another element in CspA induction is the so-called "downstream box" (DB; Mitta et al., 1997). This element is found downstream of the ATG start codon of some cold shock genes and, according to its proponents, should be able to anneal to a complementary anti-downstream box at the 16S rRNA, thereby enhancing translation initiation. The existence of the downstream box

has been disputed. Sprengart et al. (1996), Etchegaray and Inouye (1999a), Etchegaray and Inouye (1999c), Etchegaray and Inouye (1999d), Mironova et al. (1999), and Xia et al. (2001a) are in favor, while O'Connor et al. (1999), Resch et al. (1996), Bläsi et al. (1999), La Teana et al. (2000), and Rocha et al. (2000) are against. Apparently, Moll et al. (2001) finally rejected the concept of the downstream box.

Cold Shock and the Degradosome

Recently, the mRNA-decay machinery of bacteria came into focus. In general, adaptation to low temperature after cold shock includes the establishment of a new equilibrium of the transcriptome following changes in transcription and mRNA decay rates, both of which are important for gene regulation in bacteria. The mRNA content of the cell is therefore not only regulated by cold shock induction or repression of certain genes, but also by stabilization or destabilization (depending on the specific mRNA and on the usage of different subsets of RNases; Mohanty and Kushner, 2003; Polissi et al., 2003). The subsets include PNPase and RNase H, which are cold shock induced, and RNase II or RNase E, which are not (Cairrão et al., 2003).

The induction of CspA is mainly due to an increase in mRNA stability. Its half-life is 12 s at 37°C, but between 15 min and 30 min at 15°C in *E. coli* (Tanabe et al., 1992; Jiang et al., 1993; Fang et al., 1997; Gualerzi et al., 2003). If the coding region of *cspA* is fused to the constitutive promoter *lpp*, it is still cold inducible. This observation is explained by a strong vulnerability of the transcript to RNase E degradation at 37°C. Even if the *cspA* promoter is turned on constitutively, CspA can only be synthesized if the transcript is stabilized, perhaps by CspE (Fang et al., 1997; Feng et al., 2001). As in *E. coli*, the transcripts of *cspB* and *cspC* in *B. subtilis* are also dramatically stabilized, having a half-life of 1 min at 37°C and more than 30 min at 15°C (Kaan et al., 1999). A similar observation was made in *Rhodobacter capsulatus* with a *cspA* transcript half-life of around 4 min at 32°C and 47 min at 10°C (Jäger et al., 2004).

Downregulation of MCSP mRNA is an important step, at least in enterobacteria, before growth can resume. This phenomenon is mainly due to the exceptionally strong ability of MCSP mRNAs to initiate at the ribosome. Therefore MCSP mRNA outcompetes bulk mRNA and thus prevents growth (Neuhaus et al., 2000b; Xia et al., 2001a; Yamanaka and Inouye, 2001b). In the above-mentioned PNPase-deficient strains, the decay of *cspA* mRNA is delayed, subsequently preventing re-growth (Neuhaus et al., 2000b). In *Yersinia enterocolitica*, the *cspA* tandem mRNA is cleaved at multiple specific cut sites, with an AGUAAA consensus (termed "cold shock cut box") to downregulate the MCSP mRNA. After these initial cleaving steps, the fragments are removed rapidly and growth can resume (Neuhaus et al., 2003). Cleavage of the *cspA* transcript within the coding sequence and subsequent rapid removal of the fragments was also found in *Rhodobacter capsulatus* (a member of the alpha-proteobacteria), but no consensus cut sequence could be detected (Jäger et al., 2004). CspE was found to interfere with both the PNPase and RNase E of the degradosome machinery, inhibiting internal cleavage and removal of the poly(A) tails from mRNAs, thus stabilizing particular mRNAs (Feng et al., 2001).

Bacteria without PNPase, which is a secondary cold shock protein, are cold sensitive (Clarke and Dowds, 1994; Goverde et al., 1998; Bae et al., 2000; Zangrossi et al., 2000). Curiously, this appears not to be true for *Pseudomonas putida*, indicating surprising differences between some species (Favaro and Deho, 2003). The cold-temperature induction of PNPase in *E. coli* occurs by reversal of its autoregulation. At 37°C, ribonuclease III cleaves the leader of the pnp mRNA, whereupon PNPase represses its own translation via unknown mechanisms. This latter step is inhibited after cold shock (Beran and Simons, 2001; Mathy et al., 2001).

RNase H was recently found to be a cold shock protein, too. This protein, posttranscriptionally regulated by mRNA stabilization due to PNPase activity, subsequently regulates maturation of other mRNAs (especially small stable RNAs) by its exonuclease abilities. An RNase H mutant produces smaller colonies when grown at lower temperatures (Cairrão et al., 2003).

Other Cold-Inducible Proteins

CspA induces and is part of the cold stimulon, directly or indirectly regulating 30 proteins, such as H-NS or GyrA (Madan Babu and Teichmann, 2003; Martinez-Antonio and Collado-Vides, 2003). H-NS is a histone-like nucleoid protein acting on DNA bending (La Teana et al., 1991; Brandi et al., 1994; Giangrossi et al., 2001b) and GyrA is part of topoisomerase II (Maxwell and Howells, 1999). Those promoters of secondary cold shock proteins contain one or more of the so-called "Y-box motif" CCAAT. Recognized by CspA, this Y-box motif subsequently activates transcription of the protein (Qoronfleh et al., 1992). This is true at least for H-NS, GyrA, and possibly other proteins (La Teana et al., 1991; Jones et al., 1992b). The enhanced level of GyrA together with H-NS, and HUβ increases the negative supercoiling of plasmids and chromosomal DNA (Goldstein and Drlica, 1984; Giangrossi

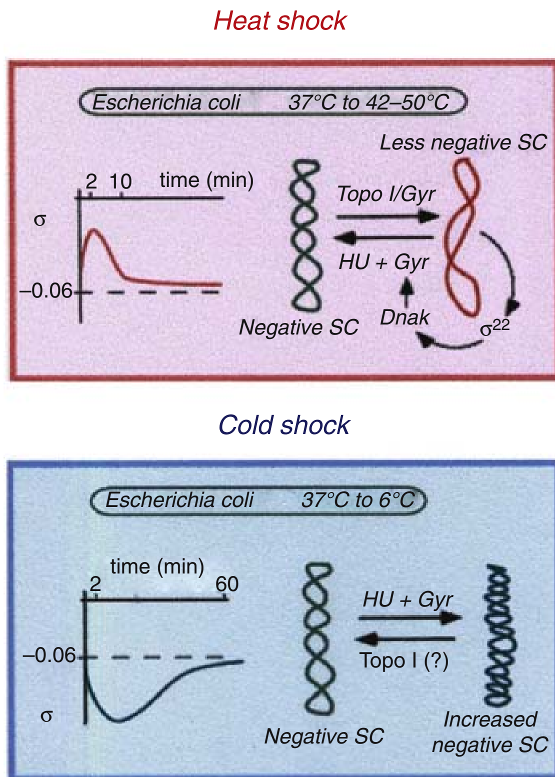


Fig. 6. Effects of heat shock and cold shock on plasmid DNA. The left side of each panel shows the variation in the plasmid-specific linking difference (σ = δLk/Lko), dependent on the time of exposure to the shock temperature. SC, supercoiling; Topo, topoisomerase; Gyr, gyrase; and HU, a small, basic, heat-stable DNA-binding protein. From Lopez-Garcia and Forterre (1999).

et al., 2002). Why GyrB is not induced is unclear, but the induction of GyrA seems to be sufficient to increase the DNA twisting after cold shock. The DNA twisting itself regulates at least the induction of recA, the gene of another cold shock protein found in *E. coli* (Hulton et al., 1990; La Teana et al., 1991; Wang and Syvanen, 1992; Brandi et al., 1996; Hurme and Rhen, 1998). However, the increase in negative supercoiling was found to be transient after cold shock (Fig. 6). This shows that the open complex formation of transcription turned cold-insensitive after the adaptation of the entire system to low temperature (Krispin and Allmansberger, 1995; Lopez-Garcia and Forterre, 1999). In addition, most promoters of the –10/–35 type "close" in vitro below 15°C, which in turn may prevent protein synthesis for vital proteins below a certain threshold temperature (Minakhin and Severinov, 2003; Severinov and Darst, 1997). (Actually, the lowest temperature reported for growth in *E. coli* is 7°C; Kawamoto et al., 1989). Also, replication (Atlung and Hansen, 1999; Nyborg et al., 2000), protein folding (Seaton and Vickery, 1994; Kandror and Goldberg, 1997; Vickery et al., 1997), and antitermination (Bae et al., 2000) are cold-regulated. A list of cold-inducible proteins from *E. coli* is shown in Table 3. The list of cold shock induced genes has been extended recently by a genome-wide transcriptional analysis of cold shocked *E. coli* cells. New genes found by this study include transport or metabolism of diverse sugars and molecular chaperones (mopA, mopB, htpG, and ppiA). However, not all cold shock genes are displayed in this study (e.g. genes of the nusA-pnp operon), since some mRNA's might be to unstable to be detected by this method (Phadtare and Inouye, 2004b).

Some cold-inducible proteins from *B. subtilis* indicate that very different physiological processes such as chemotaxis (CheY), sugar uptake (Hpr), translation (ribosomal proteins S6, L7 and L12), protein folding (PPiB), and general metabolism (CysK, HvC, Gap and triosephosphate isomerase) are temperature regulated (Graumann et al., 1996b). A list of cold-stress induced proteins in *B. subtilis* described has been published by Graumann and Marahiel (1999b), and transcriptional profiling of the cold shock response in this organism was conducted by Beckering et al. (2002) and Kaan et al. (2002).

Antitermination, which is mediated by CspA and other cold-shock induced Csps, was proposed to induce the genes of secondary cold-induced proteins (such as NusA, InfB, RbfA and Pnp) located in the region of the *metY-rpsO* operon. These Csps probably prevent secondary structure formation in the nascent RNA, which causes antitermination in ρ-independent terminator regions. The read-through produces a higher transcript level, which, in turn, increases the translation of such proteins (Bae et al., 2000; Zangrossi et al., 2000). *nusA* is an essential gene and NusA protein governs transcriptional elongation, pausing, termination and antitermination. The core RNA polymerase associates with the sigma factor (*sigA*) to form the holoenzyme that is capable of promoter recognition. As the polymerase complex enters the transcriptional elongation phase, NusA replaces SigA in the complex (Gopal et al., 2001). RbfA associates with the 30S subunit of the ribosome, enabling 16S rRNA maturation and interaction with mRNA (Xia et al., 2003).

Another ribosome associated cold shock protein was discovered recently. Yfia is associated with the ribosomes in *E. coli* (as long as the growth is arrested) and disappears afterwards (Agafonov et al., 2001; Rak et al., 2002).

A random observation of a cold-sensitive laboratory strain of *E. coli* led to the discovery of BipA. BipA was originally described as a protein induced after exposure to permeability-inducing

Table 3. *Escherichia coli* cold-inducible genes and their gene products.

Gene	Product	Reference(s)
aceE	Pyruvate dehydro genase (lipomide)	Jones et al., 1987; Qoronfleh et al., 1992
aceF	Pyruvate dehydro genase (dihydro lipoamide acetyltransferase)	VanBogelen and Neidhardt, 1990; Qoronfleh et al., 1992
ahpC	Alkyl hydroperoxidase reductase	Leblanc et al., 2003
bipA (yihK)	Ribosome associated GTPase	Pfennig and Flower, 2001
crhC	RNA helicase (CrhC)	Chamot et al., 1999
csdA/dead	Cold shock DEAD-box protein A	Jones et al., 1996
cspA	Cold shock protein A	Goldstein et al., 1990
cspB	Cold shock protein B	Etchegaray et al., 1996
cspG	Cold shock protein G	Nakashima et al., 1996
cspI	Cold shock protein I	Wang et al., 1999
des	Desaturase	Sakamoto et al., 1997b; Aguilar et al., 1998
dnaA	DNA A	Atlung and Hansen, 1999
gyrA	Gyrase subunit A	Jones et al., 1992b
hns	H-NS (histone-like protein)	La Teana et al., 1991; Brandi et al., 1994
hscBA	Hsc66 (heat shock protein homolog)	Lelivelt and Kawula, 1995
hupB	Nucleoid-assciated protein HUβ	Giangrossi et al., 2002
infA	Initiation factor-1	Giuliodori et al., 2004
infB	Initiation factor-2	Jones et al., 1987; Qoronfleh et al., 1992
infC	Initiation factor-3	Giuliodori et al., 2004
lpxP	Palmitoleoyl transferase	Carty et al., 1999
nusA	NusA	Jones et al., 1987; Qoronfleh et al., 1992
otsAB	Trehalose synthesis	Kandror et al., 2002
pnp	Polynucleotide phosphorylase	Jones et al., 1987; Qoronfleh et al., 1992; Clarke and Dowds, 1994; Wang et al., 1996; Goverde et al., 1998
rbfA	Ribosome binding factor A	Dammel and Noller, 1995; Jones and Inouye, 1996
recA	RecA	Jones et al., 1987; Qoronfleh et al., 1992
rnr	RNase H	Cairrão et al., 2003
sodA	Superoxide dismutase	Smirnova et al., 2001b
tig	Trigger factor TF	Kandror and Goldberg, 1997
ves	Major cold shock protein family	Yamada et al., 2002
yfia	Ribosome-associated cold shock response protein	Agafonov et al., 1999; Agafonov et al., 2001

protein produced by neutrophils. The function of BipA at low temperature is not known (Pfennig and Flower, 2001).

Another recent finding is the *ves* gene in *E. coli*, which is clearly cold inducible and shares some homology to *cspH*. But a mutant of this gene showed no phenotype at high or low temperature (Yamada et al., 2002).

Organisms other than *E. coli* or *B. subtilis* may exhibit an "untypical" cold shock response. For example, in *Listeria monocytogenes*, a ferritin homolog was found under cold shock conditions and, similarly in *Streptococcus thermophilus*, an iron-binding protein being a member of the Dps family (Nicodeme, 2004 #2525; Hébraud, 2000 #2662). In *Aeromonas hydrophila* no CspA-like protein was found after cold shock, but only to transiently and weakly expressed 11 kDa proteins (Imbert and Gancel, 2004).

The cold shock response is, as shown above, not a single event or a circumscribed response. Normally, cross-protection against other stresses is imprinted on the cells. A few recent reports on this finding include the induction of. barotolerance in Lactobacillus sanfranciscensis after cold stress (Scheyhing et al., 2004), NaCl tolerance in Shewanella putrefaciens (Leblanc et al., 2003), in Listeria monocytogenes sigB induction enhances freezing survival (Wemekamp-Kamphuis et al., 2004b), and survival of Vibrio parahaemolyticus after crystal violet challenge is higher after cold shock (Lin et al., 2004).

Cold Acclimation

The term "cold acclimation" is used for cells that have adapted to low temperature after cold shock and have reached logarithmic growth with a new, now longer doubling time. In cold acclimated cells the internal processes have reached new equilibria, as could be shown for the protein content in *Listeria monocytogenes* (Liu et al., 2002), or the transcriptome in *E. coli* (Polissi et al., 2003). An interesting phenomenon related to cold acclimation is filamentation as exemplified by *Salmonella*, *Escherichia* or *Pseudomonas* strains kept at low temperature (Khan et al., 2003; Mattick et al., 2003a; Mattick et al., 2003b). This is also observed in *Bacillus cereus* and *B. weihenstephanensis* (K. Neuhaus and S. Scherer, personal observation). Whether filamentation of

bacteria grown under various stresses is a response to or just an aftereffect of the stress is not clear. However, a quadruple deletion of the cold inducible MCSP in *E. coli* shows filamentation already at 15°C (Xia et al., 2001b).

Cold Acclimation Proteins

In contrast to cold shock protein (Csp) expression, cold acclimation protein (Cap) expression is at a higher level when the cold shock response has been downregulated (Hébraud and Potier, 1999). Not many reports deal with real Caps. However, both groups of cold-inducible proteins (Cips) are overlapping, since some cold-shock induced proteins have a transient maximum expression level but still show a higher level at low-temperature growth compared to growth at ambient temperatures. Our impression is, however, that the classifications given in the literature are not stringent. For instance, CspB is referred to as a cold shock protein in *B. subtilis*, but its expression is still higher at low than at ambient temperature (Weber and Marahiel, 2002; P. Graumann, personal communication; see the discussion section in Weber and Marahiel, 2002). According to Berger et al. (1997), CspB would be classified as a Cap. Therefore, the classification appears to be inconsistent and depends on the view of the particular researcher (Hébraud et al., 1994; Bayles et al., 1996; Michel et al., 1997; Mitta et al., 1997; Thieringer et al., 1998). Our simplified classification (Fig. 7) is based on Mitta et al. (1997) and Graumann and Marahiel (1999b) and the proposition that Cips are cold-induced proteins, irrespective of the kinetics of their regulation. Cips can be divided into Csps with a transient peak of induction, regardless of the levels attained in comparison to pre-shock levels. The Csps may be divided into class I Csps (with an induction more than 10-fold) and class II Csps (with an induction less than 10-fold). Cold acclimation proteins (Caps) are not strongly cold shock induced (no clear peak), but levels steadily increase after a temperature downshift and remain higher (during the period of low-temperature cellular growth) than levels at ambient temperatures.

In *E. coli* genes showing a higher transcriptional level 5 h after a cold shock to 15°C include genes encoding flagellar proteins, as well as the spermidine acetyltransferase *speG* (Phadtare and Inouye, 2004b). For *Pseudomonas fragi*, many different groups of Cips have been presented, but only four of them have been identified, and these belong to the CspA homology group. Two of them are designated "CapA" and "CapB," and the other two (also heat shock induced) are designated "TapA" and "TapB" (temperature adaptation proteins; Hébraud et al., 1994; Michel, 1996). A further study dealing explicitly with cold-acclimation proteins names the CspA-homolog from *Arthrobacter globiformis* "CapA." The protein level increases after cold shock and remains high for 24 h (Berger et al., 1997). In our opinion, the time period tested may be too short to determine whether this protein is a Csp or a Cap in this organism. When the cells were cold shocked from 25°C to 4°C, they had a subsequent lag phase of 14 h. For comparison, *Yersinia enterocolitica*, which can grow at –5°C like *A. globiformis* (Bergann et al., 1995), was cold shocked from 30°C to 10°C and had a lag of approximately 80 min. The level of the major Csps

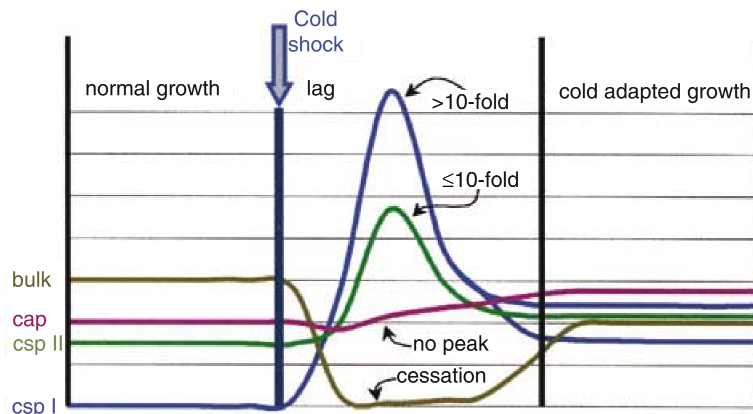


Fig. 7. Qualitative differences between different groups of cold induced proteins (Cips). Bulk proteins (brown) are also designated "housekeeping proteins." After a cold shock, they decrease. Cold shock proteins (Csps) of group I (blue) have low concentrations at ambient temperature but are highly induced (>10-fold) after cold shock. They can remain on a higher or lower level during cold-adapted growth, as indicated by a split of the blue line. Csps of group II (green) are present at normal growth and are induced less than 10-fold. Cold acclimation proteins (Caps, pink) are induced at low temperature, but have no peak after a cold shock. Modified according to Thieringer et al. (1998).

remains stable up to 3 h after cold shock. That means that the proteins are detectable at high level for more than twice as long as the lag phase (Neuhaus, 2000a). If this standard is applied to *A. globiformis*, the relatively large amounts of CspA should be detectable for 28 h, or even longer, before CspA finally disappears. A relatively recent list of cold-inducible proteins of *B. subtilis* and their classification is given by Graumann and Marahiel (1999b). They suggest several different proteins are Caps (e.g., L7/L12, L10, EFTs, EFTu, EF-G, Tig, GlnA, LeuC, ThrC, AroF and GuaB), which play different physiological roles in translation, protein-folding, and general metabolism. Recent studies suggest that in *B. subtilis* the transcription factor SigB, which is induced by cold shock, is important for continuous growth and sporulation at low temperature. Growth of a corresponding mutant is severely impaired at 15°C, but the mutant is rescued by addition of glycine betaine (Brigulla et al., 2003; Mendez et al., 2004). In *Pantoea agglomerans*, a 60-kDa protein was found to be a cryoprotective Cap; however, the nature of this protein is not known (Koda et al., 2001). A recent study with the psychrotolerant *Listeria monocytogenes* lists several genes as necessary for growth at 10°C. Most genes found were already known to participate in other stress responses, e.g., cold-adaptation (*flaA* and *flp*), regulatory adaptive responses (*rpoN*, *lhkA*, *yycJ*, *bglG*, *adaB* and *psr*), general stress (*groEL*, *clpP*, *clpB*, *flp* and *trxB*), amino acid metabolism (*hisJ*, *trpG*, *cysS* and *aroA*), cell surface alterations (*fbp*, *psr* and *flaA*), and degradative metabolism (*eutB*, *celD* and *mleA*). Four further proteins with unknown function, only present in *Listeria*, were found, too (Liu et al., 2002). As can be seen from the diversity of the listed proteins found to be enhanced during growth at low temperature, many different aspects of the cell metabolism are affected to varying degrees. Interestingly, no (classical) MCSP was found, except the well-known cold inducible *flaA* and *flp* genes (Bayles et al., 1996).

For the identification of cold acclimation genes in *Yersinia enterocolitica* during prolonged growth in the cold, a genome-wide expression analysis was performed by creating random transcriptional fusions to the *luxCDABE*-reporter. This approach allowed the direct comparison of promoter activities (by comparing cell growth rates) of various genes at normal and low temperature. Out of 5700 investigated lux-transposon mutants, approximately 100 genes were detected with enhanced promoter activity at low temperature (compared to promoter activity under optimal growth temperature). Most of these genes could be placed into functional groups like motility proteins, transport proteins, and regulatory proteins (Bresolin et al., 2004).

In any case, knowledge concerning cold acclimation proteins (which enable bacteria to grow constantly at low temperature) is limited, and further research in this area is needed. It would be especially interesting to examine psychrotolerant or psychrophilic organisms in contrast to their mesophilic counterparts. Such a comparison could be fruitful in genera containing psychrophile, mesophile, and thermophile species, such as Bacillus and others.

Compatible Solutes

An emerging result from cold shock studies is that compatible solutes (such as glycine betaine, L-proline, and similar compounds) play also an important role in adaptation to the low temperature. However, how compatible solutes protect the cells against low temperature remains unclear. Several, possibly overlapping, scenarios are conceivable according to (Kandror et al., 2002): 1) compatible solutes may act as "chemical chaperones" against low temperature denaturation or aggregation. 2) Cold stress may cause oxidative stress, too. Some compatible solutes, e.g., trehalose, act as free radical scavengers. 3) Compatible solutes protect the membrane or 4) compatible solutes are induced in expectance of a possible drop below freezing.

Listeria monocytogenes has at least two compatible solute transporters for glycine betaine: porter I is a Na/glycine betaine symporter and porter II is an ATP dependent transporter. Cold activated uptake of glycine betaine was most rapid between 7°C and 12°C (Mendum and Smith, 2002). Another compatible solute transporter is encoded by the *opuC* gene of *Listeria*, encoding for a carnitine transporter. This porter is also induced after chilling, and carnitine is also accumulated after osmotic or low temperature stress (Angelidis et al., 2002a), similar to observations made in *B. subtilis* by Brigulla et al. (2003). However, if the glycine betaine porter II is blocked, the increased carnitine uptake cannot completely restore the cryoprotective effect (Angelidis et al., 2002b). If all three compatible solute transporters are deleted, *L. monocytogenes* is severely impaired in growth at low temperature, but growth is not completely abolished (Wemekamp-Kamphuis et al., 2004a). In *E. coli*, the trehalose synthesis genes *otsAB* and the cryptic promoter (P1) of the *proU* transporter (important for mediating cytoplasmic accumulation of compatible solutes) are induced during low-temperature growth. Therefore, the cellular trehalose content increases up to eightfold after cold shock (Kandror et al., 2002; Rajkumari and

Gowrishankar, 2001). If compatible solutes are added to the medium, *L. monocytogenes* and *E. coli* survive better in cold (Dykes and Moorhead, 2001; Shahjee et al., 2002). However, the situation might be different in psychrophilic bacteria, since (Mindock et al., 2001) have found no compatible solute accumulation in such *Arthrobacter* species at 4°C.

Bacterial Cold Sensors

The reaction of bacteria towards cold stress should be as fast as possible. Currently, several processes that use a temperature signal to induce cellular processes have been suggested (for reviews, see Browse and Xin (2001) and Eriksson et al. (2002). Temperature is a factor that affects the whole bacterial cell immediately. Different mechanisms are used simultaneously to sense low temperature and these mechanisms might overlap as in the case of H-NS (see below). Note, furthermore, that many virulent bacteria have specially adapted temperature sensing mechanisms that determine whether they are inside or outside a host (for reviews, see DiRita et al., 2000; Eriksson et al., 2002; Gophna and Ron, 2003). A common threshold temperature for induction of virulence genes is around 30°C. Here, only the mechanisms involved in the cold shock or cold adaptation response (adaptation to temperature much lower than 30°C) will be discussed (see the section Pathogens in this Chapter).

DNA

In bacteria, the degree of superhelicity of the DNA varies in response to changes in the ambient temperatures. In many examples, the expression of genes is dependent on DNA conformation (Eriksson et al., 2002). Supercoiling is mainly regulated in *E. coli* by topoisomerases I and II (Drlica, 1992; Hurme and Rhen, 1998; Tse-Dinh et al., 1997). But the conformation is fine tuned by proteins such as H-NS. H-NS appears to bind curved regions of DNA and is responsible for the cold repression of bacterial genes, possibly by denying open promoter complex formation necessary for transcription (Eriksson et al., 2002; Williams and Rimsky, 1997). Similarly, the promoter of the cold inducible histone-like protein HUβ is possibly affected by temperatures that stop transcription from the promoter sites P1 to P3, but not by temperatures that allow transcription from P4. The HUβ protein affects DNA structure, fine tuning transcription of many genes at low temperature (Giangrossi et al., 2002). A further analysis of the cold shock response on the DNA level is given by Golovlev (2003).

An interesting facet is the involvement of the GATC methylation by the DNA methyltransferase Dam and its possible involvement in the cold shock response. According to this hypothesis, Dam is limited in fast growing cells (e.g. inside a host), leading to a hemi-methylated DNA which is more stable and displays a higher melting point. After shedding of *E. coli* into the environment, the cells experience a cold shock and the transcription of genes containing a GATC cluster will cease due to the high stability of hemi-methylated DNA. This effect might explain the decrease in transcription of some (many?) downregulated genes. However, this conclusions are only hypothetical so far (Riva et al., 2004).

Messenger RNA

As has been discussed in the section The Cold Shock Response, in this Chapter, the mRNA of CspA is degraded rapidly at high temperature but is stabilized at low temperature. The cold sensor, therefore, is the folding characteristics and associated resistance to degradation of the mRNA. This reaction upon temperature downshift occurs rapidly, since the folding involves the mRNA that is already synthesized. Another example is the mRNA of σ^{32}. At lower temperature, this mRNA is folded and therefore cannot be translated. At higher temperature, it is unfolded, becomes accessible to the ribosome, and the translated σ factor then switches on the heat shock response (Morita et al., 1999a; Morita et al., 1999b). A similar mechanism was described for σ^S, a stationary phase sigma factor. The transcription of *rpoS*, the gene for σ^S depends on *dsrA*, a small regulatory RNA which probably stabilizes the *rpoS* mRNA. The half life of *dsrA* is prolonged at 25°C compared to its half life at 37°C (Repoila and Gottesman, 2001). A similar system in which the mRNA acts as thermometer for thermal induction of a gene, is described by Chowdhury (2003) #2661.

Ribosome

That the ribosome may act as a sensor for both heat and cold shock has been proposed (Fig. 8). After heat shock, the A-site of the ribosome is empty; at cold shock, the A-site is blocked owing to a stop in the initiation and translation of misfolded mRNA. Both situations lead to an increase or decrease, respectively, of the stringent response regulator, guanosine 5′-triphosphate-3′-diphosphate and guanosine 5′-diphosphate-3′-diphosphate (collectively abbreviated [p]ppGpp). A decrease could be the signal for a cold shock response (VanBogelen and Neidhardt, 1990). For example, after a nutri-

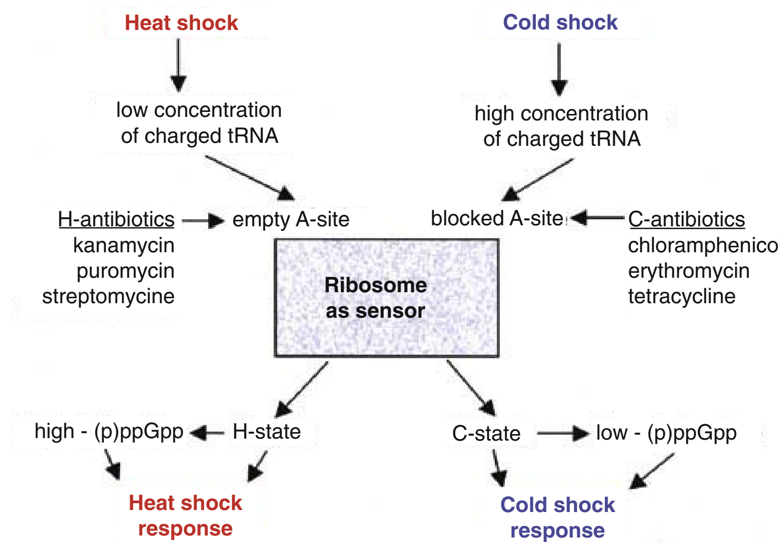


Fig. 8. Model of the ribosome as a sensor of temperature in bacteria. After a heat shock, translation proceeds faster than charged tRNA can be supplied, which may result in an empty A-site that is also affected by H-antibiotics. This could signal the ribosomal induction of the heat shock response (H-state) and increase of the guanosine 5′-triphosphate-3′-diphosphate and guanosine 5′-diphosphate-3′-diphosphate ([p]ppGpp) concentration. In contrast, cold shock leads to a reduced translational capacity of the cell and thereby a block of the A-site (as is also achieved by C-antibiotics) owing to a high concentration of charged tRNA. As a consequence, the cold shock response may be induced and the levels of (p)ppGpp are lowered, which in turn has been shown to increase the induction of cold-inducible proteins (Cips) after a cold shock. Redrawn from Graumann et al. (1996b).

tional downshift, the level of (p)ppGpp is increased and this leads to induction of DnaK and GroEL (both heat shock proteins; Schnier, 1987). Conversely, a nutritional upshift is coupled with a decrease in (p)ppGpp and leads to induction of CspA (Brandi et al., 1999a; Yamanaka and Inouye, 2001a). Additionally, a mutant lacking RelA ([p]ppGpp synthetase) and SpoT ([p]ppGpp hydrolase) is unable to produce (p)ppGpp and has a higher induction of cold shock proteins after a cold shock. This mutant phenotype seems to be preadapted to low temperature (Jones et al., 1992a).

Protein

Changes in protein conformation, namely denaturation, are more pronounced after an increase in temperature. Such misfolded proteins are bound by chaperones, subsequently inducing a heat shock response (Arsene et al., 1999; Eriksson et al., 2002). However, conformational changes in proteins because of low temperature are also used for cold adaptation in some instances.

An intriguing example of low temperature sensing by protein interaction is the aspartate chemotaxis of *E. coli*. The relevant thermosensors are transmembrane chemoreceptors or methyl-accepting chemotaxis proteins, one of them (Tap) being a cold sensor (Nara et al., 1991). During adaptation, receptor methylation (catalyzed by the methyltransferase CheR) and demethylation (catalyzed by the methylesterase CheB) regulate the histidine kinase activity of the sensors. Thermosensing may be due to the specific temperature dependency of the methylation-demethylation equilibrium (Nara et al., 1996; Nishiyama et al., 1997; Nishiyama et al., 1999a; Nishiyama et al., 1999b).

The action of H-NS (see the above section DNA) by influencing the conformation of DNA is itself to some extent dependent on the conformation of this protein. H-NS function is associated with oligomerization by means of a coiled-coil structure. This flexible structure stiffens at lower temperatures allowing better oligomerization and subsequent DNA binding of H-NS (Dorman et al., 1999; Smyth et al., 2000).

The Cytoplasmic Membrane

Another mechanism of cold-temperature sensing involves the physical state of the membrane (for reviews, see Vigh et al., 1998; Sakamoto and Murata, 2002). For instance, it has been proposed that the thylakoid membrane acts as a cellular thermometer where thermal stress is sensed and transduced into a cellular signal leading to

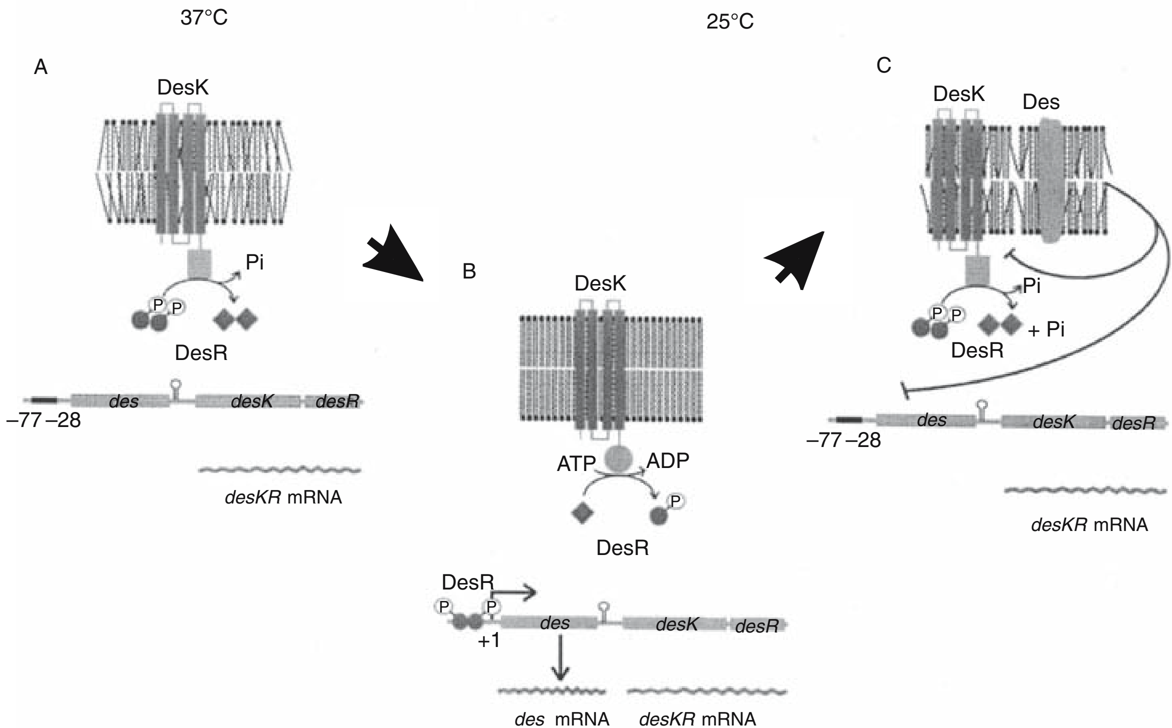


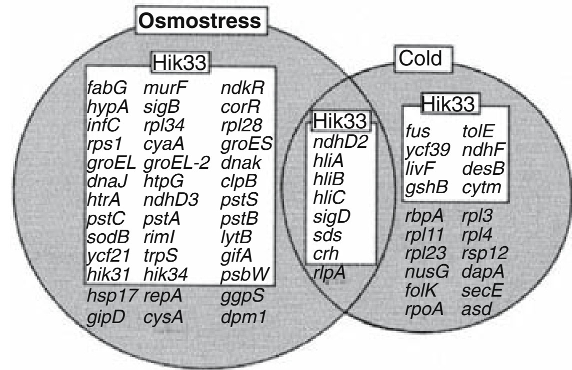
Fig. 9. Model of *des* transcriptional control by two-component temperature signal transduction proteins. It is proposed that DesK assumes different signaling states in response to a temperature-induced change in membrane fluidity. This is accomplished by regulating the ratio of kinase to phosphatase activity such that a phosphatase-dominant state is present at 37°C, when membrane lipids are disordered (A), whereas a kinase-dominant state predominates upon an increase in the proportion of ordered membrane lipids after a temperature downshift to 25°C (B). DesK-mediated phosphorylation of DesR results in transcriptional activation of *des* (B) leading to synthesis of Des, which desaturates the acyl chains of membrane phospholipids (C). These newly synthesized unsaturated fatty acids inhibit *des* transcription either by favoring DesK dephosphorylation of DesR-P or by causing dissociation of DesR-P from its binding site (C). Adapted from Aguilar et al. (2001) and Cybulski et al. (2004).

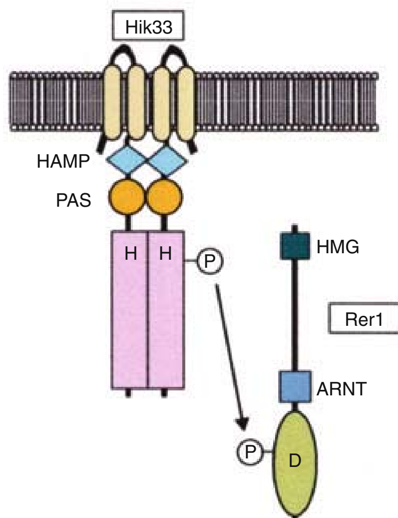
the activation of heat shock (HS) genes (Horvath et al., 1998).

In *B. subtilis*, a two-component signal transduction system was found involving a sensor kinase (DesK) and a response regulator (DesR). This system regulates the cold induction of the *des* gene coding for the D5-lipid desaturase. Unsaturated fatty acids (UFAs), which are the product of Des, act as negative signaling molecules of *des* transcription (Aguilar et al., 2001). Apparently, the physical state of the cytoplasmic membrane regulates the two-component system: DesK phosphorylates a DesR dimer after temperature downshift (which equals to ordered lipids in a more rigid membrane. The phosporylated DesR is able to bind to the promoter of *des*, inducing it thereby. After re-installing the fluid state of the membrane, DesK dephosphorylates DesR to inactivate it (Cybulski et al., 2004). The regulatory loop of DesK, DesR, Des and unsaturated fatty acids is shown in Fig. 9.

In the cyanobacterium *Synechocystis*, two histidine kinases and a response regulator have been identified which regulate several genes at cold temperature (Suzuki et al., 2000a; Suzuki et al., 2000b). Apparently, more than one cold-responding histidine kinase and probably several response regulators should be present in a cyanobacterial cell (Suzuki et al., 2001). Interestingly, the membrane bound histidine kinase senses not only cold but also osmotic stress with some overlap in the induced genes (Mikami et al., 2002). If the membranes are artificially rigidified by gene-engineering, some cold inducible genes are expressed at higher levels in this organism. However, some cold inducible genes do not respond to this intervention, suggesting that another cold sensor remains to be identified (Inaba et al., 2003). A similar two-component system is involved in upregulating phytopathogenic factors in *Pseudomonas syringae*. This system consists of the membrane-bound histidine protein kinase CorS and two transcriptional regulators, CorR and CorP, which induce virulence factors (Smirnova et al., 2002). Figure 11 summarizes the two-component sensor system from *Synechocystis* and *B. subtilis*.

Fig. 10. Osmostress-inducible and cold-inducible genes that were regulated by the sensor histidine kinase Hik33 in wild-type *Synechocystis* cells. Large and small circles enclose genes whose expression was induced by osmotic stress and cold stress, respectively. Rectangles in these circles enclose genes whose expression was regulated to a greater or lesser extent by Hik33 in cells under hyperosmotic stress and under cold stress. Genes outside rectangles appeared to be insensitive to the mutation in Hik33 in terms of their responses to the respective stresses. The rectangle in the overlapping region of the two circles encloses genes whose Hik33-regulated expression was observed under both kinds of stress. From Mikami et al. (2002).





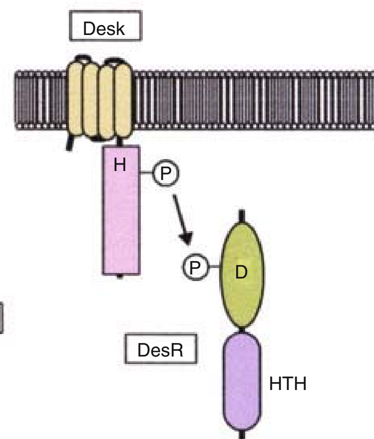


Fig. 11. Schematic representation of the predicted structures of cold-sensing histidine kinases and signal-transducing response regulators involved in the regulation of expression of fatty acid desaturases in *Synechocystis* and *B. subtilis*. (a) Hik33 and Rer1 are the histidine kinase and response regulator, respectively, in *Synechocystis* sp. PCC 6803. In Hik33, the histidine kinase domain is indicated by pink rectangles, and the histidine residue possibly involved in the phosphorylation relay reaction is indicated by "H." The HAMP (histidine-kinase-adenylyl-cyclase-methyl-binding protein phosphatase) domain and the PAS (PER-ARNT-SIM) domain (which is named after the period clock protein [PER] of *Drosophila*, the vertebrate aryl hydrocarbon receptor nuclear translocator [ARNT], and the single-minded protein [Whitby et al.] of *Drosophila*) are indicated by a blue diamond and a yellow circle, respectively. The PAS domain contributes to protein–protein interactions and, thus, Hik33 is likely to form a dimer, as shown in the figure. In Rer1, the receiver domain with a phosphorylatable aspartate residue is indicated by a green ellipse labeled "D." The amino-terminal region is assumed to form a DNA-binding domain, which contains an HMG (high-mobility-group) box and the transcriptional activation domain of the aryl hydrocarbon receptor nuclear translocator (ARNT; (Suzuki et al., 2000a). (b) DesK and DesR are the histidine kinase and the response regulator, respectively, in *B. subtilis*. The DNA-binding domain is indicated by the HTH (helix-turn-helix) motif in the carboxy-terminal region that binds to the 5'upstream region of the *des* gene (Aguilar et al., 2001). In both systems, the cold-induced phosphorylation of histidine residues and the relay of phosphorylation to aspartate residues (indicated by arrows) have not yet been demonstrated. From Sakamoto and Murata (2002).

Cold Adaptation: General Remarks

Mechanisms of cold adaptation in psychrotolerant and psychrotrophic bacteria remain poorly defined. Recent studies suggest multiple strategies to cope with low temperatures. A general conclusion drawn from such strategies is to allow more flexibility in any structures including membranes, proteins or RNAs (Dalluge et al., 1996; Dalluge et al., 1997; Saunders et al., 2003). In membranes, unsaturated fatty acids are introduced to maintain ambient membrane fluidity. Proteins from psychrophilic bacteria are less rigid in structure owing to amino acid exchange (Gerday et al., 1997), or tend to dissociate easier into nonfunctional monomers because of a

weakening in hydrophobic bonds (Jahns and Kaltwasser, 1993; Ramstein et al., 2003).

Cold Adaptation of the Cytoplasmic Membrane

General Strategies of Fatty Acid Alteration

Membrane adaptation to different growth temperatures has been a target of research for a long time (de Mendoza and Cronan, 1983). There is now a large body of data dealing with the effect of temperature on the membrane composition of many species (for reviews, see Russell, 1997; Sakamoto and Murata, 2002). The lipid composition of the cytoplasmic membrane is of great importance for many cellular processes such as nutrient uptake, electron flow in respiration or photosynthesis, ATP synthesis, and others. A biological membrane is a highly complex and dynamic structure that can switch between different physical states. If bacteria are subjected to rapid chilling or freezing, a variety of damages can occur, like damage and release of lipopolysaccharides and altering the permeability of the membrane (Boziaris and Adams, 2001; Kempler and Ray, 1978; Ray and Speck, 1973; Riva et al., 2004). Besides having such mechanical effects, temperature influences the fluidity of the membrane. The lipid composition of the membrane, in combination with the temperature, controls the phase transition from the fluid phase to the semicrystalline or solid phase (Jones et al., 2002; for a review, see, e.g., Dowhan, 1997). To grow at low temperature, cells must have cytoplasmic membranes that retain sufficient fluidity to maintain a physical state supportive of the multiple functions of the membrane-a concept that has been termed "homoviscous adaptation" (Sinensky, 1974; Suutari and Laakso, 1994).

Psychrophilic and mesophilic bacteria, as well as archaea, adjust the lipid composition of their membranes so that the proton permeability of their membranes remains within a narrow range. This phenomenon is termed "homeoproton permeability adaptation" (see Van de Vossenberg et al., 1995). The growth temperature-dependent alterations in fatty acyl chain composition are thus mainly aimed at maintaining the proton permeability of the cytoplasmic membrane at a rather constant level (Albers et al., 2000). For cold-adapted bacteria such as *Psychrobacter immobilis*, this means that a decrease of the temperature would lead to a low proton permeability, which must be counteracted by an appropriate adaptation of the membrane lipids (Fig. 12).

In general, several fatty acid changes are known to increase or decrease membrane fluidity in bacteria (for reviews, see Russell [1997] and Gounot and Russell [1999]). Most important for cold adaptation appears to be both unsaturation and chain shortening, but there are other adaptations (Table 4) which have been demonstrated experimentally. For instance, *lpxP* encodes a palmitoleoyl transferase. Palmitoleate is not present in *E. coli* grown at 30°C but comprises 11% of the fatty acid content in cells grown at 12°C. The *lpxP* gene was found to be 30-fold cold-inducible after 2 h. Thereafter, the activity gradually declines but does not disappear (Carty et al., 1999). A possible advantage suggested is that the palmitoleate content of the outer membrane provides a more effective barrier to harmful chemicals at low temperature (Vorachek-Warren et al., 2002).

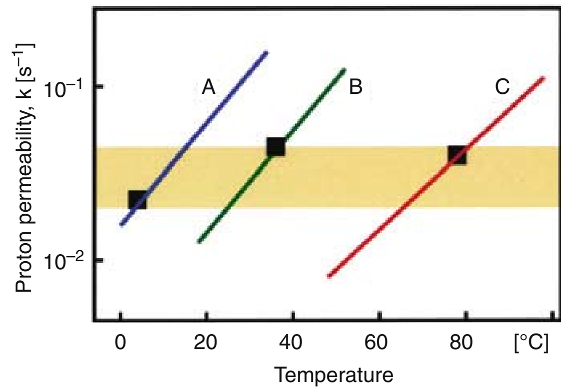


Fig. 12. Graphic representation of the proton permeability of the psychrophilic bacterium *Psychrobacter immobilis* (line A), five mesophilic species represented by line B (*Bacillus subtilis*, *Escherichia coli*, *Methanosarcina barkeri*, *Halobacterium salinarum* and *Halorubrum vacuolatum*), and the hyperthermophilic *Sulfolobus acidocaldarius*, line C. The black squares represent measured proton permeabilities and the yellow area indicates the rather narrow range within which proton permeability is maintained and growth is possible. Note that some thermophilic and hyperthermophilic bacteria have higher proton permeability. See Albers et al. (2000), which is also the source of this figure.

Table 4. Fatty acid changes influencing membrane fluidity in bacteria.

Increase of fluidity		Decrease of fluidity
Unsaturation	↔	Saturation
Cis double bond	↔	*Trans* double bond
Chain shortening	↔	Chain lengthening
Methyl branching	↔	Straight chain
Cis-unsaturation	↔	Straight chain

From Gounot and Russell (1999).

Membranes in Psychrotolerants and Psychrophiles

Recently, rather few reports have dealt with the membrane adaptation of psychrotolerant or psy-

chrophilic bacteria (Jones et al., 1997; Nichols et al., 1997; Whyte et al., 1999; Allen and Bartlett, 2000; Allen and Bartlett, 2002; Drouin et al., 2000; Edgcomb et al., 2000; Kumar et al., 2002). The psychrotolerant *Listeria monocytogenes* is a foodborne pathogen that can grow well at refrigeration temperature. Probably because of the medical importance of *Listeria monocytogenes*, some studies have been performed recently. When grown in continuous culture at 10°C in contrast to 30°C, this bacterium has a lower proportion of *anteiso*-$C_{17:0}$ and a higher proportion of *anteiso*-$C_{15:0}$ and short chain fatty acids (Jones et al., 1997). Similarly, Mastronicolis et al. (1998) found that cold shocked *L. monocytogenes* displayed increased *anteiso*-$C_{15:0}$ in all lipid classes. Studies of fatty acid profiles of wildtype and cold-sensitive, branched-chain fatty acid-deficient mutants of *L. monocytogenes* suggest that the fatty acid 12-methyltetradecanoic acid (*anteiso*-$C_{15:0}$) plays a critical role in low-temperature growth of *L. monocytogenes*, presumably by maintaining membrane fluidity. The fluidity of isolated cytoplasmic membranes of the wildtype, and a cold-sensitive mutant of *L. monocytogenes*, grown with and without the supplementation of 2-methylbutyric acid, has been studied (Annous et al., 1997; Edgcomb et al., 2000). These authors concluded that the fatty acid *anteiso*-$C_{15:0}$ imparts an essential fluidity to the *L. monocytogenes* membrane and that this fluidity permits growth at refrigeration temperatures. However, even between closely related bacteria, differences in the membrane adaptation to low temperature can be found. The *L. monocytogenes* strains Scott A and CNL 895897 show differences in their pattern of branched fatty acids in response to low temperatures. The CNL strain uses, in addition to odd-numbered branched fatty acids found in both strains, substantial amounts of even-numbered branched fatty acids, too (Chihib et al., 2003). A similar example was reported from *Sphingomonas*, where one strain used unsaturated fatty acids and the other strain shifted from even-chain to odd-chain fatty acids (Männistö and Puhakka, 2001a).

The production of increased proportions of membrane unsaturated fatty acids correlates with bacterial growth at low temperature or high pressure (Allen and Bartlett, 2002). Allen et al. (1999) characterized the fatty acids produced by

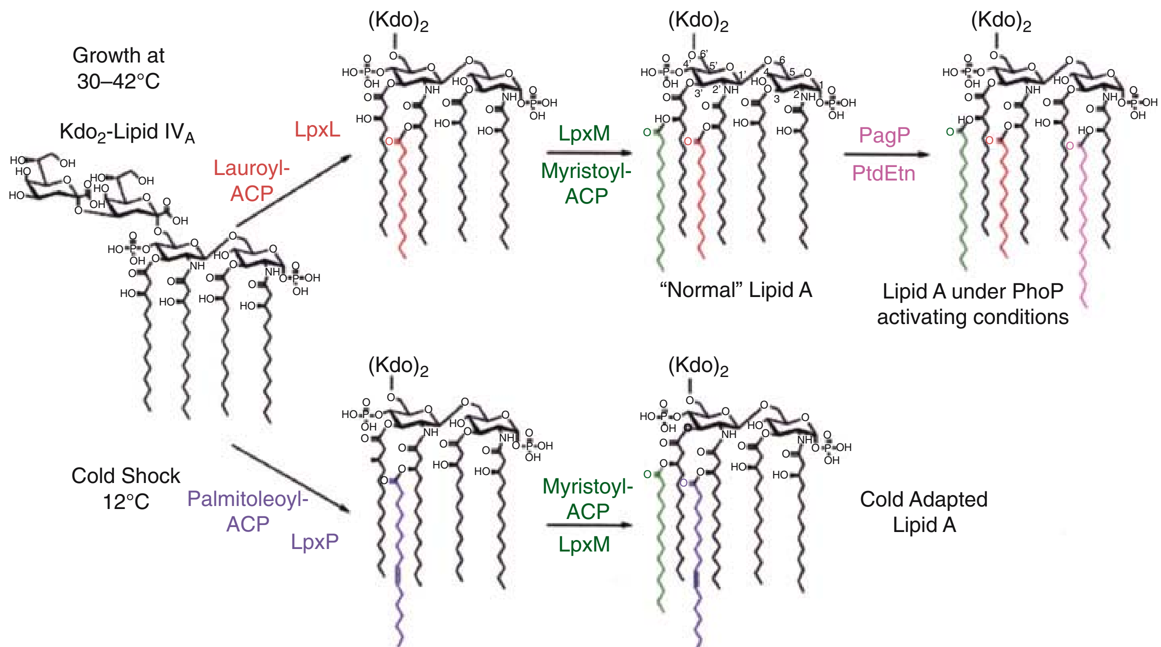


Fig. 13. Biosynthesis of Kdo2 -lipid A during cold shock. In cells grown at 30°C or above, the key precursor Kdo2 -lipid IVA is utilized solely by the lauroyltransferase LpxL (Clementz et al., 1996). However, in cold-shocked cells, an additional acyltransferase, designated "LpxP" (Carty et al., 1999), is induced, which is proposed to incorporate palmitoleate at the same site normally reserved for laurate. In wildtype cells, the action of LpxL and LpxP is followed rapidly by the myristoyltransferase, LpxM, generating hexa-acylated lipid A (Brozek and Raetz, 1990; Clementz et al., 1997). About two-thirds of the hexa-acylated lipid A isolated from cells grown overnight at 12°C contains palmitoleate, and the remainder contains laurate. When the PhoP/PhoQ system is activated or when cells are grown on ammonium metavanadate, a portion of the lipid A molecules contain a palmitate residue at position 2, which is incorporated by the outer membrane enzyme PagP using glycerophospholipids as palmitate donors (Bishop et al., 2000). From Vorachek-Warren et al. (2002).

the deep-sea bacterium *Photobacterium profundum* grown at various temperatures and pressures. In addition, oleic acid-auxotrophic mutants were isolated. One of these mutants, strain EA3, was deficient in the production of monounsaturated fatty acids and was both low-temperature sensitive and high-pressure sensitive in the absence of exogenous 18:1 fatty acid. The authors conclude that monounsaturated but not polyunsaturated fatty acids are required for growth of *P. profundum* both at high pressure and low temperature.

Note, however, that the fatty acid content does not always change dramatically in response to temperature. Könneke and Widdel (2003) examined a range of sulfate-reducing bacteria in their response of the fatty acid content (unsaturated vs. saturated). The highest levels of *cis*-unsaturated fatty acids was measured in the psychrophilic species, but a substantial response in increasing amounts of unsaturated fatty acids at low temperature was only found in the genus *Desulfobacter*. All other genera (*Desulfofaba*, *Desulfofrigus*, *Desulfotalea*, *Desulforhopalus*, *Desulfococcus*, *Desulfosarcina* and *Desulfovibrio*) responded only with slight changes (Könneke and Widdel, 2003). A similar result was formerly reported about psychrotrophic *Pseudomonas* species (Bhakoo and Herbert, 1980).

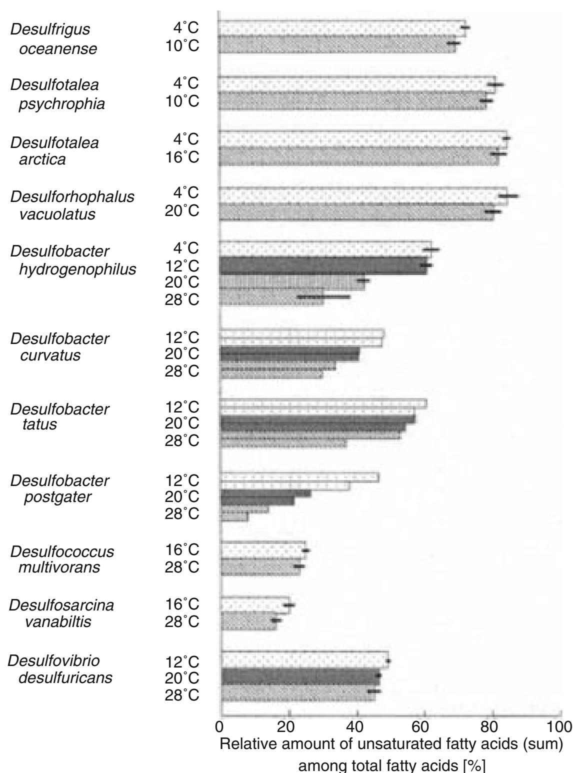


Fig. 14. Proportions of unsaturated fatty acids among total fatty acids in psychrophilic and mesophilic species of sulfate-reducing bacteria grown at different temperatures. Analyses were carried out when cells were still growing and had reached three-quarters of the maximum (final) optical density. Note that only in *Desulfobacter* species a substantial increase in unsaturated fatty acids is visible. In all other species examined virtually no increase in unsaturated fatty acids can be found. Adapted from Könneke and Widdel (2003).

Differences Between Closely Related Mesophilic and Psychrotolerant Strains

Randomly selected strains of a bacterial collection of marine sea-ice bacteria from Antarctica were analyzed to obtain a profile of the membrane fatty acids. Results showed that short-chain saturated and unsaturated fatty acids were more common in the psychrotolerants when compared to psychrophiles. In contrast, branched-chain fatty acids were more abundant in the psychrophiles (Rotert et al., 1993). Such observations raise the question of whether differences in the capability of membrane adaptation to low temperature between closely related psychrotolerant and mesophilic strains (i.e., belonging to the same species) are responsible for the thermal type.

Some species commonly harbor psychrotolerant as well as mesophilic strains. One example is *Rhizobium leguminosarum*, which is known as a mesophilic species growing poorly at temperatures under 10°C (Graham, 1992). However, psychrotolerant strains have been isolated from the Arctic legumes *Astragalus* and *Oxytropis*, and nitrogenase activity in Arctic nodules was detectable down to 0°C. The minimal and maximal growth temperature of isolates was 0°C and 27–30°C, respectively (for a review on Arctic rhizobia, see Prévost et al., 1999). Psychrotolerant and mesophilic strains have also been isolated from the legume species *Lathyus japonicus* and *L. pratensis* (Drouin et al., 2000). These authors have determined the fatty acid profiles after growth at 25°C, at 5°C, and after cold shock from 25°C to 5°C. Interestingly, the degree of psychrotolerance of the strains did not correlate with their fatty acid composition.

There is a vast body of literature concerning mesophilic and psychrotolerant isolates of the *Bacillus cereus*, which is a toxin producer (Granum and Lund, 1997; Dietrich et al., 1999; Prüß et al., 1999) in food (Mayr, 1999), and in soil (Von Stetten et al., 1999). All species of the *Bacillus cereus* group are so similar that they should be within the same species (Helgason et al., 2000). However, because members of the *Bacillus cereus* group are more or less medically important, placing them in a single taxon appears not to be sensible. Therefore, psychrotolerant

isolates have been described as the new species *Bacillus weihenstephanensis* (Lechner et al., 1998). The difference in growth rate of the mesophilic and psychrotolerant strains is shown in Fig. 15. Very little is known about the physiological and genetic basis of cold adaptation of psychrotolerant strains of the *Bacillus cereus* group relative to mesophilic strains. We therefore analyzed the fatty acid composition (among other parameters) of a mesophilic *Bacillus cereus* and a very closely related psychrotolerant *B. weihenstephanensis*. *Bacillus* is known to have a branched-chain fatty acid profile (Table 5). *Iso*- and *anteiso*-branched fatty acids are predominant, which is a characteristic observed in all species of *Bacillus* studied so far (e.g., Kämpfer, 1994). In both strains, *iso*-branched fatty acids increased about 6–7% at 12°C (in comparison to their amounts at 25°C) because of an increase of *i*-13:0 and *i*-16:0. A further increase of *i*-16:0 in the psychrotolerant strain at 7°C increased the level of branched *iso* fatty acids to nearly 50% of the total fatty acids. Upon lowering the temperature, straight-chain fatty acid and monounsaturated fatty acid levels decreased in response to changes in C16 fatty acid levels. These data confirm the hypothesis that bacilli adapt to decreasing environmental temperature by replacing the saturated straight-chain acids with the lower melting point branched-chain acids, or by changing to fatty acid branching instead of fatty acid unsaturation (Suutari and Laakso, 1992). Kaneda (1991) reported that mainly 12- and 13-methyltetradecanoic acids (= *a*-15:0 and *i*-15:0) controlled the fluidity of membranes with branched-chain fatty acids. Indeed, these fatty acids constituted the major fraction in our study at 25°C but decreased at 12°C. At this latter temperature, an increase of *i*-13:0 was observed, which may also play a role in the control of membrane fluidity. In addition, we found some unidentified fatty acids in both strains. Especially, two fatty acids with retention times of 27 and 29 min increased in both strains to high levels upon lowering the temperature.

We did not observe a correlation of the minimum growth temperature with the fatty acid composition. The nearly identical fatty acid pattern of the mesophilic and psychrotolerant *B. cereus* indicate that differences in lipid-dependent membrane architecture may not be responsible for the substantially different growth rates of these strains at 12°C.

Carotenoids in Membranes

Evidence is emerging that carotenoids in the cytoplasmic membrane also play a role in cold adaptation of some species (Jagannadham et al., 1996; Chattopadhyay et al., 1997). In vitro studies with synthetic membranes of phosphatidylcholine demonstrated that the major pigments zeaxanthin, β-cryptoxanthin and β-carotene were bound to these membranes and decreased their fluidity (Jagannadham et al., 2000). In this respect it is interesting that Gram-positive bacteria collected from the Antarctic region show a predominance of pigmented isolates. In *Arthrobacter agilis*, collected from the Antarctic sea ice, pigmentation is due to a C-50 carotenoid induced at low temperature. Hypothetically, such carotenoids stabilize the membrane, since such C-50 carotenoids are only reported from other extremophiles and archea, coping with salt, cold and radiation stress (Fong et al. [2001] and references therein). This speculation fits the observation reported by Varkonyi et al. (2002) that some carotenoids are only low-temperature induced in the thylakoid membranes of the cyanobacterium *Cylondrospermopsis raciborskii*, possibly protecting them from reactive radicals. However, carotenoid-mediated stabilization of membranes and decrease in membrane fluidity seems to offset the increase in membrane fluidity accompanying fatty acid changes in low temperature habitats. Further research is needed to elucidate the interplay between carotenoids and fatty acids at low temperature and the exact role of the former.

Differences Between Thermotypes of Archaea as an Example

A comparison of different thermotypes of microorganisms is possible by comparing single features, e.g., complete genomes or the MCSPs of different *Bacillus* species (Morra et al., 2003; Zeeb and Balbach, 2003a; Zeeb et al., 2003b; Zhou and Dong, 2003). Such an approach was chosen by Saunders et al. (2003), with interesting results for some Archaea: Comparative genomics between the two cold-adapted *Methanogenium frigidum* and *Methanococcoides burtonii* and other mesophile or (hyper-)thermophile Archaea revealed trends in amino acid and tRNA composition and structural features of proteins, which are to some extent applicable to eubacteria. Proteins from the cold-adapted Archaea are characterized by a higher content of noncharged polar amino acids, particularly Gln and Thr, and a lower content of hydrophobic amino acids, particularly Leu. Sequence data from nine methanogen genomes (optimal growth temperature 15–98°C) were used to generate 1111 modeled protein structures. Analysis of the models from the cold-adapted Archaea showed a strong tendency in the solvent-accessible area for more Gln, Thr, and hydrophobic residues and fewer charged residues. A cold shock domain (CSD) protein (CspA homolog) in *M. frigidum*,

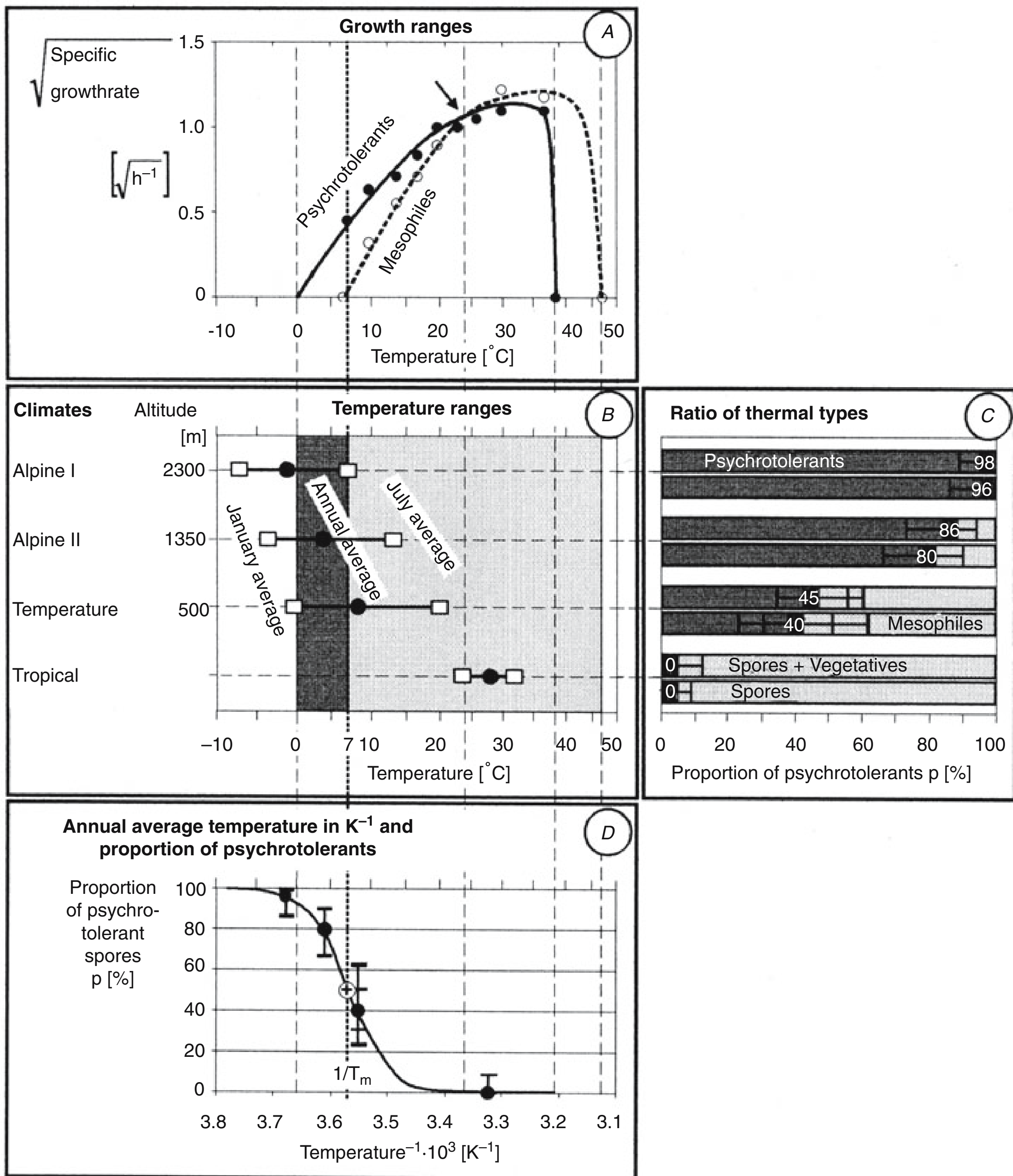


Fig. 15. Difference in growth rate of a mesophilic *Bacillus cereus* and a psychrotolerant *Bacillus weihenstephanensis* strain, and influence of different climates on the ratio of mesophilic and psychrotolerant *cspA* genotypes. To obtain a holistic view, the diagrams have common axes. (B) and (C) share the climate axis, and (A), (B) and (D) share the temperature axes. Temperatures are displayed as both °C and K-1. A) Comparison between the growth ranges of psychrotolerant and mesophilic strains. B) Temperature ranges of a tropical climate, and a climatic sequence, consisting of one temperate climate at 500 m altitude and two temperate high-mountain climates at 1350 m and 2300 m altitude designated as alpine II and alpine I. January and July average temperatures (□) and annual average temperatures (●) are indicated. The white area indicates temperatures below 0°C, where no substantial growth occurs; the darker area marks the range between 0°C and 7°C, where only psychrotolerant strains grow well, and the brighter area highlights the growth range of the mesophilic strains. C) The ratios (in %) of psychrotolerant (dark bar) to mesophilic (bright bar) *cspA* genotypes are displayed for each climate. The upper bar of each pair gives this ratio for the total population, consisting of spores and vegetative cells; the lower bar shows the ratio for spores only. The 95% confidence intervals of the individual assays are indicated by error bars. The bold outer error bars of the temperate sample indicate the estimated intraclimatic mean variation. D) Proportion of psychrotolerant spores over the annual average temperature. This relation can be described by a tangens hyperbolicus function, with its point of inflection () shifted to 7°C. From Von Stetten et al. (1999).

Table 5. Major fatty acids of a mesophilic *Bacillus cereus* WSBC 10030 and a psychrotolerant *Bacillus weihenstephanensis* WSBC 10226 grown at different temperatures in percent.

		Mesophilic strain grown at		Psychrotolerant strain grown at		
RT[b,c]	Fatty acid	12°C	25°C	7°C	12°C	25°C
12.95	*i*-12:0	0.9	1.0	1.6	2.1	1.4
16.37	*i*-13:0	13.4	8.5	14.7	15.3	8.9
19.82	*i*-14:0	4.9	5.6	3.8	4.1	3.4
23.32	*i*-15:0	16.6	18.1	7.5	10.1	13.3
26.48	*i*-16:0	7.0	2.0	18.0	6.8	2.6
29.86	*i*-17:0	2.4	3.1	0.8	2.5	5.1
	Branched, iso	*45.2*	*38.3*	*46.4*	*40.9*	*34.7*
16.64	*a*-13:0	3.9	3.7	6.2	8.8	5.3
23.60	*a*-15:0	7.2	10.2	6.5	8.9	9.0
26.63	*a*-16:0	1.6	4.1	0.0	1.6	3.6
	Branched, anteiso	*13.7*	*20.2*	*13.2*	*20.7*	*20.9*
14.19	12:0	0.4	0.8	1.9	1.8	1.4
21.12	14:0	3.7	5.6	4.4	4.3	4.9
27.88	16:0	7.8	10.4	2.9	5.5	8.1
	Straight, even	*11.9*	*16.8*	*9.2*	*11.6*	*14.4*
27.38	16:1	7.5	10.3	4.3	8.4	11.6
33.35	18:1	1.4	1.5	1.8	1.0	1.7
	Unsaturated, even	*8.9*	*11.8*	*6.1*	*9.4*	*13.3*
25.71	u.i.	1.4	1.2	1.9	1.0	1.4
27.00	u.i.[b]	8.7	4.9	10.1	5.7	3.3
29.00	u.i.[b]	3.7	2.2	7.7	5.3	4.9
29.27	u.i.[b]	0.7	0.7	1.1	1.2	1.4
	Unidentified	*14.5*	*9.0*	*20.8*	*13.2*	*11.0*

The data in the column RT are retention times in minutes. All other numbers are percent of the individual fatty acids. The lines in italic reflect the sum of the indicated type of fatty acid and the lines below show the values of some specific fatty acids within each group.

Abbreviations: WSBC, Weihenstephan Bacillus Collection, Microbial Ecology Group Weihenstephan; RT, retention time; and u.i., unidentified.

[a]Thomas Kaplan and Siegfried Scherer, unpublished results.

[b]Small peaks, representing less than 1% of total fatty acids are not listed.

[c]See Byun et al. (2003).

two hypothetical proteins with CSD-folds in *M. burtonii*, and a unique winged helix DNA-binding domain protein in *M. burtonii* were identified. This suggests that these types of nucleic acid binding proteins play a critical role in cold-adapted Archaea. Structural analysis of tRNA sequences from the Archaea indicated that G+C content is the major factor influencing tRNA stability in hyperthermophiles but not in the psychrophiles, mesophiles or moderate thermo-philes. Below an optimal growth temperature of 60°C, the G+C content in tRNA was largely unchanged, indicating that any requirement for flexibility of tRNA in psychrophiles is mediated by other means.

Recently, a proteomic determination of the cold adaptation in the Antartic archaeon, *Methanococcoides burtonii* has been undertaken. By this approach many proteins necessary for growth at low temperature were described, however, the function and interplay of these proteins are still mostly unknown (Goodchild et al., 2004).

Response of Desaturases to Low Temperature

Both anaerobic and aerobic mechanisms are responsible for the synthesis of unsaturated fatty acids (UFA) in bacteria. The anaerobic pathway, elucidated in detail for *E. coli*, produces *cis*-UFA by a specific 2,3-dehydrase acting at the C-10 level (for a review, see Cronan and Rock, 1996). A second mechanism is the introduction of double bonds into the fatty acids. The reaction is catalyzed by oxygen-dependent desaturation of the full-length fatty acid chain, either as an acyl-thioester or as a phospholipid fatty acid moiety, and requires a specific electron transport chain (see references in Aguilar et al., 1998).

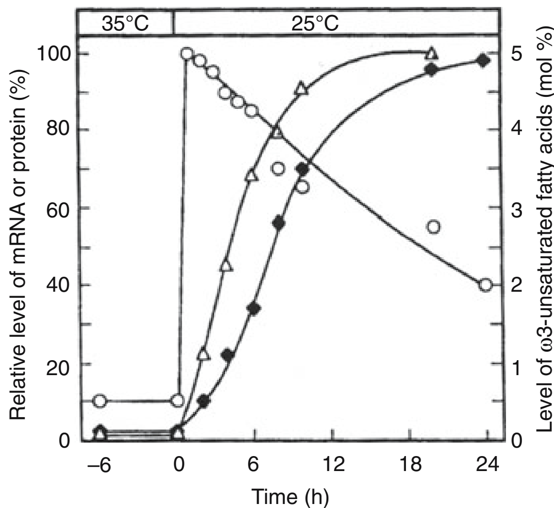


Fig. 16. Changes of levels of *desB* mRNA (open circles), the encoded 3-desaturase (open triangles) and ω3-unsaturated fatty acids (closed diamonds) in *Synechocystis* after a temperature downshift from 35°C to 25°C. From Los and Murata (1999).

The molecular basis of the response of fatty acid adaptation to cold shock has been studied in some detail in unicellular cyanobacteria (Los et al., 1997; Sakamoto and Bryant, 1997a; Sakamoto et al., 1997b; Los and Murata, 1999). In cyanobacteria, four desaturase genes (*desA–desD*) have been reported; *desA*, *B* and *D* have been demonstrated to be cold-inducible in *Synechocystis* (cf. Fig. 16). In addition, *desC* mRNA has been reported to be upregulated within 15 min upon cold shock in *Synechococcus*. This upregulation appears to be due to an increased stability of *des* mRNA at low temperature. A series of mutants was generated by targeted mutagenesis of individual desaturases (Tasaka et al., 1996). Inactivation of *desA* plus *desD* in *Synechocystis* lead to a cold-sensitive phenotype that prevented this mutant from propagating at 20°C. Clearly, the desaturation of membrane lipids is an important factor in acclimation to low temperature.

In contrast to cyanobacteria, *B. subtilis* has only a single desaturase gene, which was described mainly by two groups (Aguilar et al., 1998; Aguilar et al., 1999; Weber et al., 2001b). Cold shock induction of *des* occurs within 30 min and is almost exclusively controlled at the level of transcription, but unlike the situation in cyanobacteria, the stability of mRNA is not increased. Apparently, the *des* gene product is the only component of the *B. subtilis* desaturation system that is regulated by growth temperature. It is a typical transient cold shock induction, which would imply that desaturation does not occur through de novo synthesis of fatty acids.

Surprisingly, a *des* null mutant of *B. subtilis* has no phenotype even when cells are cold-shocked. However, this depends on the presence of isoleucine. In the absence of isoleucine, these mutants were cold sensitive. These data have been interpreted to mean that exogenous isoleucine triggers the switch from *iso*- to *anteiso*-branched saturated fatty acids, providing the organism with a second means to adapt membrane fluidity to low temperature (Klein et al., 1999; Fig. 17).

Adaptation of Protein Synthesis to Low Temperature

Protein Synthesis and the Cold Shock Response

The discovery by Broeze et al. (1978) that the initiation of mRNA transcription is impaired at low temperature indicated that the ribosome is a target of cold shock (Hurme and Rhen, 1998; Perrot et al., 2000). Acting as an RNA chaperone, CspA facilitates initiation and elongation of translation after cold shock (Jiang et al., 1997). Also, a ribosomal protein S21 homolog, which is encoded by *rpsU*, is cold induced in *Sinorhizobium meliloti* (O'Connell and Thomashow, 2000a) as well as in the cyanobacterium *Anabaena variabilis* (Sato, 1994; Sato et al., 1997). This protein may facilitate the binding of mRNA to the ribosome. Interestingly, *rpsU* is located downstream of *cspA* in *S. meliloti*. It may thus help the ribosome to function at low temperatures in the same way as other cold shock proteins (such as RbfA; Jones and Inouye, 1996; Huang et al., 2003). Possibly, small cold shock proteins (e.g., CspA), which appear to be synthesized continuously in some organisms (Graumann et al., 1997; Yamanaka et al., 1999b), may help render the ribosomes able to participate in translation at cold temperatures (i.e., transform them into cold-insensitive ribosomes), but this has not been demonstrated experimentally until now. In any case, by tagging CspB in *B. subtilis* with the green fluorescent protein, and ribosomal protein L1 with the blue fluorescent protein, CspB and ribosomes were seen to colocalize in the cell (Mascarenhas et al., 2001; Weber et al., 2001c).

The level of inactive ribosomes determines the extent of the expression of the cold shock response. Once a balanced translational capacity is achieved, the cold shock response is repressed. At least four proteins (RbfA, initiation factor [IF] 2, CsdA/DeaD, and pY/Yfia) have been proposed as mediators the ribosome's transformation into a cold-insensitive state (Jones and

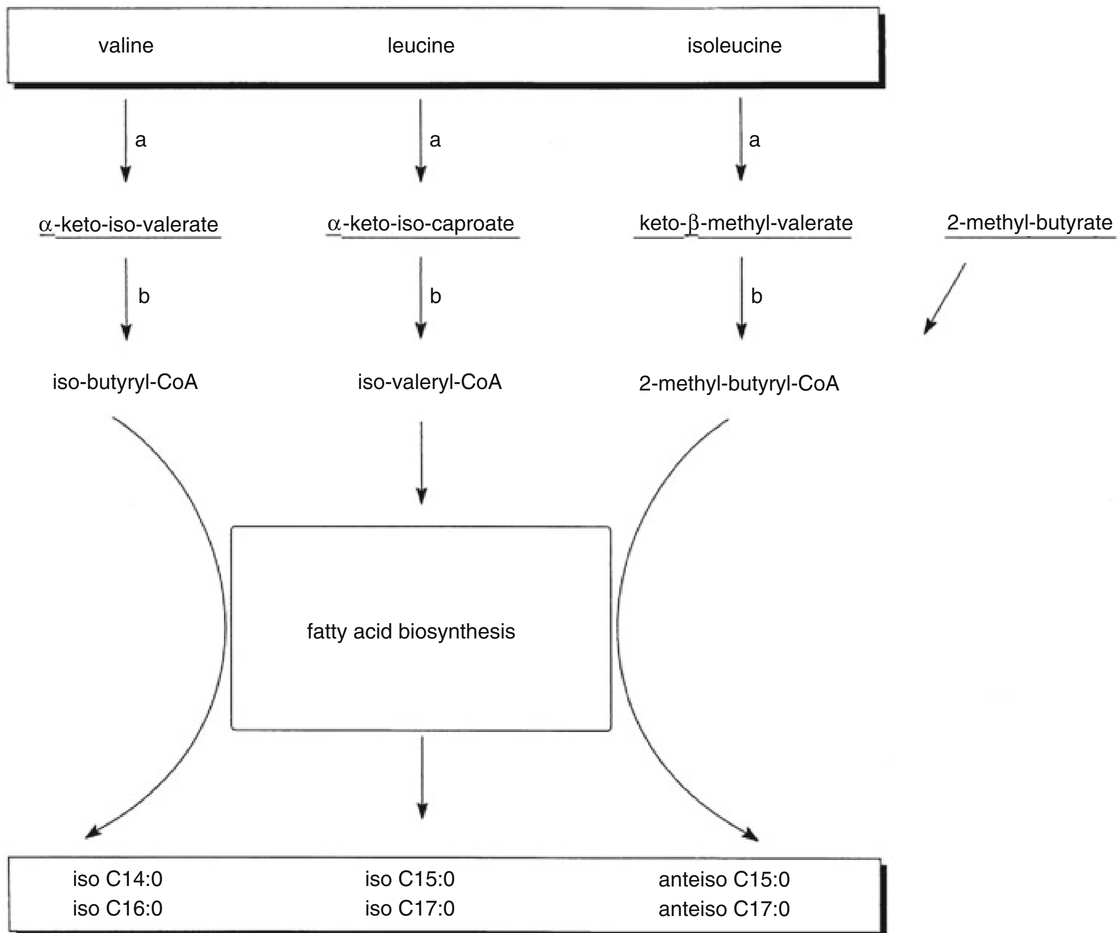


Fig. 17. Schematic representing branched chain fatty acid biosynthesis in *Bacillus subtilis* and its dependence on external supply of valine, leucine and isoleucine. The isoleucine-based pathway offers one possible avenue for membrane adaptation to low temperatures. From Klein et al. (1999).

Inouye, 1996). The ribosomal binding factor A (RbfA) was found to be a suppressor of a cold-sensitive mutation in the 16S rRNA. Cells lacking RbfA exhibit a cold-sensitive phenotype (Dammel and Noller, 1993; Dammel and Noller, 1995), perhaps because the 16S RNA is not processed properly (Bylund et al., 1998). CsdA was found in a 2D-gel analysis of 70S ribosomes from cold-shocked *E. coli* and designated "cold shock DEAD-box protein A." This protein is a homolog of the DEAD-box helicases and possesses RNA unwinding activity. Again, a CsdA mutant is impaired in growth at low temperatures and has the cold-sensitive phenotype of elongated cells (Jones et al., 1996). Recently, it was reported that CsdA is involved in biogenesis of 50S ribosomal subunits. Presumably the RNA helicase activity of CsdA permits a structural rearrangement during 50S biogenesis at low temperature (Charollais et al., 2004). Finally, IF2 is needed for initiation of mRNA translation at the ribosome (Moreno et al., 2000). New data about a ribosome modification after cold shock were published by Agafonov et al. (2001). Ribosomes of cold shocked *E. coli* are shown to be associated with a protein called "PY" or "Yfia" (Rak et al., 2002). However, this protein apparently disappears when the growth arrest is resolved (Kalinin et al., 2002). PY blocks the P as well as the A site of the ribosome, inhibiting translation initiation during cold shock but not under normal growth conditions. Only cold shock genes such as *cspA* may be able to override PY inhibition. By blocking the translation of all but cold shock proteins, the cell diverts all translation factors to the synthesis of cold shock proteins, thus ensuring its survival in the cold (Vila-Sanjurjo et al., 2004). This finding might explain the initiation inhibition after cold shock, originally observed by (Broeze et al., 1978).

O'Connell et al. (2000b) screened cold-shock gene loci in *Sinorhizobium meliloti* by using a

luxAB reporter transposon. Unexpectedly, they found that the transposon of many cold-inducible mutants was inserted in the 16S and 23S rRNA genes. Subsequent experiments confirmed that transcription of all three *rrn* operons of this bacterium is induced by cold shock. Since the number of ribosomes is usually positively correlated with growth rate, one would expect ribosome synthesis inhibition when growth at low temperature is downregulated. However, the cell may upregulate ribosome synthesis because protein synthesis is severely inhibited at low temperature but is needed for survival.

Cold Adaptation of the Ribosome

As has been described above, protein synthesis of mesophilic bacteria is a target of the cold shock response. For protein synthesis, a proper function of tRNA is essential. Many posttranscriptional modifications of tRNA are known. The study of three psychrophilic bacteria from the genera *Moritella* and *Vibrio* revealed that, among other posttranslational modifications, these organisms contained 40–70% more dihydrouridine than did mesophilic bacteria (Dalluge et al., 1997). Nuclear magnetic resonance studies showed that dihydrouridine leads to a higher local flexibility of RNA molecules (Dalluge et al., 1996). Apparently, therefore, the role of the elevated content of this modified nucleoside is to increase local conformational flexibility of tRNA under low temperature conditions where thermal motions and intermolecular interactions of biomolecules are compromised. Interestingly, downstream of the cold inducible *rbfA* gene, a pseudouridine tRNA synthase gene (*trueB*) is located in *E. coli* (S. Scherer and K. Neuhaus, unpublished observations).

In 1969, ribosomes prepared by Nash and Grant (1969) from a psychrophilic *Candida gelida* were inactivated rapidly at 40°C, whereas the ribosomes from a mesophilic *Candida utilis* were unaffected by a similar treatment. Ribosomes prepared by Szer (1970) from a psychrophilic *Pseudomonas* were functional at 0°C and contained a factor which could be washed off, leaving the ribosomes functional at 25–37°C but not at lower temperature. The ribosomes of the mesophile *E. coli* became activated at low temperature upon addition of this factor. Both authors thus concluded that the ribosomes of cold-adapted microorganisms should be structurally different from mesophilic ones, but the identity of the proteins involved is still unknown.

Cold-adapted microorganisms may therefore have structurally different ribosomes when compared to mesophilic bacteria. The comparison of mesophilic and psychrotolerant isolates from the *B. cereus* group showed a systematic difference in the structure of 16S rRNA (Lechner et al., 1998; Von Stetten et al., 1998). Interestingly, both signatures systematically contain A or T in psychrotolerant strains, and G or C in mesophilic strains. One may therefore speculate that the flexibility of the ribosome at low temperature may be increased in some parts of the molecule in the psychrotolerant isolates. However, the occurrence of specific sequence motifs in psychrotolerant strains is not necessarily due to a positive selection pressure associated with this ribosome's function but could be a consequence of neutral drift processes. Therefore, further analysis of the genomic DNA from a wide range of isolates was undertaken (Prüß et al., 1999). This analysis showed that *B. cereus* group strains have between 6 and 10 copies of 16S rDNA. Moreover, a number of these environmental strains have both rDNA operons with psychrotolerant signatures and rDNA operons with mesophilic signatures. The ability of these isolates to grow at low temperatures correlates with the prevalence of rDNA operons having psychrotolerant signatures, indicating specific nucleotides within the 16S rRNA play a role in psychrotolerance (Fig. 18). In vivo measurement of protein synthesis in a psychrotolerant *B. weihenstephanensis* and a mesophilic *B. cereus* clearly showed that ^{35}S-methionine incorporation at low temperature occurs faster by a factor of four (T. Kaplan et al., unpublished data).

Protein Structure and Enzyme Activity

In general, the temperature optima of enzymes from cold-adapted bacteria have been reported to be well above the growth optimum (e.g., Reichhardt, 1998), but those of extracellular enzymes from Arctic and sea ice bacteria have been reported to be as low as 15–20°C (Huston et al., 2000). Usually, the enzyme activity at low temperature is comparatively high (Sun et al., 1998) and the thermostability of cold adapted enzymes is reduced significantly. An example of temperature-dependent activity of the same enzyme isolated from a psychrophilic, mesophilic and thermophilic bacterium is shown in Fig. 19. However, investigation of the molecular basis of cold-active enzymes from psychrophiles has only recently received increased research attention owing to novel opportunities for biotechnological exploitation (Russell, 1998). The application of these enzymes offers considerable potential to the biotechnology industry, for example, in the detergent and food industries, for the production of fine chemicals, and in bioremediation processes (Gerday et al., 2000).

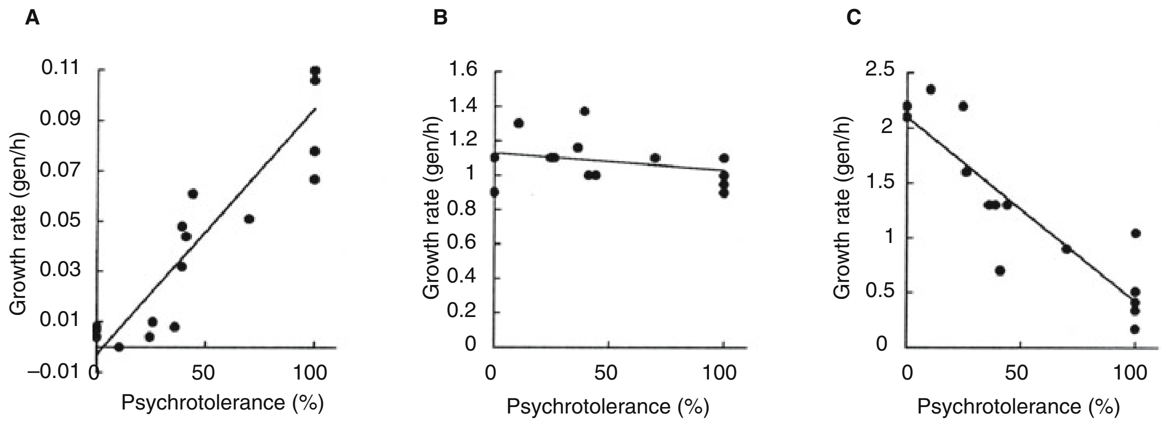


Fig. 18. Comparison of growth rates and psychrotolerance indices. Cultures were grown at 10°C (A), 28°C (B), and 42°C (C). The growth rates are plotted against the percentage of psychrotolerant signatures. A psychrotolerance index of 50% means that one half of the operons of this strain carries the psychrotolerant signature and the other half carries the mesophilic signature. The experiment was done two to six times, and the mean of the populations were determined. Data are from Prüß et al. (1999).

Chemical reactions are characterized by a strong dependency of the reaction velocity on the reaction temperature. The decrease of the rate constant can be described by the Svante Arrhenius equation

$$K = A\ e^{-Ea/RT}$$

where R and T represent the molar gas constant and absolute temperature, respectively, and A is the frequency factor. Typically, a decrease of the reaction temperature by 10°C will lead to a decrease of the reaction rate by a factor of 1.5–4 (Q_{10} value). Notably, the greater the activation energy E_a, the stronger is the temperature dependency of the reaction rate (the reaction rate constant is K). Reactions with low activation energies will only slightly depend on the reaction temperature.

The influence of the reaction temperature on the reaction rate is more complicated when enzyme-catalyzed reactions are considered (for reviews, see Gerday et al., 1999; Lonhienne et al., 2000; Feller, 2003a; Lonhienne et al., 2000). In this case, substrate concentration, enzyme concentration as well as the enzyme-substrate interaction play an important role. At nonsaturating substrate concentration, the reaction velocity depends also on the K_m, which is influenced by the nature of the interaction of the enzyme with the substrate. An electrostatic interaction will be weakened by an increase in temperature, while the hydrophobic interactions tend to be stabilized. Therefore, the reaction velocity of enzymes will be differentially influenced by temperature because of the relative contribution of electrostatic versus hydrophobic forces. Such factors

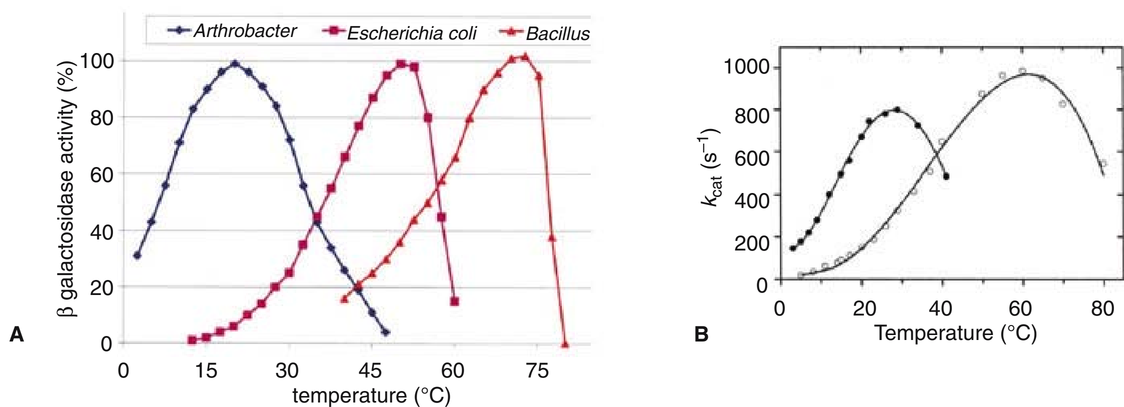


Fig. 19. Graphic comparison of the thermodependence of enzymes. A) β-Galactosidase from the psychrophile *Arthrobacter* D2 (blue), the mesophile *Escherichia coli* (violet) and a thermophilic *Bacillus* (red). Adapted from Brenchley (1996). B) α-Amylase from a psychrophilic (●) and a mesophilic (○) organism.

have to be considered to understand the cold adaptation of enzymes.

Generally, one would assume that enzyme stability, flexibility, and activity have to be properly adjusted to the low temperature. Another aspect of proteins from psychrotrophic organisms is not only the increased thermolability of the protein itself, but also the increased dissociation of monomers or heterodimers. As examples the H-NS-like protein from the psychrophilic *Psychrobacter* should be mentioned. The α-helical domain of this protein displays weaker intermolecular interactions, which may account for the low thermal stability at 37°C (Tendeng et al., 2003). In *E. coli*, H-NS has two isoforms, HUα and the cold-inducible isoform HUβ. HUβ-homodimers show weaker intermolecular interactions (Ramstein et al., 2003).

Recently, many cold active enzymes have been purified and characterized, both at the biochemical and structural level. This section does not cover the biochemical and biophysical aspects; instead, the reader is referred to recent reviews (Gerday et al., 2000; Russell, 2000; Lonhienne et al., 2000; D'Amico et al., 2002a; Feller, 2003a; Feller and Gerday, 2003b) or to Table 6, which lists recent studies of psychrotrophic enzymes. In summary, several adaptations to low temperature are found in different enzymes from psychrophilic bacteria (Table 7). Notably, in no case have all of these adaptations been realized in one protein; each protein has a couple of such changes which is sufficient to render the enzyme cold active. The rules governing their adaptation to cold appear to be relatively diverse, and they are only beginning to be understood.

Due to the widespread occurrence in all thermal types and the relative small size, CspB from *Bacillus* species is a favored model protein for examining thermostabilization, folding and similar structural effects. The findings obtained on CspB might be generalized on other protein families occuring in different thermal types of bacteria, but are beyond the scope of this review. The

Table 6. Recent publications dealing with cold adapted enzymes from psychrotrophic organisms.

Enzyme characterized	Species	Reference(s)
3-Isopropylmalate dehydrogenase	*Vibrio* sp. I5	Svingor et al., 2001
adenylate kinase	*Bacillus globisporus*	Bae and Phillips, 2004
alcohol dehydrogenase	*Moraxella* sp. TAE123	Liang et al., 2004
Alkaline phosphatase	*Shewanella* sp.	Murakawa et al., 2002
Alkaline phosphatase	*Vibrio* sp. AP	Asgeirsson and Andresson, 2001
aminopeptidase	*Colwellia psychrerythraea* strain 34H	Huston et al., 2004
Chitinase	*Alteromonas* sp. O-7	Orikoshi et al., 2003
Chitobiase	*Arthrobacter* sp. TAD1	Lonhienne et al., 2001
Citrate synthase	*Arthrobacter* sp. DS2-3R	Gerike et al., 2001; Kumar and Nussinov, 2004
Dihydrofolate reductase	*Moritella profunda*	Xu et al., 2003b
Esterase	*Psychrobacter* sp. Ant300	Kulakova et al., 2004
Family 8 xylanase	*Pseudoalteromonas planktis*	Van Petegem et al., 2003
Glutamate dehydrogenase	*Psychrobacter* sp. TAD1	Camardella et al., 2002
hydrolytic enzymes	divers	Groudieva et al., 2004
Isocitrate lyase	*Colwellia maris*	Watanabe et al., 2001; Yoneta et al., 2004; Watanabe and Takada, 2004
Lipase	*Pseudomonas fragi*	Alquati et al., 2002
L-Threonine dehydrogenase	*Cytophaga* sp. KUC-1	Kazuoka et al., 2003
malate dehydrogenase	*Moritella* sp. strain 5710	Saito and Nakayama, 2004
Malate synthase	*Colwellia maris*	Watanabe et al., 2001
NAD^+ dependent dehydrogenases	*Shewanella* PA-43	Irwin et al., 2001b
Omithine carbamoyltransferase	*Moritella abyssi*	Xu et al., 2003a
Pectate lyase	*Pseudoalteromonas haloplanktis*	Truong et al., 2001
peptidyl-prolyl cis-trans isomerase	*Shewanella* sp. SIB1	Suzuki et al., 2004
proteases	*Pseudomonas* sp.	Vazquez et al., 2004
Protein-tyrosine phosphatase	*Shewanella* sp.	Tsuruta et al., 2004
Replication protein Rep	*Psychrobacter* sp. TA144	Duilio et al., 2001
Serine hydrolase	*Acinetobacter* sp. No. 6	Suzuki et al., 2002
Serine peptidase	*Shewanella* sp. PA-43	Irwin et al., 2001a
Subtilisin-like serine protease	*Vibrio* sp. PA44	Arnorsdottir et al., 2002
α-Amylase	*Pseudoalteromonas haloplanktis*	Claverie et al., 2003; D'Amico et al., 2002b
β-Galactosidase	*Arthrobacter psychrolactophilus*	Nakagawa et al., 2003
β-Galactosidase	*Arthrobacter* sp. SB	Hoyoux et al., 2001
β-Galactosidase	*Pseudoalteromonas haloplanktis*	Fernandes et al., 2002
β-Galactosidase	*Pseudoalteromonas* sp. TAE 79b	Coker et al., 2003

Table 7. Adaptation of cold active enzymes in comparison to their mesophilic counterparts.

- More polar and less hydrophobic residues
- Additional glycine residues and low arginine/lysine ratio
- Fewer hydrogen bonds, aromatic interactions, and ion pairs
- Lack of or fewer salt bridges
- Additional or extended surface loop(s) with increased polar residues, or decreased proline content (improves solvent interactions), or both
- Modified alpha helix dipole interactions
- Reduced hydrophobic interactions between subunits
- Weaker calcium binding

From Russell (2000) and Arnorsdottir et al. (2002).

reader may consult Garcia-Mira et al. (2004), Makhatadze et al. (2004), Garofoli et al. (2004), and similar publications.

Metabolic Activity and Growth

Bacteria experiencing a cold shock normally adapt by induction of the cold shock response and cold shock acclimation proteins. If they are kept at temperatures below the minimal growth temperature, cells tend to die over time. Estuarine and marine *Vibrio* species seem to disappear under low temperature conditions (e.g., below 15°C) from their habitat but reappear with increasing temperatures. Such organisms enter a so called viable but not culturable (VBNC) state. During this VBNC state, the cells become coccoid, whereas normally they are rod shaped and their metabolic activity is maintained. A resuscitation is possible by shifting the culture to higher temperature (e.g., to 37°C for 24 h) before plating (Carroll et al., 2001; Datta and Bhadra, 2003; Johnston and Brown, 2002). Other organisms are also known to enter a VBNC state after exposure to low temperature, as e.g., *Aeromonas hydrophila* (Mary et al., 2002). The latter strain was reported not to have a Csp similar to CspA of *E. coli*, which might contribute to the entering of a VBNC state (Imbert and Gancel, 2004). The VBNC condition might allow such organisms to become resistant and dormant below temperatures permissive for their growth and survive with a minimal metabolic rate. The lowest temperatures at which metabolically active bacterial communities exist has been reported to be –12°C to –17°C (Carpenter et al., 2000).

Motility

In any environment, bacteria use motility to either find nutrients or associate with a surface. Whether bacteria move in subzero environments, such as sea ice, is unclear since the lowest temperature tested for motility or chemotaxis is 5°C. Though *Colwellia psychrerythraea* was shown to be motile at –10°C, the minimum temperature for its growth is reported as –5°C. The swimming speed dropped to 28 μm/s before ceasing (Junge et al., 2003).

Nutrient Uptake

Nutrient uptake is a basic prerequisite for growth. Algae as well as bacteria have a reduced affinity for nitrate at low temperature (Nedwell and Rutter, 1994), and on the basis of the different nitrate uptake and ammonium uptake responses to temperature, dependency on ammonium as an inorganic nitrogen source is suggested to increase at low temperatures (Ray et al., 1999). Apparently, mesophilic bacteria have an enhanced substrate requirement at minimal growth temperatures (Wiebe et al., 1992). Therefore, one would expect that high activity of transport systems at low temperatures is a prime target of cold adaptation of psychrophilic bacteria (Russell, 1990a). Some reports state that sugar transport is largely independent of temperature in psychrophilic yeast and psychrotolerant bacteria (for a review, see Herbert, 1986). To our knowledge, only one transport system from a psychrophilic bacterium has been studied so far. The $^{14}CH_3NH_3^+$ uptake activity of a psychrophilic marine bacterium *Vibrio* sp. was markedly higher at 0–15°C, and the apparent K_m value for the uptake of $^{14}CH_3NH_3^+$ did not change significantly over the temperature range 0–25°C. Thus, the NH_4^+ transport system of this bacterium was highly active at low temperatures (Chou et al., 1999). Assessment of the temperature dependency of this system (Fig. 20) demonstrated its unusual psychrophilic properties.

Carbon Metabolism and Electron Flow

Carbon metabolism and electron flow is also affected by temperature. In chilling-sensitive cold-stressed plants, a decrease in temperature inhibits respiration, but not much is known about this response in bacteria. Cold stress seems to induce changes in the carbon flow of a given organism, either by increasing cold sensitive key enzymes necessary for certain metabolic pathways or by switching to alternative pathways or cold adapted isoenzymes.

Cold stress induces a change from respiratory metabolism to anaerobic lactate formation in psychrotrophic *Rhizobium* strains (Sardesai and Babu, 2000). Analysis of specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the pentose phosphate pathway showed the upward regulation of alternate pathways of carbohydrate metabolism under cold stress, resulting in rapidly

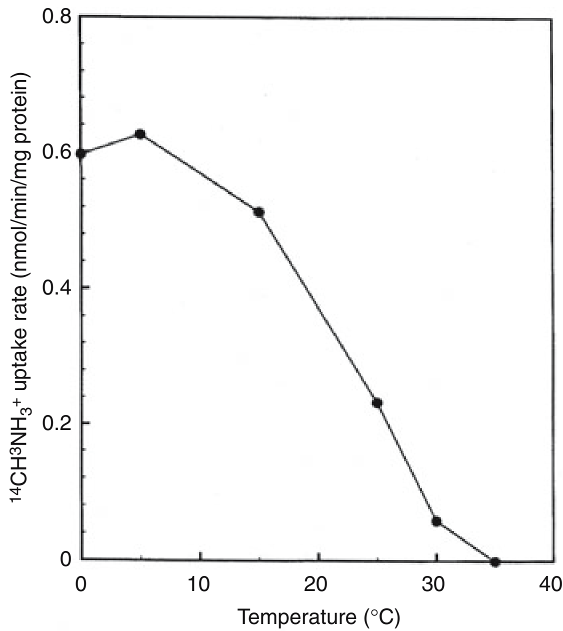


Fig. 20. Temperature dependence of the cold-adapted ammonium uptake system of the psychrophilic marine bacterium *Vibrio* sp. strain ABE-1. From Chou et al. (1999).

generated energy to overcome the stress. The glycolytic activity was also markedly stimulated by a factor of 2.5 in *Lactococcus lactis* upon cold shock from 30°C to 10°C (Wouters et al., 2000a). Upward regulation of malate dehydrogenase suggests that it is a critical input for cold tolerance (Sardesai and Babu, 2001). The cold-stress mediated decrease in the poly-β-hydroxybutyrate (PHB) in a psychrotolerant *Rhizobium* was due to an inhibition of PHB synthesis rather than an increase in its breakdown (Sardesai and Babu, 2001). A downshift in temperature had marked effects on carbon and electron flow in a methanogenic archaeal community in rice field soil, leading to a dominance of psychrotolerant homoacetogenesis (Fey and Conrad, 2000).

Glucose oxidation was also found to be temperature-regulated. At low growth temperature, *Pseudomonas fluorescens* accumulated 2-ketogluconate in the medium as the major oxidation product of glucose. At 30°C, no 2-ketogluconate was excreted at any time (Lynch et al., 1975a; Lynch et al., 1975b). Also, a marked effect of temperature on diauxic growth with glucose and organic acids was observed in this bacterium. Organic acids were preferentially used at 30°C during the first growth phase, and glucose utilization was delayed until onset of the second growth phase. At 5°C, glucose utilization was not repressed during the first growth phase (Lynch and Franklin, 1978). Another psychrotrophic member of this genus, *Pseudomonas syringae*, upregulates urocanase for histidine utilization upon a temperature downshift (Janiyani and Ray, 2002). To cope with cold, psychrophilic *Colwellia maris* expresses a thermolabile isocitrate lyase. This isocitrate lyase is able to utilize its substrate at lower temperatures because of a lower temperature optimum. The same organism has two isocitrate dehydrogenase isoenzymes: one with mesophilic (IDH-I) and the other with psychrophilic characteristics (Ochiai et al., 1979). Accordingly, both the tricarboxylic acid and glyoxylate cycles are important for growth in cold (Watanabe et al., 2002).

A lipase produced by a psychrotrophic *Pseudomonas* strain was found to have the lowest temperature optimum of 35°C in vitro, to have higher activity at low temperature, and to be thermolable compared to other lipases from the same enzyme subfamily (Rashid et al., 2001). The authors conclude that this lipase has adapted to function within the growth range of its host (i.e., –5°C to 35°C).

The psychrotrophic *Acinetobacter* sp. HH1-1 undergoes several metabolic changes in adaptation of its carbon metabolism if exposed to low temperature: 1) Isocitrate lyase is mainly found in the culture supernatant at low temperature. Whether this is due to leakage, as the authors of the study suggested, or to active transport is not clear. 2) The cell associated esterase activity increases and seems to be important for growth at low temperature. 3) Extracellular lipolytic enzymes and production of extracellular polysaccharide are negatively affected at lower temperatures (Barbaro et al., 2001).

Growth Rates

Many more physiological processes are adapted to low temperature in psychrophilic microorganisms. Examples are histidine utilization (Kannan et al., 1998), sulfate reduction (Knoblauch and Jørgensen, 1999a; Knoblauch et al., 1999b), transcription (Ray et al., 1999; Uma et al., 1999), adaptation of the outer membrane of Gram-negative bacteria (Ray et al., 1994; De et al., 1997), reduction of the polar polysaccharide capsular layer (Mindock et al., 2001), carotenoid synthesis (Chattopadhyay et al., 1997), or exoenzyme secretion. The latter is even maximal at –2°C to +4°C in four psychrophilic Antarctic bacterial strains (Feller et al., 1994).

In toto, numerous cold-adapted physiological reactions contribute to, and determine, the growth rate of cold-adapted bacteria (see also the section The Cold Shock Response and Cold Adaptation in this Chapter). As a result, at low temperature, growth rate is higher than in mesophiles, and the lower limit is lower. In principle, the lower growth limit is determined by the

freezing temperature of the cytosol. Most cells remain unfrozen at –10°C to –15°C because of the physical properties of the aqueous solvent systems inside and outside the cells (see Russell, 1990a). These physical boundaries thus determine the absolute lower growth temperature limit in general. The deepest temperature of metabolic activity in bacteria has been reported as –17°C (Carpenter et al., 2000).

The maximal specific growth rate of a psychrotolerant *Pseudomonas fluorescens* with respect to temperature was studied, yielding an Arrhenius plot with a drastic change in slope at 17°C (Fig. 21). Over the cold domain (0–17°C), the temperature characteristic was twofold higher than over the suboptimal domain (17–30°C; Guillou and Guespin-Michel, 1996). The protein content was also measured over the entire temperature range and the authors suggest that, below 17°C, protein degradation is inhibited. This influence of low temperature on protein turnover has also been reported for a psychrotolerant *Arthobacter globiformis* (Potier, 1990) and could be an explanation for the higher temperature characteristic of the Arrhenius plot in the low-temperature range. A biphasic behavior of the growth rate Arrhenius plot was also reported for a *Pseudomonas putida* strain (Chablain et al., 1997). It is, however, too early to suggest that this may be a general feature of cold-adapted strains.

Brenchley (1996) noted that only a few studies have been reported on growth rates at low temperatures. Table 8 lists some doubling times at low temperatures that have been compiled from

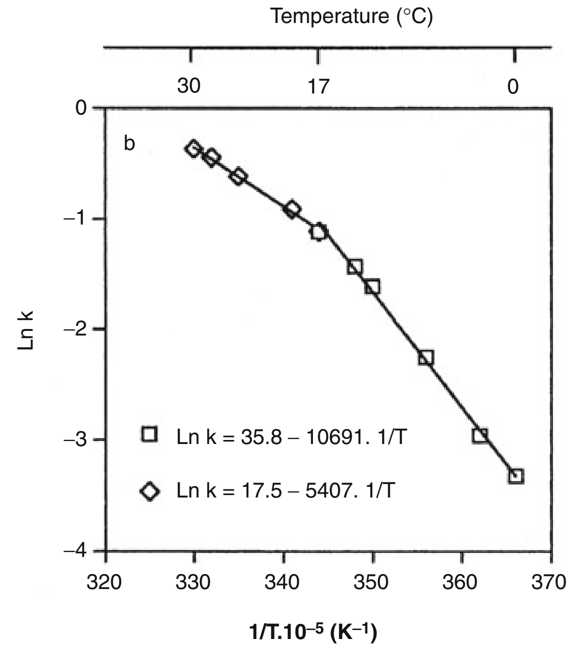


Fig. 21. Biphasic Arrhenius plot of the growth rate of *Pseudomonas fluorescens*. From Guillou and Guespin-Michel (1996).

Table 8. Selected doubling times of cold adapted bacteria.

Species	TT	Temp. (°C)	dt	Reference(s)
Psychrobacter sp. Str. 1	Pt	–10	39d	Bakermans et al., 2003
Frigoribacterium aff. Faeni	Pt	–10	294d	Bakermans et al., 2003
Rhodococcus sp.	Pt	–10	370d	Bakermans et al., 2003
Bacillus psychrophilus	Pp	–5	7h	Morita, 1975
Bacillus sp.	Pp	–2	48h	Inniss, 1975
Pseudomonas fluorescens	Pt	0	28h	Guillou and Guespin-Michel, 1996
Methanogenium frigidum	Pp	0	42d	Franzmann et al., 1997
Yersinia enterocolitica	Pt	0	27h	Neuhaus, 2000a
Carnobacterium funditum	Pt	1	19h	Franzmann et al., 1991
Vibrio marinus	Pp	3	4h	Morita and Albright, 1965
Leuconostoc mesenteroides	Pt	4	24h	Hamasaki et al., 2003
Leuconostoc citreum	Pt	4	52h	Hamasaki et al., 2003
Bacillus sp.	Nd	5	8h	Brenchley, 1996
Psychromonas antarcticus	Pp	5	36h	Mountfort et al., 1998
Rhodoferax antarcticus	Pp	5	60h	Madigan et al., 2000
Pseudomonas sp.	Pt	10	3h	Morita, 1975
Clostridium gasigenes	Pp	10	9h	Broda et al., 2000
Bacillus weihenstephanensis	Pt	10	11h	Prüß et al., 1999
Clostridium algidixylanolyticum	Pt	10	20h	Broda et al., 2000
Desulfotalea psychrophila	Pp	10	27h	Knoblauch et al., 1999b
Desulfofrigus fragile	Pp	10	169h	Knoblauch et al., 1999b
Enterococcus faecalis	Mp	10	50h	Thammavongs et al., 1996
Bacillus cereus	Mp	10	90h	Prüß et al., 1999
Methanogenium frigidum	Pp	15	5d	Franzmann et al., 1997
Yersinia enterocolitica	Pt	15	4h	Neuhaus, 2000a

Abbreviations: TT, thermal type; Pp, psychrophilic; Pt, psychrotolerant; Mp, mesophilic; Nd, not determined; and dt, doubling time.

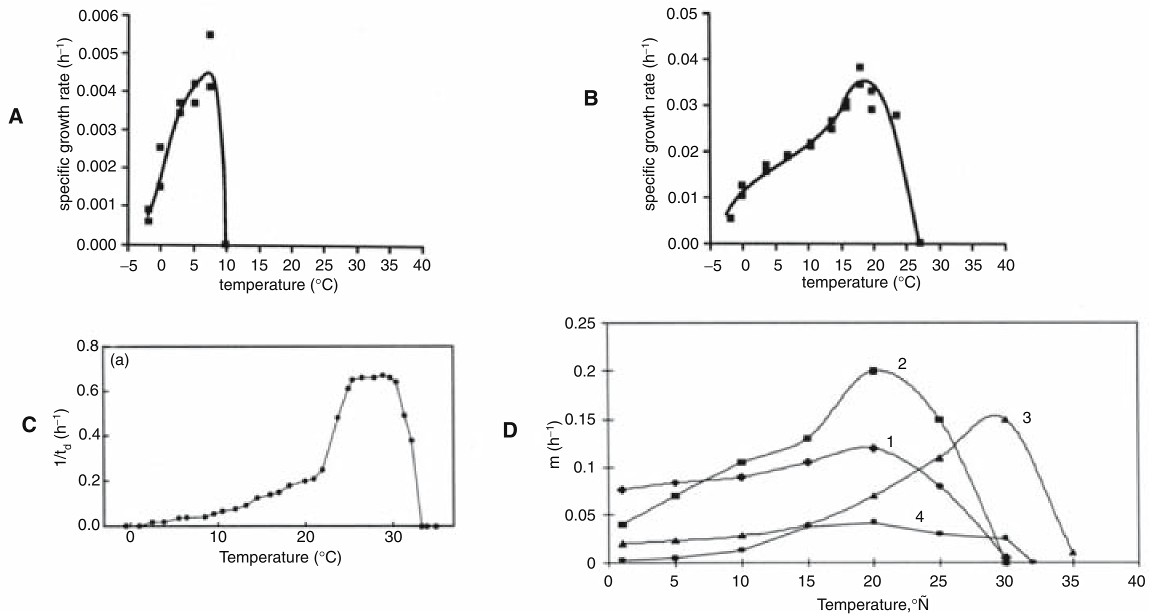


Fig. 22. Thermodependence of growth rates in psychrophilic and psychrotolerant species of bacteria. A) *Desulfofaba* sp. is an extreme psychrophile. B) *Desulfofrigus* sp. is a moderate psychrophile. C) *Clostridium algidixylanolyticum* is a typical psychrotolerant. D) Different species of *Acetobacterium* are compared. 1) *A. bakii* (psychrotolerant, from pond sediments); 2) *A. paludosum* (psychrotolerant, from fen); 3) *A. fimetarium* (mesophile, from manure); and 4) *A. tundrae* (psychrophile, from tundra). From Broda et al. (2000), Knoblauch and Jørgensen (1999a), and Nozhevnikova et al. (2001b).

this and other literature. This list gives a rough idea only, and is not a systematic survey. Three plots of growth rate versus temperature are shown in Fig. 22 for psychrotolerant and psychrophilic strains. The kind of mathematical equation that will describe these functions (and why this would be the case) has been discussed widely, but no firm conclusion could be reached (for a review, see, e.g., Berry and Foegeding [1997] and Gounot [1991]). Please note the very different ranges of growth rates shown in Fig. 22. Note also that the terms psychrophilic and psychrotolerant are defined by the growth ranges and by no means reflect growth rates. For instance, a psychrotolerant *Pseudomonas* has a doubling time of 3 h at 10°C, while a psychrophilic *Desulfotalea* species grows with a doubling time of 27 h at the same temperature. The growth rate depends on the substrate used in the experiment, among other factors. More important, some bacteria such as *Methanogenium* or *Desulfofrigus* are notoriously slow-growing organisms, irrespective of the growth temperature.

Cell Wall

A possibly overlooked phenomenon in response to low temperature might be an increase in cell wall thickness. This has been reported for a cold resistant *Pseudomonas fluorescens*, which showed a 2-fold increase in cell wall thickness, compared to its parent strain, not cold adapted (Khan et al., 2003). Our own observations during RNA extraction from cold shocked and non-cold shocked *Bacillus* strains also pointed in such a direction. Cold shocked cells give significantly reduced yields of RNA, with the same protocol. However, similar yields are obtained by elongated bead beating or sonification (pers. observations).

Environment and Applied Aspects

The rising interest in cold-adapted microorganisms is fueled by a diverse range of aims connected to their explorations. The answers to the following questions will increase understanding of geo-microbiological processes: Which organisms are found in which environment? And, how does low temperature (Stougaard et al., 2002) and climate (Bidle et al., 2002) influence microbial communities? Other questions deal with treatment of contaminated soil or water, usage of cold-adapted enzymes in technical applications (reviewed by Cavicchioli et al., 2002b), influence of temperature on pathogens (often in relation to food), and finally, usage of cold induced promoters for protein production.

A general remark about the occurrence of microbes in the environment was made by Martinus Beijerinck (1851–1931): "Everything is everywhere; the environment selects." This statement applies to microbial thermotypes.

Wherever a cold environment is found, e.g., a cold spring (Rudolph et al., 2001), some alpine meadows (Von Stetten et al., 1999), or a refrigerator somewhere in a jungle village, one can be sure that psychroactive microorganisms can be isolated (M. Neuhaus, personal communication). Conversely, hyperthermophilic organisms have been found in soils worldwide, including permafrost regions (Marchant et al., 2002), and some researchers would extend the search area for extremophile microorganisms into extraterrestrial space (Cavicchioli, 2002a; Mitrofanov et al., 2003). The following sections summarize or mention only recent publications in connection with the aims stated above, and one should be aware that each field overlaps.

Environmental Aspects

Investigating the ecology of bacteria and archaea is vital to understanding the functioning of the global biochemical cycles. Sulfate reducing bacteria and methanogenic archaea are important terminal oxidizers in the anaerobic mineralization of organic matter and can be seen as ecological equivalents, mineralizing organic matter to CO_2 or to CO_2 and CH_4 in, respectively, high- and low-sulfate environments (Purdy et al., 2003). Methanogenesis is also important as a possible climate influence. Methane has a high "green house" effect on the atmosphere. This may explain the great interest in the methanogenesis that occurs in low–temperature environments, which include the sea, the permafrost regions, and deep lakes (Simankova et al., 2003). In low temperature sediments in the Antarctic, *Desulfotalea-Desulforhopalus fulforhopalus* versus *Methanosaeta* appear to be the most abundant species of those groups (Purdy et al., 2003). Ecophysiological processes may change in anaerobic systems under extreme conditions (e.g., freezing). In low-sulfate sediments, H_2-driven methanogenesis was found to be mediated by sulfate reduction. After freezing, both methanogenesis and sulfate reduction decreased. In high-sulfate sediments, sulfate reduction was a major process in frozen and unfrozen samples (Mountfort et al., 2003). In deep lake sediments, a community of psychrophilic methanogens was found, with maximal rates of methane production occurring at 6°C (Nozhevnikova et al., 2003). However, permafrost sediments and other cold environments could also be a sink for methane, since methanotrophic (methane consuming) bacteria have been found in permafrost sediments of Siberia (Khmelenina et al., 2002) and elsewhere (for a review, see Trotsenko and Khmelenina, 2002). In the same environment, anabiotic (dormant) cyst-like bacteria were found in sediment samples drawn from a depth of 50–80 m (Dmitriev et al., 2001), and a variety of psychroactive bacteria have been found at a depth of 11–24 m. Interestingly, few Gram-negative isolates could grow at –10°C (the permanent temperature of the sampling site), but all of these isolates grew optimally at around 25–30°C. Therefore, they have to be classified as psychrotolerant (Bakermans et al., 2003).

Removal of soil contamination is an important issue, especially at low temperature. Petroleum hydrocarbons are the most widespread contaminants in the environment. Cold adapted bacteria able to biodegrade such hydrocarbons are already present in pristine soils but increase as a result of the contamination (Margesin et al., 2003). Similarly, psychrotrophic bacteria from the genera *Shewanella* and *Arthrobacter* have been isolated from oil-reservoir water and have potential for use in bioremediation (Kato et al., 2001). Obviously, microorganisms exist which are able to degrade the hydrocarbons and other organic wastes under such conditions (Männistö et al., 2001b; Männistö et al., 2001c; Baraniecki et al., 2002; Eriksson et al., 2001; Eriksson et al., 2003; Soares et al., 2003; Thomassin-Lacroix et al., 2001; Thomassin-Lacroix et al., 2002).

The poles of the earth (including habitats like sea ice, deep lakes, and similar places) have come into focus. Many scientists are excited by the finding of the big reservoir of liquid water beneath the Antarctic ice shield known as Lake Vostok (Fig. 23). To date, the biological resource of this lake remains untapped, since procedures to remove samples without introducing contamination are still under discussion (Gavaghan, 2002). However, other habitats of Antarctica were examined, and in a permanently frozen

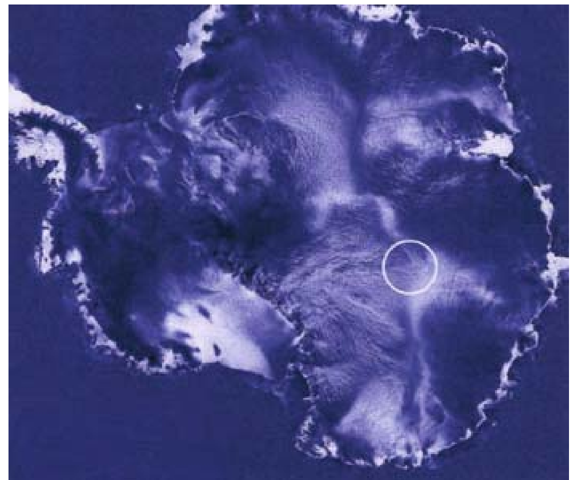


Fig. 23. Lake Vostok (ringed) has lain undisturbed below the ice sheets of Antarctica for many years. From Gavaghan (2002).

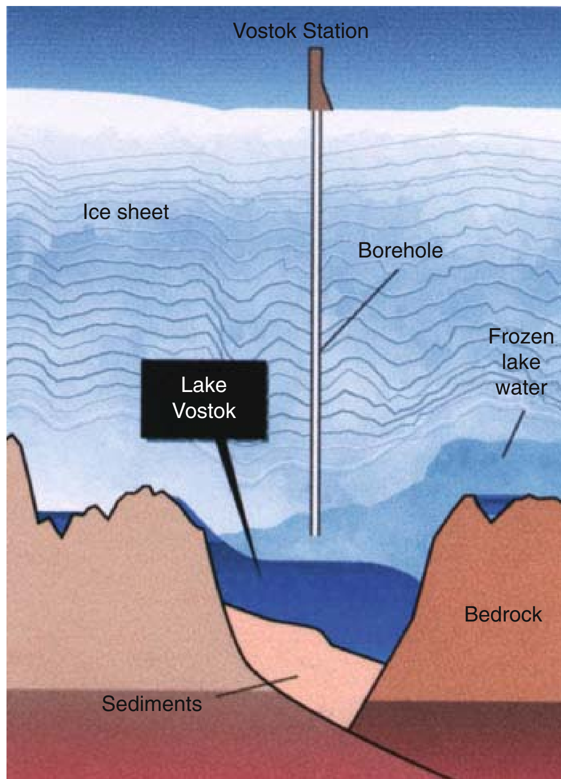


Fig. 24. Schematic view of the borehole to Lake Vostok. It extends beneath Vostok station into areas of frozen lake water but does not reach the lake. From Gavaghan (2002).

Antarctic Lake, a diverse range of phototrophic purple bacteria was found (Karr et al., 2003). This finding is surprising because organisms in Antarctic habitats commonly face continuous low temperatures, as well as poor light conditions and nutrient limitations, making Antarctica appear deserted. But many adapted organisms are thriving below the snow, playing a most fundamental role in the polar ecosystem (for reviews, see Laybourn-Parry [2002]; Thomas and Dieckmann [2002b], and Rossi et al. [2003]).

Technical Uses

An important step in wastewater treatments is the removal of water pollutants by microorganisms. But even in a moderate climate, wastewater temperature may drop to 10°C or 15°C in winter, eventually inhibiting growth of the microbial flora. Under certain conditions low temperature might be beneficial (e.g., low temperature reduces the number of bacteria introduced into the sea by Antarctic research stations; Hughes and Blenkharn, 2003), but mainly it poses a challenge to modern wastewater treatment facilities. Different technical solutions have been proposed to treat wastewater successfully at 13°C (for a review, see Lettinga et al., 2001). Another problem is the huge amount of solids entering sewage treatment facilities. Anaerobic digestion might decrease the amount, but this leads to fouling and biogas (including significant amounts of methane) emission (Nozhevnikova et al., 2001a). Conversely, psychrotolerant nitrifying bacteria may pose a threat to drinking water quality in cold climates (Lipponen et al., 2002).

For extraction of artificially expressed proteins, either for the laboratory (Moran et al., 2001) or for technical uses (Tutino et al., 2001), cold adapted organisms or promoters activated at low temperatures might have certain advantages for the production of thermolabile, toxic, or proteolytically sensitive proteins, for increasing proper folding, increased solubility, or stability (Gonzalez et al., 2003; Mujacic et al., 1999; Takeuchi et al., 2003; Tutino et al., 2001). For more information about the usage of cold inducible promoters in *E. coli*, the reader is referred to the review by Baneyx (1999) or the methodological papers by Baneyx and Mujacic (2003), Qing et al. (2004), and Duilio et al. (2004).

Food Production and Protection

Psychrophilic and psychrotolerant microorganisms are of great importance to the food industry. These organisms are used for direct production, e.g., of dairy products, on the one hand, and may spoil cold stored food or be pathogenic on the other (Russell, 2002).

Lactic acid bacteria (LAB) are traditionally used to produce fermented food, but this heterogeneous group is also used for other purposes, e.g., as probiotics and bioprotectives. Because of the importance and amount of food processed with LAB, a huge body of literature has accumulated that is focused on the cold shock response and cold survival. Different methods are applied to enhance survival of LAB after cold shock or freezing of starter cultures. Exopolysaccharides, overproduction of MCSPs, and employment of other stressful conditions enhances survival of LAB after chilling or freezing (Derzelle et al., 2003; Hong and Marshall, 2001; Maus and Ingham, 2003; Serror et al., 2003a). The discovery of new thermosensitive replicons and two transposons by Serror et al. (2003b) added to the toolbox for manipulating *Lactobacillus* species. For a review of the stress responses in LAB, including cold shock, see Van de Guchte et al. (2002) and the extensive literature survey provided by Carr et al. (2002).

Several studies have been published on the occurrence of psychrotrophic and psychrophilic

bacteria in food matrices. The publications can be divided into those dealing with spoiling organisms and those dealing with survival of certain pathogens under different low temperature conditions. A few recent publications in this field of low temperature research will be briefly mentioned. The smoky odor in chocolate milk stored at 4–9°C is due to guaicol produced by the spoiling organism *Rahnella aquatilis*. This is the first identification of an organism responsible for this type of spoilage (Jensen et al., 2001). Psychrotolerant LAB have been identified as causative agents for spoilage in cooked meat products (Hamasaki et al., 2003). The main flora of cold stored pork meat was found to be *Pseudomonas*, *Aeromonas* and *Acinetobacter* species (Olsson et al., 2003). Neither cold nor carbon dioxide induce a viable but nonculturable state in *Listeria monocytogenes* (Li et al., 2003). However, reduction in the number of this organism is achieved by using essential oils and freezing (Cressy et al., 2003). *Bacillus cereus* can be controlled in chilled dairy products by adding variacin, a lantibiotic produced by *Kocuria varians* (O'Mahony et al., 2001). However, the resistance of *Salmonella* and *E. coli* OH157:H7 (EHEC) to other stresses was increased by subjecting cells to cold temperature beforehand (Bollman et al., 2001; Gawande and Bhagwat, 2002). This increased resistance can be used to advantage in that more viable counts can be obtained by classical plating methods, thereby increasing the sensitivity of pathogen detection (Sol et al., 2002). Other organisms of particular concern able to grow at low temperature include *Clostridium perfringens*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Yersinia enterocolitica*, etc. (Chan et al., 2001; Guentert and Linton, 2003; Harrison et al., 2000; Kalinowski et al., 2003; Steele and Wright, 2001; Zhao et al., 2003). Interestingly, the source of psychrophilic clostridia spoiling vacuum packed chilled meat products is most likely soil particles and fecal material introduced at the abattoir (Boerema et al., 2003).

Reduction or elimination of pathogenic psychrotolerant bacteria is a major aim of food processing. However, the ecology of the pathogen growing in the food matrix is often poorly understood. An elegant in situ method for monitoring a pathogenic *Yersinia enterocolitica* in cheese samples was reported by (Maoz et al., 2002). A full-length *luxCDABE* operon was introduced in the genome of this organism, which carried a constitutive promoter. The emitted light, corresponding to colony forming unit (cfu) counts, was monitored with a sensitive, charge coupled device (CCD) camera. This system does not need the addition of any further substance like antibiotics (to maintain a plasmid) or substrate for the light producing LuxAB enzymes. The influence of, e.g., bioprotective cultures and other means to control the pathogen, can be monitored in situ without laborious cfu plate countings. This technique can be used to monitor other pathogenic organisms (Francis et al., 2000).

Pathogens

Low temperature in connection with pathogens again includes many different facets. Survival of psychrotolerant pathogenic organisms in food has been mentioned in the section above. In this section, some recent findings about the connection between low temperature and pathogens and virulence are highlighted. Temperature regulation of virulence factors has been reviewed by DiRita et al. (2000) and Konkel and Tilly (2000).

Many pathogens regulate virulence genes via temperature sensing mechanisms (see the section Bacterial Cold Sensors in this Chapter). *Yersinia enterocolitica* contains a virulence plasmid, which carries multiple regions of intrinsic curvature. These bends are detectable at 30°C but melt at 37°C, the temperature at which the cells undergo phenotypic switching (Rohde et al., 1999). Other examples of such a behavior can be found in *Vibrio salmonicida*. Disease of Atlantic salmon occurs only if the water temperature is below 10°C. An important virulence factor of this species might be iron siderophores and other iron uptake systems expressed only at the low temperature (Colquhoun and Sorum, 2001). E.g. the main cold shock protein in *L. monocytogenes* seems to be a ferritin-like protein. A similar finding has been reported from *Streptococcus thermophilus*, also expressing an iron binding protein after cold shock (Hébraud, 2000 #2662; Nicodeme, 2004 #2525). In *L. monocytogenes*, the transcriptional activator PrfA controls many virulence genes. The mRNA of this activator acts as thermometer. The 5′ untranslated region renders the ribosome binding site inaccessible at lower temperature (e.g., 30°C) and then switches to an accessible form at higher temperature (e.g., 37°C; Johansson et al., 2002). However, findings showed virulence gene expression at 30°C and below in artificially infected *Drosophila melagonaster* (Mansfield et al., 2003).

Phytopathogens (e.g., *Pseudomonas syringae*) were also found to regulate virulence factors in response to temperature. The phytotoxin coronatine mimics the plant hormone jasmonate. The biosynthesis cluster is regulated by a two-component system, probably sensing membrane fluidity (Smirnova et al., 2002). Thermoregulated expression of virulence factors in plant-associated bacteria has been reviewed by Smirnova et al. (2001a). Conversely, a distinct

Table 9. Recent reviews in connection with life at low temperatures in bacteria

Scope	Title	Reference(s)
Enzymes	Molecular basis of cold adaptation	D'Amico et al., 2002a
Enzymes	Molecular adaptations to cold in psychrophilic enzymes	Feller, 2003a
Enzymes	Psychrophilic enzymes: hot topics in cold adaptation	Feller and Gerday, 2003b
Enzymes	Cold-adapted enzymes: from fundamentals to biotechnology	Gerday et al., 2000
Enzymes	Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility	Lonhienne et al., 2000
Enzymes	Toward a molecular understanding of cold activity of enzymes from psychrophiles	Russell, 2000
Food	A review of aerobic and psychrotrophic plate count procedures for fresh meat and poultry products	Jay, 2002
Food	Bacterial membranes: the effects of chill storage and food processing. An overview	Russell, 2002
Food	Stress responses in lactic acid bacteria	Van de Guchte et al., 2002
Food	The role of cold-shock proteins in low-temperature adaptation of food-related bacteria	Wouters et al., 2000b
General	Cold shock response and adaptation at near-freezing temperature in microorganisms	Inouye and Phadtare, 2004
General	Biology of extremophilic and extremotolerant methanotrophs	Trotsenko and Khmelenina, 2002
General	Life at low temperature	Neuhaus and Scherer, 2004
General	Extremophiles 2002	Rossi et al., 2003
Geology	Extremophiles and the search for extraterrestrial life	Cavicchioli, 2002a
Geology	Life in the deep freeze	Gavaghan, 2002
Geology	Survival mechanisms in Antarctic lakes	Laybourn-Parry, 2002
Geology	Antarctic Sea ice—a habitat for extremophiles	Thomas and Dieckmann, 2002b
Molecular	Temperature sensing and cold acclimation	Browse and Xin, 2001
Molecular	The link between bacterial radiation resistance and cold adaptation	Chattopadhyay, 2002
Molecular	Environmental sensing mechanisms in *Bordetella*	Coote, 2001
Molecular	Low-temperature sensors in bacteria	Eriksson et al., 2002
Molecular	Bacterial cold shock proteins	Ermolenko and Makhatadze, 2002
Molecular	Bacterial cold-shock response at the level of DNA transcription, translation and chromosome dynamics	Golovlev, 2003
Molecular	Transcriptional and post-transcriptional control of cold-shock genes	Gualerzi et al., 2003
Molecular	Conservation of the cold shock domain protein family in plants	Karlson and Imai, 2003
Molecular	Identifying global regulators in transcriptional regulatory networks in bacteria	Martinez-Antonio and Collado-Vides, 2003
Molecular	Recent developments in bacteria cold-shock response	Phadtare, 2004a
Molecular	Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress	Sakamoto and Murata, 2002
Molecular	Biliproteins and phycobilisomes from cyanobacteria and red algae at the extremes of habitat	Samsonoff and MacColl, 2001
Molecular	Control of transcription termination in bacteria by RNA-binding proteins that modulate RNA structures	Stülke, 2002
Molecular	Cold adaptation of archaeal elongation factor 2 (EF-2) proteins.	Thomas and Cavicchioli, 2002a
Molecular	Coping with the cold: the cold shock response in the Gram-positive soil bacterium *Bacillus subtilis*	Weber and Marahiel, 2002
Molecular	Bacterial cold shock responses	Weber and Marahiel, 2003
Molecular	Molecular components of physiological stress responses in *Escherichia coli*	Wick and Egli, 2004
Pathogens	Virulence gene regulation inside and outside	DiRita et al., 2000
Pathogens	Temperature-regulated expression of bacterial virulence genes	Konkel and Tilly, 2000
Technique	Cold-inducible promoters for heterologous protein expression	Baneyx and Mujacic, 2003
Technique	Low-temperature extremophiles and their applications	Cavicchioli et al., 2002b
Technique	Challenge of psychrophilic anaerobic wastewater treatment	Lettinga et al., 2001

group of psychrotrophic *Pseudomonas* species can protect plants by disease-suppression or growth promotion (Johansson and Wright, 2003; Katiyar and Goel, 2003; Mishra and Goel, 1999). One could speculate that this increased plant resistance is due to CspA, which was shown to be a highly active elicitor of tobacco defense responses (Felix and Boller, 2003).

Recently, the general involvement of cold shock genes in virulence has been suggested. Cold-inducible RNases (PNPase and RNase H) were found to be important for full virulence of *Shigella* and enteroinvasive *E. coli* (see Cairrão et al. [2003] and references therein). The PerR regulon of Streptococcus pyogenes is needed for full virulence and contains a Csp (Brenot et al., 2005). Two studies showed a connection between susceptibility to certain antimicrobial substances and the cold shock proteins in *Staphylococcus aureus*: Methicillin resistant *S. aureus* can be treated with the detergent Triton X-100, which reduces the methicillin-resistance. The more resistant a particular strain was before treatment the more its resistance decreased. Comparative proteomics revealed that the MCSPs CspABC of such methicillin-resistant strains (unlike methicillin-sensitive strains) were highly induced (Cordwell et al., 2002). Insertion of a transposon in the *cspA* gene increases the resistance to an antimicrobial peptide of human cathepsin G in the same bacterium (Katzif et al., 2003). Possibly, increased MCSP levels increase the susceptibility to antibacterial substances in *S. aureus*, but more evidence is needed to define the role that CspA plays in such substance resistance. This finding is for some reason contrary to the cross-resistance to other various stresses, which normally increases after induction of the cold shock response and vice versa.

Concluding Remarks

The large number of proteins synthesized upon cold shock as well as in cold acclimation of psychrotolerant microorganisms (see the references in the sections The Cold Shock Response and Cold Acclimation in this Chapter) is clear evidence that many cellular processes contribute to a bacterium's capacity for growth at low temperature. Also obvious is that adaptations at the structural level of rRNA and proteins as well as transient adaptations in the pattern of gene expression are involved in cold adaptation. In toto, a variety of processes thus affect the fitness of cold-adapted bacteria, which, in turn, are important to understand the role of these organisms in their habitat. However, our understanding of the molecular structure of cold adaptation is still in its initial stage. Consequently, our understanding of the evolution of cold tolerance is also quite sketchy. The frequent presence of cold tolerant and mesophilic strains in the same genus or even in the same species suggests that the evolution of psychrotolerance is most probably a multiple step process, which may have occurred many times in parallel.

Even though this review tries to cover many aspects of bacterial life at low temperature, not every area can be covered in detail. Many specialized reviews have been written in recent years. Most of these reviews were cited in the appropriate section above, but to simplify the search for a specific topic, the following table lists reviews or similar articles with a broader scope (Table 9).

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Prokaryotes (2006) 2:263–282
DOI: 10.1007/0-387-30742-7_9

CHAPTER 1.9

Life at High Salt Concentrations

AHARON OREN

Introduction

A great variety of prokaryotes, Bacteria as well as Archaea, can be found in saline and hypersaline environments. These microorganisms are adapted to life at high salt concentrations and to the high osmotic pressure of their environment resulting from the high salinity. This chapter presents a general overview of the hypersaline environments as biotopes for prokaryotic life, the types of organisms encountered in them, and the mechanisms the different groups of prokaryotes have developed to cope with the special requirements of life in the presence of molar concentrations of salt. More detailed information on the variety of halophilic organisms can be found in the specific chapters that deal with the different taxonomic groups.

Saline and Hypersaline Habitats

The greatest part of the biosphere is saline. The waters of the oceans and seas that cover most of the earth's surface contain around 35 g dissolved salts per liter. Higher salt concentrations are often encountered in near-shore environments such as salt marshes, sabkhas and lagoons, under conditions in which evaporation is rapid and water exchange with the open sea is slow. Still higher concentrations of salts, up to saturation of NaCl and beyond, exist in natural inland salt lakes such as the Dead Sea on the border between Israel and Jordan (with total dissolved salts of about 340 g/liter), the Great Salt Lake in Utah (up to 333 g/liter in 1975, although values have decreased since 1975 due to the positive water balance of the lake), and many others. Gradients of increasing salt concentrations are found in the man-made evaporation ponds and crystallizer basins of multi-pond solar saltern systems near tropical and subtropical shores worldwide. All these environments, from seawater salinity to NaCl-saturated brines, are potential habitats for prokaryotic life (Brock, 1979; Rodriguez-Valera, 1988, 1993). Additional hypersaline environments inhabited by salt-tolerating (halotolerant) and salt-loving (halophilic) microorganisms are salted food products such as salted fish, animal hides treated with salt for their preservation, saline soils, and subterranean brines that are often associated with oil fields.

The properties of hypersaline environments as habitats for halophilic and halotolerant prokaryotes are primarily defined according to the total salt concentration. However, also the ionic composition is a key factor determining the properties of the environment as a biotope. Brines that originated by evaporation of seawater (so-called thalassohaline brines) reflect the ionic composition of the sea, at least during the first stages of evaporation (Fig. 1). The ionic composition starts to change significantly when evaporation proceeds to the stage at which the solubility limit of $CaSO_4$ is reached and gypsum precipitates (at a total salt concentration above 100–120 g/liter). The brines that enter saltern crystallizer ponds in multi-pond salterns are thus depleted in calcium and to a minor extent in sulfate. During the subsequent precipitation of NaCl as halite, the ionic composition changes again, and the relative concentrations of K^+ and Mg^{2+} increase. The Great Salt Lake, Utah, though since long detached from the world ocean, still reflects in its ionic composition the seawater that contributed its salt, and therefore its waters can still be classified as thalassohaline. Thalassohaline brines are characterized by neutral or slightly alkaline pH values (7–8).

In other hypersaline environments, the ionic composition may greatly differ from that of seawater ("athalassohaline environments"). The Dead Sea is a prime example of an athalassohaline lake. Here divalent cations dominate, with concentrations of Mg^{2+} (1.89 M) and Ca^{2+} (0.45 M) exceeding those of Na^+ (1.56 M) and K^+ (0.20 M) (1998 values). As a result of the high Ca^{2+} concentration the solubility of sulfate is low, and monovalent anions (Cl^- and Br^-) make up more than 99.9% of the anion sum (Fig. 1). The pH of the Dead Sea brine is relatively low, around 5.8–6.0.

Alkaline athalassohaline brines are relatively abundant. Alkaline "soda lakes" are present in

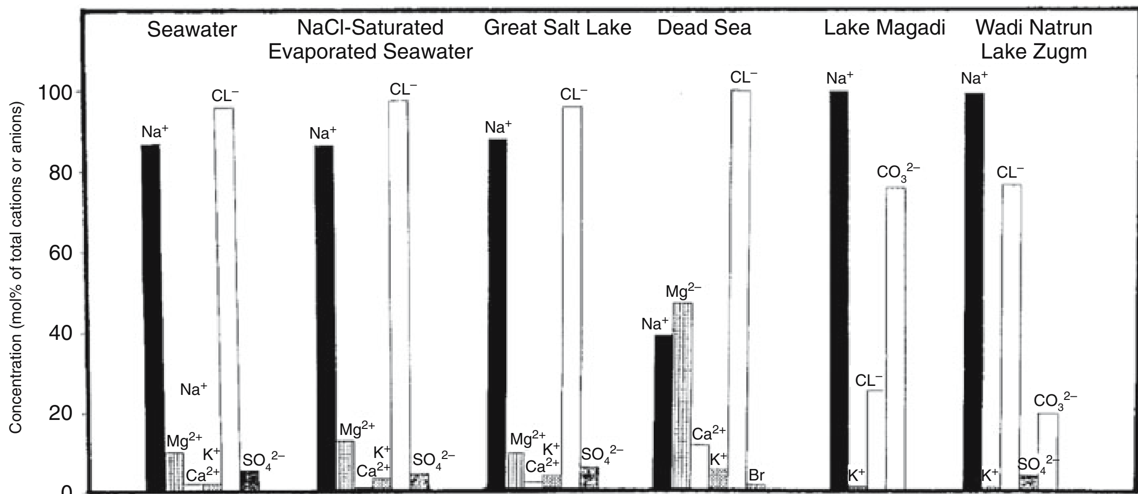

Fig. 1. The ionic composition of seawater and selected hypersaline environments. The bars represent the mol fraction of different cations and anions in the brines. Data for seawater, saltern brines and Great Salt Lake were derived from Javor (1989), and data on Lake Magadi (analyses for 1976) and on Lake Zugm, Wadi Natrun, Egypt, were from Grant and Tindall (1986) and from Grant et al. (1998a), respectively. Dead Sea data (deep water, 1998) were obtained from Michael Beyth (the Israel Ministry of National Infrastructures, personal communication).

diverse geographic locations such as in East Africa (Lake Magadi and other lakes in Kenya and Tanganyika), in the Wadi Natrun in Egypt, and in California, Nevada, India, Tibet, China, and elsewhere. Here the salt composition is dominated by monovalent cations. Because of the high pH (up to 10–11 and higher) the solubility of the divalent cations Mg^{2+} and Ca^{2+} is very low, and the concentrations of these ions may be below the detection limit. Carbonate and bicarbonate ions contribute a significant part of the anion sum in such lakes, in addition to chloride and sulfate.

Classification and Phylogeny of Prokaryotes Living at High Salt Concentrations

Microorganisms adapted to life at high salt concentrations are widespread, both within the bacterial and the archaeal domain. As a result, highly diverse prokaryote communities can be found at all salt concentrations from seawater up to about 340–350 g/liter (brines saturated with NaCl) in both thalassohaline and athalassohaline environments. A few microorganisms can adapt to life over the whole salt concentration range from near fresh water to halite saturation. *Halomonas elongata* is a well-known example of such a bacterium (Vreeland et al., 1980). In most cases, however, each organism has a relatively restricted salt concentration range enabling growth. Some bacteria are adapted to life in saturated and near-saturated brines, being unable to grow and even survive at NaCl concentrations below 15–20%. Most representatives of the halophilic Archaea of the order Halobacteriales show such a behavior. Others thrive at an intermediate salt concentration range. Salt requirement and tolerance may be temperature-dependent, and many cases have been described in which both salt tolerance and requirement are enhanced at increased temperatures (see e.g., Mullakhanbhai and Larsen, 1975).

Different classification schemes have been designed to define the salt relationships of microorganisms. All such schemes are artificial to some extent. Because of the continuum of properties found within the prokaryote world there will always be organisms that cannot unequivocally be classified within any of the groups defined. The most widely accepted classification according to salt dependence and salt tolerance is that of Kushner (1978, 1985), given in a slightly modified form in Table 1. This scheme recognizes different degrees of salt dependence (slightly, moderately, and extremely halophilic). In addition, halotolerant microorganisms exist that, while not requiring high salt concentrations for growth, are able to grow at high concentrations of NaCl and of other salts. *Staphylococcus* species present a good example for this category, as they grow well both in the absence of salt and at NaCl concentrations as high as 10–15% and even higher, a property often exploited in the design of selective and diagnostic growth media. It should be noted that classification should be based not only on

Table 1. Classification of microorganisms according to their response to salt.

Category	Properties	Examples
Non-halophilic	Grows best in media containing less than 0.2M salt	Most freshwater bacteria
Slight halophile	Grows best in media containing 0.2–0.5M salt	Most marine bacteria
Moderate halophile	Grows best in media containing 0.5–2.5M salt	*Salinivibrio costicola*
Borderline extreme halophile	Grows best in media containing 1.5–4.0M salt	*Halorhodospira halophila*
Extreme halophile	Grows best in media containing 2.5–5.2M salt	*Halobacterium salinarum*
Halotolerant	Non-halophile which can tolerate salt; if the growth range extends above 2.5M salt, it may be considered extremely halotolerant	*Staphylococcus aureus*

the behavior toward NaCl but to other ions as well, especially for organisms adapted to life in athalassohaline environments (Edgerton and Brimblecombe, 1981).

The table is based on classification schemes proposed by Kushner (1978, 1985).

Halophilic behavior is found all over the phylogenetic tree of the prokaryotes, both within the Archaea and the Bacteria. Within the archaeal domain, growth at salt concentrations above 15–20% has been documented not only in the Halobacteriales but also in the methanogenic genus *Methanohalophilus* (family Methanosarcinaceae). Most halophilic Bacteria characterized belong to the γ-subdivision of the Proteobacteria, but moderate halophiles can also be found in other subgroups of the Proteobacteria, the low G+C and the high G+C Gram-positive Bacteria, the cyanobacterial branch, the *Flavobacterium* branch, and the Spirochetes (Fig. 2) (Ventosa et al., 1998).

The archaeal order of the Halobacteriales contains the extreme halophiles par excellence. These are highly specialized microorganisms, most of which will not grow at total salt concentrations below 2.5–3 M. When suspended in solutions containing less than 1–2 M salt, cells are irreversibly damaged, and many species will lyse. Some of the early studies on this unique group of prokaryotes were summarized in Larsen's classic essay on "the halobacteria's confusion to biology" (Larsen, 1973), and a full account of their properties can be found elsewhere (e.g., Kushner, 1985; Oren, 1994; Tindall and Trüper, 1986).

The presence of dense communities of members of the Halobacteriales in hypersaline environments often can be observed with the unaided eye thanks to the bright red, orange, or purple coloration of most representatives of the group and to the extremely high community densities at which these Archaea may develop. The occurrence of red hues has been documented for the north arm of the Great Salt Lake (Post, 1977), the Dead Sea (Oren, 1988a), and hypersaline alkaline lakes such as Lake Magadi, Kenya (Grant and Tindall, 1986). Red colored brines also are present typically during the final stages of the evaporation of seawater in solar saltern crystallizer ponds (Borowitzka, 1981; Javor, 1989; Oren, 1993, 1994) (Figs. 3–6). Sometimes other types of microorganisms may also contribute to the color of the brine, such as the β-carotene-rich, green halophilic alga *Dunaliella salina* in saltern ponds (Fig. 6), or photosynthetic purple bacteria of the genus *Ectothiorhodospira*

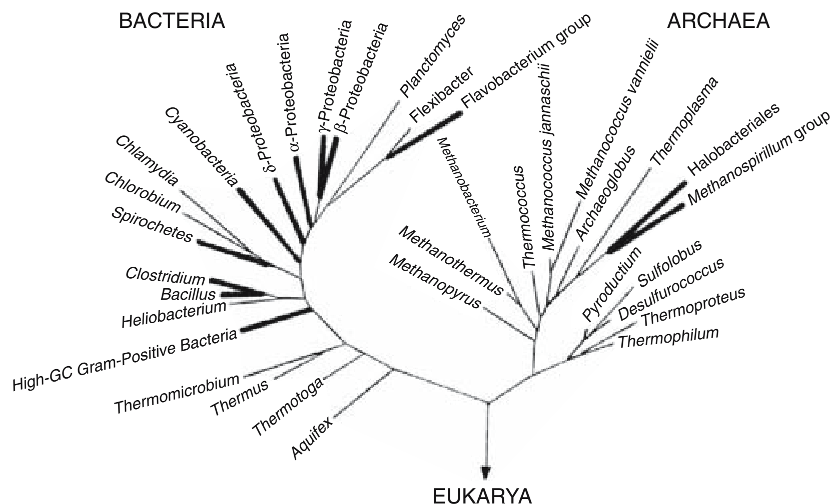

Fig. 2. Phylogenetic tree of the Bacteria and the Archaea, based on 16S rRNA sequence comparisons, indicating the distribution of halophilism. Bold lines indicate branches containing representatives able to grow at or near optimal rates at NaCl concentrations exceeding 15%.

Fig. 3. Saltern crystallizer pond of the Israel Salt Company at the Red Sea coast near Eilat at a total dissolved salt concentration of about 340 g/liter, colored red by halophilic Archaea.

Fig. 5. Saltern ponds of the Cargill Solar Salt Works (Newark, CA), showing a crystallizer pond colored brightly red by halophilic Archaea (courtesy of Carol D. Litchfield, George Mason University, Fairfax, VA).

or *Halorhodospira*, which may contribute at least part of the red coloration of the brines in the alkaline lakes of the Wadi Natrun, Egypt (Jannasch, 1957).

Halophilic Archaea may survive for prolonged periods within halite crystals. This property has aroused considerable interest in recent years, following the isolation of viable halophilic Archaea from salt collected from salt mines dating from the Triassic (195–225 million years B.P.) and Permian (225–270 million years B.P.) periods (Norton et al., 1993). A variety of halophilic Archaea was recently isolated from the Permian Salado salt formation near Carlsbad, New Mexico, including many unknown types (Vreeland et al., 1998). Controversy still exists over whether these bacteria were trapped within the crystals, where they retained their viability, or whether these cells may have entered the salt more recently, during disturbances of the salt layer that were caused by natural phenomena or human activity. Vreeland and Powers (1999) give a critical discussion of the intriguing findings of viable prokaryotic cells within ancient salt deposits. Recently, the heterogeneous 16S rRNA genes in *Haloarcula* isolates from ancient salt deposits have been compared with those of modern strains in the expectation that there are fewer differences between the genes of truly ancient *Haloarcula* than in modern strains if the gene multiplicity originated by duplication. No indications were found that the genes from present-day strains are more divergent than the ancient ones (Grant et al., 1998b).

Fig. 4. Saltern crystallizer pond of the Israel Salt Company at the Mediterranean coast near Atlit at a total dissolved salt concentration of about 340 g/liter, colored red by halophilic Archaea.

Fig. 6. Brines in crystallizer pond of the Cargill Solar Salt Works (Newark, CA), colored in part red due to dense communities of halophilic Archaea (foreground) and in part showing a more orange color imparted by the β-carotene-rich unicellular green alga *Dunaliella salina*. The total dissolved salt at the time (February 1997, following a period of heavy rains) was about 250 g/liter.

Other taxonomically coherent groups consisting solely or mainly of halophilic microorganisms are the order Haloanaerobiales and the family Halomonadaceae. The Haloanaerobiales form an order of moderately halophilic anaerobic bacteria within the low G+C branch of the Gram-positive Bacteria (Rainey et al., 1995). As discussed below and elsewhere (Oren, 1992), this group is of special interest because the mechanism of salt adaptation used by its members resembles that of the aerobic halophilic Archaea rather than that of the other halophilic or halotolerant Bacteria. The family of the Halomonadaceae (γ-subgroup of the Proteobacteria) contains some of the most versatile prokaryotes with respect to their adaptability to a wide range of salt concentrations. The adaptations of some of its representatives to salt have been studied extensively (Franzmann et al., 1988b; Ventosa et al., 1998).

Thermophilic, Psychrophilic, and Alkaliphilic Halophiles

Among the halophilic prokaryotes some are adapted to other forms of environmental stress in addition to salt stress. Thus, thermophilic, psychrophilic, and alkaliphilic halophiles are known. No acidophilic halophiles have been described as yet. The Dead Sea with a pH of about 6.0 is probably the most acidic environment in which mass development of halophilic Archaea has been reported (Oren, 1988a).

Most aerobic halophilic Archaea of the order Halobacteriales have rather high temperature optima, in the range between 35 and 50°C and sometimes even higher. Growth at high temperatures may be an adaptation to the often relatively high temperatures of salt lakes in tropical areas.

Within the anaerobic Bacteria of the order Haloanaerobiales several moderately thermophilic representatives were described. *Halothermothrix orenii*, the first truly thermophilic halophile discovered, was isolated from Chott El Guettar, a warm saline lake in Tunisia. It grows optimally at 60°C and up to 68°C at salt concentrations as high as 200 g/liter (Cayol et al., 1994). *Acetohalobium arabaticum* strain Z-7492 has a temperature optimum of 55°C (Kevbrin et al., 1995).

Cold-adapted halophiles also occur. The halophilic Archaeon *Halorubrum lacusprofundi* was isolated from Deep Lake, Antarctica, a hypersaline lake in which the water temperature varies according to the season between below zero to +11.5°C. The isolate grows optimally at 31–37°C, but slow growth does occur down to temperatures of 4°C (Franzmann et al., 1988a). In addition, a variety of halophilic and halotolerant Bacteria was isolated from different salt lakes in Antarctica (Dobson et al., 1991; Franzmann, 1991; McMeekin and Franzmann, 1988; McMeekin et al., 1993).

Halophilic Archaea of the order Halobacteriales are abundant in hypersaline soda lakes such as Lake Magadi (Kenya) (Grant and Tindall, 1986; Tindall and Trüper, 1986; Tindall et al., 1980, 1984), the Wadi Natrun lakes (Egypt) (Imhoff et al., 1978, 1979; Soliman and Trüper, 1982), and soda lakes in China (Wang and Tang, 1989) and India (Upasani and Desai, 1990). They may impart a red color to such lakes. These environments are characterized by salinity at or close to halite saturation and contain, in addition, high concentrations of carbonates. The pH values are around 10–11 (Grant and Tindall, 1986). Also anaerobic halophilic alkaliphiles occur in such environments. Lake Magadi was shown to harbor a varied anaerobic community, including cellulolytic, proteolytic, saccharolytic, and homoacetogenic bacteria (Shiba and Horikoshi, 1988; Zhilina and Zavarzin, 1994; Zhilina et al., 1996). The homoacetogen *Natroniella acetigena* was isolated from this environment. Its pH optimum is 9.8–10.0, and it can grow up to pH 10.7 (Zhilina et al., 1996). Anaerobes were also isolated from the alkaline saline Big Soda Lake, Nevada (Shiba and Horikoshi, 1988; Shiba et al., 1989).

Metabolic Diversity of Halophilic Microorganisms

A survey of the halophilic microorganisms for metabolic diversity shows that many, but not all types of dissimilatory metabolism known within the prokaryotic world, can function in hypersaline environments as well. Figure 7 presents an overview of the functional diversity of halophilic prokaryotes, based both on laboratory experiments with isolated cultures and on measurements of the processes as they occur in nature.

Oxygenic photosynthesis by cyanobacteria can occur almost up to NaCl saturation. Though the main planktonic primary producers in most hypersaline environments are eukaryotic algae of the genus *Dunaliella* (Javor, 1989; Oren, 1988a, 1994; Post, 1977), cyanobacteria such as *Aphanothece halophytica* [*Cyanothece*; for a discussion of the problems in the taxonomy of the "Halothece" group see Garcia-Pichel et al. (1998)] are often found abundantly in benthic microbial mats that cover the shallow sediments of salt lakes and saltern ponds, especially in the salinity range between 150–250 g/liter (Oren,

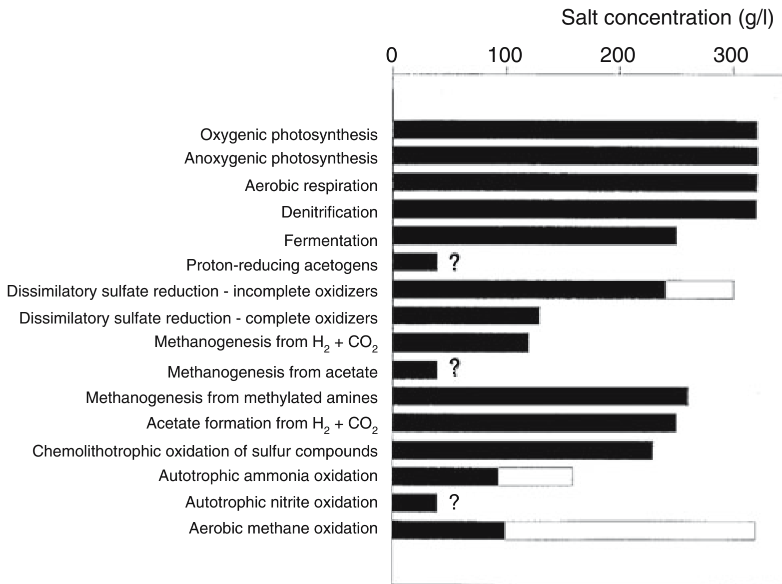

Fig. 7. Approximate upper salt concentration limits for the occurrence of selected microbial processes. Values presented are based in part on laboratory studies of pure cultures (black bars) and on activity measurements of natural microbial communities in hypersaline environments (white bars). Data were derived in part from Brandt and Ingvorsen (1998) , Ollivier et al. (1998), Oremland and King (1989), Oren (1988b, 1999), Sokolov and Trotsenko (1995), Zhilina and Zavarzin (1990), and many additional sources.

Fig. 8. A crust of gypsum densely populated with cyanobacteria (*Aphanothece halophytica* [*Cyanothece*; see Garcia-Pichel et al., 1998] and others) in a saltern evaporation pond of the Israel Salt Company at the Red Sea coast near Eilat at a total dissolved salt concentration of 286 g/liter. The author is sampling the carotenoid-rich upper unicellular cyanobacterial layer and the green layer of filamentous and unicellular cyanobacteria below.

1999). Figure 8 shows an example of a dense benthic community of unicellular cyanobacteria living within a gypsum crust of a saltern pond at a salt concentration of 286 g/liter.

Anoxygenic photosynthetic prokaryotes also abound up to the highest salt concentrations. Examples are representatives of the genus *Halorhodospira*, which contains species such as *Halorhodospira halochloris* and *Halorhodospira halophila* that can be classified as borderline extreme halophiles (see Table 1). *Halorhodospira* cells were documented to impart a bright red color to the alkaline hypersaline lakes of Wadi Natrun, Egypt (Jannasch, 1957). Additional halophilic purple bacteria have been characterized, such as *Halochromatium* and *Thiohalocapsa* (Caumette et al., 1991, 1997; Imhoff et al., 1998). There is also a report that green sulfur bacteria (*Chlorobium* or a relative) may occur at high salt concentrations (Anderson, 1958), but details are lacking.

Most halophilic prokaryotes that have been isolated and studied are aerobic chemoorganotrophs. Aerobic breakdown of organic compounds is possible at salinities up to NaCl saturation. Both halophilic Archaea of the order Halobacteriales and different types of Bacteria may be involved in the breakdown of organic compounds in hypersaline environments. In addition to simple compounds such as sugars, amino acids, etc., also a number of unusual substrates can be degraded at high salt concentrations. Aliphatic and aromatic hydrocarbons, including even- and odd-carbon-number saturated hydrocarbons; saturated isoprenoid alkanes (pristane); different aromatic compounds, including benzoate, cinnamate, and phenylpropionate; and long-chain fatty acids, such as palmitic acid, have been shown to serve as sole carbon and energy sources for certain isolates of halophilic Archaea (Bertrand et al., 1990; Emerson et al., 1994; Kulichevskaya et al., 1991). Degradation of hexadecane was shown in the Great Salt Lake up to a salinity of 172 g/liter (Ward and Brock, 1978). Other unusual compounds shown to be degraded or transformed at high salt concentrations are formaldehyde (Azachi et al., 1995) and organophosphorus compounds (DeFrank and Cheng, 1991). Oren et al. (1992) presented an overview of the potential for breakdown of unusual compounds, including industrial pollutants, at high salt concentrations.

Oxygen is poorly soluble in concentrated brines, and therefore it is not surprising to find a considerable variety of anaerobic halophilic heterotrophs. Many representatives of the aerobic halophilic Archaea of the order Halobacteriales can grow anaerobically by using nitrate as electron acceptor (Mancinelli and Hochstein, 1986). Other potential electron acceptors used by many species are dimethylsulfoxide, trimethylamine N-oxide (Oren and Trüper, 1990) and fumarate (Oren, 1991).

Halobacterium salinarum, but none of the many other aerobic halophilic Archaea tested (Oren and Litchfield, 1999), is able to grow fermentatively on L-arginine (Hartmann et al., 1980). However, the group of halophilic microorganisms that have specialized in anaerobic fermentative growth is that of the Haloanaerobiales (low G+C branch of the Gram-positive Bacteria) (Oren, 1992). Different sugars and in some cases also amino acids are fermented to products such as acetate, ethanol, butyrate, hydrogen, and carbon dioxide (Lowe et al., 1993; Mermelstein and Zeikus, 1998; Oren, 1992; Rainey et al., 1995).

In low-salt anaerobic environments breakdown of organic compounds is completed by the cooperative action of a variety of microbial processes, including fermentation, dissimilatory sulfate reduction, methanogenesis, and possibly also activity of proton-reducing acetogens that degrade compounds such as ethanol, butyrate, and others to hydrogen and acetate. Not all these processes have been identified as yet in anaerobic hypersaline environments (Oren, 1988b). For example, no reports on the occurrence and activity of proton-reducing acetogens in hypersaline environments have been published as yet. This lack of information may be due to the difficulty in handling these intriguing bacteria, which are being studied by a very small number of microbiologists only. Thus any claim that such halophilic organisms do not occur in nature may be premature.

Dissimilatory sulfate reduction occurs up to quite high salt concentrations. Black, sulfide-containing sediments are often found on the bottom of salt lakes and saltern ponds almost up to NaCl saturation. A number of halophilic sulfate reducers have been isolated in recent years. The most salt-tolerant isolate thus far is *Desulfohalobium retbaense*, isolated from Lake Retba in Senegal, which was documented to grow at NaCl concentrations of up to 24% (Ollivier et al., 1991). Other halophilic isolates such as *Desulfovibrio halophilus* and *Desulfovibrio oxyclinae* tolerate NaCl concentrations of up to 18–22.5% only (Caumette, 1993; Caumette et al., 1991; Krekeler et al., 1997; Ollivier et al., 1994). Most halophilic and halotolerant sulfate reducers isolated are incomplete oxidizers that grow on lactate and produce acetate. Only very recently was the first halophilic acetate-oxidizing sulfate-reducing bacterium isolated: *Desulfobacter halotolerans* was obtained from the bottom sediments of the Great Salt Lake (Brandt and Ingvorsen, 1997). This organism has a rather restricted salt range, being unable to grow above 13% NaCl. Possibly, bioenergetic constraints define the upper salinity limit at which the different dissimilatory processes can occur (Oren, unpublished data). The sulfate reducers are Proteobacteria that use organic compatible solutes to provide osmotic balance, a strategy that is energetically much more expensive than the use of inorganic ions for that purpose (see below). Accumulation of trehalose and glycine betaine was documented in *Desulfovibriohalophilus* (Welsh et al., 1996). Dissimilatory sulfate reduction provides relatively little energy, and therefore the need to spend a substantial part of the available energy for the production of organic osmotic solutes may set the upper limit to the salt concentration at which these bacteria can grow. The oxidation of lactate to acetate and CO_2 yields much more energy (2 $Lactate^-$ + SO_4^{2-} $\rightarrow$ 2 $Acetate^-$ + $2HCO_3^-$ + HS^- + H^+ ; $\Delta G^{o\prime} = -160.1$ kJ) than the oxidation of acetate with sulfate as electron acceptor ($Acetate^-$ + SO_4^{2-} $\rightarrow$ $2HCO_3^-$ + HS^-; $\Delta G^{o\prime} = -47.7$ kJ). This difference may possibly explain the apparent lack of complete oxidizers at the highest salt concentration range.

The main methanogenic processes in freshwater environments are the reduction of CO_2 with hydrogen and the aceticlastic split. Neither of these reactions has been shown to occur at high salt concentrations. Solar Lake (Sinai) sediments (70–74 g/liter salt) did not show any methanogenesis from acetate or from $H_2 + CO_2$ (Giani et al., 1984). The highest salt concentration at which methanogenesis from $H_2 + CO_2$ was demonstrated in nature was 88 g/liter (Mono Lake, CA) (Oremland and King, 1989). The most halotolerant isolate that grows on $H_2 + CO_2$ is *Methanocalculus halotolerans* obtained from an oil well. This organism grows up to 12% NaCl with an optimum at 5% (Ollivier et al., 1998). The upper salinity boundary for the use of acetate as methanogenic substrate is probably even lower, but available data are few. To my knowledge, no cultures of aceticlastic methanogens are extant that grow above 4–5% NaCl.

Energetic constraints may explain the apparent lack of truly halophilic methanogens that grow on $H_2 + CO_2$ or on acetate. In contrast to the aerobic halophilic Archaea of the order Halobacteriales which contain inorganic ions for osmotic stabilization, the methanogens use the energetically more expensive option of synthesizing organic osmotic solutes (see Table 5). The aceticlastic split yields very little energy

($\Delta G^{o\prime}$ = –31.1 kJ per mol acetate). The free energy yield during growth on hydrogen is –34 kJ per mol of hydrogen, not much higher than that on acetate.

Methanogenesis does occur, however, at much higher salt concentrations. The most salt-tolerant methanogens known in culture are *Methanohalobium evestigatum* and *Methanohalophilus portucalensis*, which grow in up to 25–26% NaCl (Boone et al., 1993; Lai and Gunsalus, 1992; Zhilina and Zavarzin, 1987). Additional moderately halophilic methanogens have been isolated, growing optimally at 4–12% salt (e.g., *Methanohalophilus mahii*, *Methanohalophilus halophilus*, *Methanohalophilus portocalensis*, and *Methanohalophilus zhilinae*). The energy sources used by these methanogens are methylated amines, methanol, and dimethylsulfide (Oremland and King, 1989; Zhilina and Zavarzin, 1987, 1990; see also the review paper by Ollivier et al., 1994). The substrates, such as trimethylamine and dimethylsulfide, used by these bacteria in their natural environment are largely derived from microbial degradation of methylated compounds that serve as organic osmotic solutes in many halophilic microorganisms (Oremland and King, 1989; Zhilina and Zavarzin, 1990; see also Table 5). Thermodynamic calculations show that the energy yield on methylated amines is relatively large (between –92 and –191 kJ per mol of substrate transformed), and this may explain, at least in part, why growth of methanogenic Archaea on methylated amines may occur up to high salt concentrations.

While, as discussed above, methanogens growing on $H_2 + CO_2$ appear to be absent in hypersaline environments, halophilic homoacetogenic bacteria that use the same substrates for the production of acetate have been isolated (Zavarzin et al., 1994; Zhilina and Zavarzin, 1990; Zhilina et al., 1996). *Acetohalobium arabaticum* is able to grow between 10–25% NaCl with an optimum at 15–18% (Zhilina and Zavarzin, 1990). At first sight, reaction thermodynamics do not explain why halophilic homoacetogenic bacteria do occur when CO_2-reducing methanogens do not, as the acetogenic reaction yields even less energy than the methanogenic reaction (–26.1 kJ and –31.1 kJ per hydrogen oxidized, respectively). However, the halophilic homoacetogens belong to the order Haloanaerobiales (Rainey et al., 1995; Zhilina et al., 1996), a group that uses the energetically cheaper option of accumulating inorganic ions to establish osmotic balance (Oren, 1986; Oren et al., 1997; Rengpipat et al., 1988).

Halophilic aerobic chemoautotrophic bacteria that obtain their energy from the oxidation of reduced sulfur compounds are known. *Thiobacillus halophilus*, isolated from a hypersaline lake in Western Australia, grows in as much as 24% NaCl (Wood and Kelly, 1991). However, autotrophic oxidation of NH_4^+ to NO_2^- was never demonstrated above 150 g/liter salt, and the salt limit for the oxidation of NO_2^- to NO_3^- may be even lower (Rubentschik, 1929). To my knowledge no halophilic or halotolerant ammonia- or nitrite-oxidizing bacteria are extant in culture that are able to grow at salinities significantly exceeding those of seawater. *Nitrosococcus halophilus*, with an optimum at 4% NaCl and a maximum at 9.4% may be the most halophilic strain isolated to date (Koops et al., 1996). An attempt to demonstrate nitrification in a microcosm simulation of the microbiology of the Great Salt Lake at a total salt concentration above 30% yielded negative results (Post and Stube, 1988). Lack of energy source is probably not the main reason: ammonia, and not nitrate, is the dominant inorganic nitrogen species in most or all hypersaline water bodies, and it generally occurs in quite high concentrations, in the Dead Sea even in the millimolar range. Energetic constraints may be the cause for the apparent lack of halophilic nitrifying bacteria in nature, as only very small amounts of energy are gained from the oxidation of ammonia and of nitrite.

Thermodynamic constraints cannot explain the apparent lack of aerobic methane oxidation in hypersaline environments. Methane oxidation is a highly exergonic process ($CH_4 + 2O_2 \rightarrow HCO_3^- + H^+ + H_2O$; $\Delta G^{o\prime}$ = –813.1 kJ). However, even in an environment with a relatively low salinity such as the epilimnion of Solar Lake, Sinai, during winter stratification (about 9% salt), no methane oxidation could be measured in spite of the availability of both methane and oxygen (Conrad et al., 1995). Recent reports on the occurrence of methane oxidation in sediments of hypersaline reservoirs in Ukraina and Tuva (up to 330 g/liter total dissolved salts) and on the isolation of halophilic methanotrophs from these environments (Kalyuzhnaya et al., 1998; Khmelenina et al., 1996, 1997; Sokolov and Trotsenko 1995) indicate that the existence of halophilic methane oxidizers is at least thermodynamically feasible, and that the earlier reported lack of methane oxidation in other hypersaline environments (Conrad et al., 1995; Slobodkin and Zavarzin, 1992) should have other reasons.

Physiological and Biochemical Properties of Halophilic Prokaryotes

As biological membranes are permeable to water, any microorganism living at high salt concentrations has to maintain its intracellular environment at least isoosmotic with the salt concentration in its environment, and even

hyperosmotic when a turgor pressure has to be maintained (Brown, 1976, 1990; Csonka, 1989; Vreeland, 1987). Two fundamentally different strategies exist that enable halophilic and halotolerant prokaryotes to cope with the osmotic stress exerted by the high ionic strength of their hypersaline environment. The first option, used by the aerobic Archaea of the order Halobacteriales and by the anaerobic Bacteria of the order Haloanaerobiales, is based on the accumulation of high concentrations of inorganic ions in the cytoplasm. In most cases, K^+ rather than Na^+ is the dominant intracellular cation, and Cl^- is the dominant anion. Presence of molar concentrations of inorganic ions requires special adaptations of the entire intracellular enzymatic machinery. The "salt-in" strategy permits little flexibility and adaptability to changing conditions, as many salt-adapted enzymes and structural proteins require the continuous presence of high salt for activity and stability. The adaptive evolution of proteins and salinity-mediated selection of their properties has recently been reviewed (Dennis and Shimmin, 1997).

The second strategy is to prevent high salt concentrations from reaching the cytoplasm, and maintaining "conventional" enzymes and other proteins, not specifically designed to function at high ionic strength. Low intracellular ionic concentrations are maintained by active pumping of ions out of the cells. Osmotic equilibrium is provided by organic solutes that are either produced by the cells or accumulated from the medium (Kempf and Bremer, 1998). Such "compatible" solutes are low-molecular-weight organic compounds, soluble in water at high concentrations, and not inhibitory to enzymatic activities even in the molar concentration range. The intracellular concentrations of the organic solutes are regulated according to the salinity of the external medium. Thus, the use of organic osmotic solutes provides a great deal of flexibility and adaptability to an often, wide range of salt concentrations, with the possibility of rapid adaptation to changes in the salinity of the medium. The strategy of maintaining isoosmotic concentrations of organic osmotic solutes is used by most halophilic and halotolerant Bacteria (with the exception of the Haloanaerobiales, as stated above) and by the halophilic methanogenic Archaea. Halophilic eukaryotic microorganisms also use organic compatible solutes for osmotic stabilization. Under certain conditions, the alkaliphilic members of the Halobacteriales also make use of an organic osmoticum (2-sulfotrehalose) to aid in the achievement of osmotic equilibrium with the environment (Desmarais et al., 1997).

The "Salt-In" Strategy

Analyses of intracellular ionic concentrations in different aerobic halophilic Archaea show that these microorganisms maintain extremely high salt concentrations inside their cells. Moreover, the ionic composition of their intracellular milieu differs greatly from that of the outside medium, with K^+ being the main intracellular cation (Table 2).

The representatives of the order Haloanaerobiales (low G+C Gram-positive branch of the Bacteria) display a number of physiological and biochemical properties that are characteristic for the halophilic aerobic Archaea, rather than for the moderately halophilic aerobic Bacteria which use the organic solute strategy. No organic osmotic solutes have been found as yet in this group of anaerobic halophilic fermentative Bacteria (Mermelstein and Zeikus, 1998; Oren, 1986; Oren et al., 1997; Rengpipat et al., 1988). High concentrations of Na^+, K^+ and Cl^- were measured inside the cells of *Haloanaerobium praevalens*, *Haloanaerobium acetoethylicum*, and *Halobacteroides halobius*, high enough to be at least isotonic with the medium (Table 3). In exponen-

Table 2. Estimates of intracellular ionic concentrations in aerobic halophilic Archaea of the order Halobacteriales.

	Medium concentration				Intracellular concentration			
Species	Na^+	K^+	Mg^{2+}	Cl^-	Na^+	K^+	Mg^{2+}	Cl^-
Halobacterium salinarum	4.0	0.032			1.37	4.57		3.61
Halobacterium salinarum[a]	3.7	0.013	0.1		1.63	2.94		
Halobacterium salinarum[a]	3.33	0.05	0.13		0.80	5.32	0.12	
Haloarcula marismortui[b]	3.9	0.004–0.007	0.15	3.9	1.2–3.0	3.77–5.5		2.3–4.2
Haloarcula marismortui[a]	3.9	0.001–0.004	0.15	3.9	1.6–2.1	3.7–4.0		3.2–4.1
Haloarcula marismortui[c]	3.9	0.0075–	0.15	3.9	0.5–0.7	3.7–4.0		2.3–2.9
Halococcus morrhuae	4.0	0.032			3.17	2.03		3.66

[a]Late exponential growth phase cells; [b]Early exponential growth phase cells; [c]Stationary growth phase cells. For additional information see text.

Data were derived from Christian and Waltho (1962), Ginzburg et al. (1970), Lanyi and Silverman (1972), and Matheson et al. (1976). All concentrations are in molar units, except those relating to *Haloarcula marismortui*, which are expressed in molal units.

Table 3. Intracellular ionic concentrations of halophilic anaerobic Bacteria of the order Haloanaerobiales.

Species	Medium concentration			Intracellular concentration		
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Haloanaerobium praevalens	0.99	0.013	1.07	0.44	0.96	
	2.22	0.013	2.30	1.52	1.59	2.24
Haloanaerobium praevalens[a]	2.22	0.013	2.30	0.44	1.14	1.26
	3.08	0.013	3.16	2.63	2.05	3.28
Haloanaerobium acetoethylicum	1.16	0.032	1.40	0.92	0.24	1.20
	2.52	0.034	2.70	1.50	0.78	2.50
Halobacteroides halobius	1.56	0.013		0.54	0.92	

[a]Data obtained by X-ray microanalysis in the electron microscope. Values probably underestimate the true values.
Data were derived from Oren (1986), Oren et al. (1997), and Rengpipat et al. (1988).

tially growing cells, K^+ was the major cation. Stationary phase cells showed a high variability among individual cells, part of the cells containing high concentrations of NaCl rather than of KCl (Oren et al., 1997).

The huge potassium concentration gradient over the cytoplasmic membrane (often up to three orders of magnitude) and also the generally large sodium gradient present can be created and maintained only at the expense of energy. Also the chloride ion is far from thermodynamic equilibrium, as the presence of an inside negative membrane potential would tend to expel Cl^- from the cell. The peculiar ionic composition of the cells' cytoplasm and the concentration gradients over the cell membrane are the result of the cooperative action of different ion pumps, antiporters, and other transport proteins. The most important ones are summarized in Fig. 9, and the numbers in square brackets in the explanation below refer to the different parts of that figure.

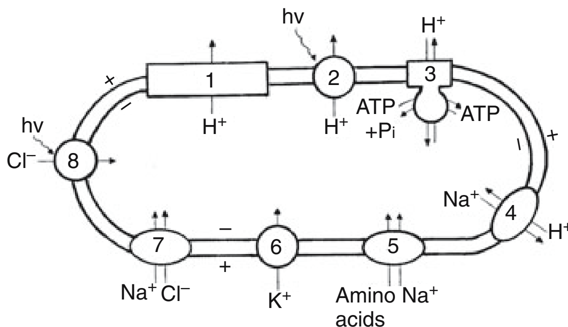

Fig. 9. Ion movements in the aerobic halophilic Archaea (order Halobacteriales): [1] proton extrusion via respiratory electron transport; [2] light-driven proton extrusion mediated by bacteriorhodopsin; [3] ATP formation by ATP synthase, driven by the proton gradient. Alternatively, this system can serve to generate a proton gradient at the expense of ATP during fermentative growth on arginine. [4] electrogenic sodium/proton antiporter; [5] sodium gradient-driven inward amino acids transport; [6] potassium uniport, driven by the membrane potential; [7] light-independent chloride transport system, probably coupled with inward transport of sodium. [8] halorhodopsin, the primary, light-driven chloride pump. For details see text.

In the Halobacteriales, respiratory electron transport with oxygen or other electron acceptors is accompanied by the extrusion of protons [1], generating a primary proton electrochemical gradient (acidic outside, alkaline inside, positive outside, negative inside). Those species that contain the retinal protein bacteriorhodopsin in their membranes may also use light energy for the direct generation of the proton electrochemical gradient [2]. The primary proton gradient is the driving force for all energy-requiring processes within the cell. Thus, ATP formation is mediated by the membrane-bound ATP synthase that couples phosphorylation of ADP with an inward flux of H^+ [3]. The membrane ATP synthase may also be used in the reverse direction, the build-up of a proton electrochemical gradient at the expense of ATP. This process is relevant in cases in which ATP formation by substrate-level phosphorylation is the primary energy-yielding process in the cell. This is the case (e.g., in *Halobacterium salinarum*) when growing anaerobically by fermentation of arginine (Hartmann et al., 1980), or in the anaerobic Bacteria of the order Haloanaerobiales, which obtain their energy by fermentation of sugars or amino acids (Oren, 1992).

The membranes of all halophiles investigated possess high activities of Na^+/H^+ antiporters, which use the proton electrochemical gradient as the driving force for the extrusion of Na^+ from the cell [4] (Hamaide et al., 1983; Lanyi and MacDonald, 1976; Luisi et al., 1980). In *Halobacterium salinarum*, the antiporter was shown to be electrogenic and probably has a stoichiometry of 2 H^+/Na^+ (Lanyi and Silverman, 1979). In addition to its function of keeping intracellular Na^+ concentrations at the desired low levels, the Na^+/H^+ antiporter activity can be expected to play an important role in the regulation of the intracellular pH.

The sodium gradient thus established can in its turn be used to drive certain endergonic processes. Thus, many of the membrane transport systems for amino acids and other compounds in the aerobic halophilic Archaea are energized by

cotransport with Na^+ ions [5]. The same is true for many moderately halophilic Bacteria, which also maintain a relatively low intracellular Na^+ concentration (Shindler et al., 1977; Ventosa et al., 1998). The Na^+ gradient thus serves to some extent as an energy reserve.

It is generally accepted that the negative inside membrane potential is the driving force for the massive K^+ accumulation. The membranes of halophilic Archaea were found to be highly permeable to potassium. K^+ ions probably enter the cells via a uniport system in response to the membrane potential (Wagner et al., 1978) [6]. K^+ enters the cell as Na^+ is ejected by the electrogenic Na^+/H^+ antiporter, thus maintaining electroneutrality. Evidence for such a mechanism was found in experiments showing accumulation of radioactively labeled rubidium (a potassium analog) ions in right-side-out vesicles of *Halobacterium salinarum* as a reaction to sodium extrusion, following excitation of the light-driven primary proton pump bacteriorhodopsin (Kanner and Racker, 1975). In addition, a K^+ transport system analogous to the Kdp system (the P-type K^+-translocating ATPase) of *Escherichia coli* was detected in *Haloferax volcanii* (Meury and Kohiyama, 1989). This system requires ATP for activation.

The high internal Cl^- concentration is not in equilibrium with the large negative-inside electrical potential that accompanies the H^+ circulation and the Na^+ efflux. Thus, electrical potential-driven passive chloride movement can result only in a loss of chloride from the cells rather than in the required uptake. An increase in the amount of intracellular Cl^- is essential if the cells should increase their volume during growth and cell division. It has been suggested that during growth the net flux of ions should result in K^+ uptake and excess Na^+ loss, and that Cl^- uptake should be equal to the difference, and thereby provide a net gain of intracellular KCl commensurate with the gain in intracellular volume (Lanyi, 1986; Schobert and Lanyi, 1982).

Two energy-dependent inward chloride pumps have been identified in *Halobacterium* cells. The first is a light-independent transport system, which is probably driven by symport with Na^+ (Duschl and Wagner, 1986) [7]. The second is light-driven, and is based on the retinal protein halorhodopsin, a primary inward Cl^- pump present in *Halobacterium salinarum*, in *Natronomonas pharaonis*, and probably also in additional halophilic Archaea (Lanyi, 1986; Schobert and Lanyi, 1982) [8].

The presence of molar concentrations of salts is generally devastating to proteins and other macromolecules. It causes aggregation or collapse of the protein structure by enhancing hydrophobic interactions within and between protein molecules and interferes with essential electrostatic interactions within or between macromolecules by charge shielding. And because of salt ion hydration, it reduces the availability of free water below the level required to sustain essential biological processes (Dennis and Shimmin, 1997; Zaccai and Eisenberg, 1991). The presence of high intracellular salt concentrations thus requires special adaptations of the whole enzymatic machinery of the cell. Cells thus adapted are able to function in the presence of high salt. However, these adaptations make the cells strictly dependent on the continuous presence of high salt concentrations for the maintenance of structural integrity and viability (Ebel et al., 1999; Eisenberg, 1995; Eisenberg and Wachtel, 1987; Eisenberg et al., 1992; Lanyi, 1974). As a result, the aerobic halophilic Archaea display little flexibility and adaptability to changes in the external salt concentration.

Most enzymes and other proteins of the Halobacteriales denature when suspended in solutions containing less than 1–2 M salt. Many enzymes are more active in the presence of KCl than of NaCl, agreeing well with the finding that K^+ is intracellularly the dominating cation. "Salting-out" salts stabilize, while "salting-in" salts inactivate halophilic enzymes. The behavior of different salts coincides with the lyotropic Hofmeister series (Lanyi, 1974). Similarly, intracellular enzymes from the fermentative anaerobic Bacteria (order Haloanaerobiales) generally function better in the presence of molar concentrations of salts than in salt-free medium, and they can be expected to be fully active at the actual salt concentrations present in the cytoplasm (Oren and Gurevich, 1993; Rengpipat et al., 1988; Zavarzin et al., 1994).

Most proteins of the Halobacteriales contain a large excess of the acidic amino acids, glutamate and aspartate, and few basic amino acids, lysine and arginine. The high content of acidic side groups was first recognized during analyses of the bulk protein of *Halobacterium* and *Halococcus* (Reistad, 1970). The malate dehydrogenase of *Haloarcula marismortui* has a 10.4 mol% excess of acidic residues and the cell envelope glycoprotein of *Halobacterium salinarum* even 19–20 mol%. Owing to the high acidity of the proteins of the halophilic Archaea, isoelectric focusing is of little use for protein characterization and isolation. In *Halococcus salifodinae*, all proteins were found to have isoelectric points between 3.8 and 4.5 (Denner et al., 1994). The bulk cellular protein of the members of the Haloanaerobiales tested is also highly acidic (Oren, 1986).

It has been argued that the excess of acidic residues may be a major factor determining the halophilic character of the proteins: excess of

negative charges on the protein surface makes the structure unstable because of the mutual repulsion of the side groups. Only when high concentrations of cations are added to shield the negative charges can the protein maintain its proper conformation required for structural stability and enzymatic activity.

Shielding of negative charges by cations undoubtedly plays an important part in the effects of salt on the enzymes and other proteins of the halophiles. A theoretical analysis of the contribution of electrostatic interactions in *Haloarcula marismortui* ferredoxin and malate dehydrogenase shows that the repulsive interactions between the acidic residues at the protein surface are a major factor in the destabilization of halophilic proteins in low-salt conditions (Elcock and McCammon, 1998). However, Lanyi (1974) and Lanyi and Stevenson (1970) stated that all the effects of salts cannot be due to charge-shielding action alone, as the concentrations required are too high. Maximal electrostatic-charge shielding would be achieved already in about 0.1 M salt or 0.5 M at most, and in even much lower concentrations of divalent cations. However, a high content especially of glutamate may be favorable, as glutamate has the greatest water binding ability of any amino acid residue. This may have important implications when considering the need of any functional protein to maintain a proper hydration shell.

Another prominent feature of the proteins of the Halobacteriales is their low content of hydrophobic amino acid residues, generally offset by an increased content of the borderline hydrophobic amino acids, serine and threonine (Lanyi, 1974). The requirement for extremely high salt concentrations for structural stability of the proteins can probably to a large extent be attributed to the low content of hydrophobic residues and the accordingly weak hydrophobic interactions within the protein molecules. High salt is then needed to maintain the weak hydrophobic interactions. Entropy increases when non-polar groups turn away from the water phase and interact with each other to form hydrophobic interactions. These interactions seem to be driven more by an avoidance of water than by an active attraction between the non-polar molecules (Lanyi, 1974). At higher salt concentrations, new hydrophobic interactions are formed having insufficient stability in water, and the molecule assumes a more tightly folded conformation. The possible involvement of the weak hydrophobic interactions in the salt requirement of the halophilic proteins is supported by the finding that certain enzymes from halophilic Archaea (including threonine deaminase, aspartate carbamoyltransferase, and alanine dehydrogenase) show cold lability: their maximal stability is reached at temperatures greater than 0°C and decreases at lower temperatures. The effect may be considered in terms of water structure: at lower temperature the size of the cluster of water molecules is increased, and hydrophobic groups can interact more easily, breaking the hydrophobic interactions (Lanyi, 1974).

Detailed studies of the malate dehydrogenase of *Haloarcula marismortui* have contributed much valuable information on the possible mechanisms involved in the halophilic behavior of proteins. Techniques such as velocity sedimentation, light scattering, neutron scattering, and circular dichroism measurements have been used to obtain information on the structural changes occurring as a function of changing salt concentrations and the hydration properties of the protein (Eisenberg, 1995; Eisenberg and Wachtel, 1987; Mevarech and Neumann, 1977; Pundak and Eisenberg, 1981; Pundak et al., 1981). These studies have shown that the halophilic properties of the enzyme are related to its capacity of associating with unusually high amounts of salts, and led to the formulation of a thermodynamic "solvation-stabilization model," in which the halophilic protein has adapted to bind hydrated ions cooperatively via a network of acidic groups on its surface (Ebel et al., 1999).

X-ray diffraction studies on crystals of the halophilic malate dehydrogenase and the ferredoxin of *Haloarcula marismortui* and the dihydrofolate reductase of *Haloferax volcanii* have added much important information (Dym et al., 1995; Frolow et al., 1996; Pieper et al., 1998). These studies showed how the carboxylic groups on the acidic residues are used to sequester, organize, and arrange a tight network of water and hydrated K^+ ions at the surface of the protein, and how an unusually large number of internal salt bridges between strategically located basic amino acid residues are formed to give the protein its internal structural rigidity. These salt bridges appear to be important determinants in the stabilization of the three-dimensional structure of halophilic proteins. Intervening solvent molecules shield the negative charges of the carboxylic acid groups on the protein surface from each other.

Comparison of the *Haloarcula marismortui* ferredoxin with the plant-type 2Fe-2S ferredoxin showed that the surface of the halophilic protein is coated with acidic residues, except for the vicinity of the iron-sulfur cluster, and that the halophilic protein contains two additional helices near the N-terminus. These helices form a separate hyperacidic domain, postulated to provide extra surface carboxylates for solvation. Bound water molecules on the protein surface have on the average 40% more hydrogen bonds than in a typical non-halophilic protein crystal

structure. These water molecules are thus tightly bound within the hydration shell by protein-water and water-water hydrogen bonds and by hydration of interspersed K^+ ions (Frolow et al., 1996).

A recent study of the glutamate dehydrogenase of *Halobacterium salinarum* showed the surface of the molecule being covered with acidic residues and displaying a significant reduction in exposed hydrophobic character as compared to non-halophilic counterparts. The low lysine content helps to increase the overall negative charge on the protein surface but also serves to decrease the hydrophobic fraction of the solvent-accessible surface (Britton et al., 1998).

The "Low Salt-In" Strategy

The second option, realized in most halophilic and halotolerant representatives of the Bacteria and also in the halophilic methanogenic Archaea, involves the maintenance of a cytoplasm much lower in salt than the outside medium. Table 4 summarizes estimates of intracellular salt concentrations in a number of aerobic halophilic Bacteria. While in some cases the apparent intracellular ionic concentrations are in the molar range (possibly in part due to technical difficulties related to the exact assessment of the intracellular water volumes in cell pellets) (Ventosa et al., 1998), it is clear that the intracellular salt concentrations are generally insufficient to provide osmotic balance. Generally the intracellular Na^+ concentrations are kept low. Outward-directed sodium transporters in the cytoplasmic membrane (in most cases electrogenic Na^+/H^+ antiporters) are highly important both in maintaining the proper intracellular ionic environment and in pH regulation (Hamaide et al., 1983; Ventosa et al., 1998). Outward-directed Na^+ transporters may include primary respiration-driven Na^+ pumps detected in some moderate halophiles such as *Salinivibrio costicola* (Tokuda and Unemoto, 1983).

Organic compatible solutes make up the major part of the osmotically active compounds in the cells' cytoplasm. Compatible solutes are polar, highly soluble molecules, most of them uncharged or zwitterionic at the physiological pH. The list of compounds known to be synthesized as compatible solutes by halophilic microorganisms is steadily growing (Galinski, 1993, 1995; Galinski and Trüper, 1994; Reed, 1986; Trüper et al., 1991; Ventosa et al., 1998; Wohlfarth et al., 1990). Figure 10 shows the main osmotic solutes identified thus far in prokaryotes, and Table 5 provides information on the taxonomic groups in which the different solutes have been detected.

The accumulation of "compatible" osmotic solutes achieves osmotic equilibrium while still enabling activity of "conventional", non-salt-adapted enzymes (Galinski, 1993, 1995). Many prokaryotic cells contain cocktails of different compatible solutes rather than relying on a single compound (Galinski, 1995). The concentrations of the osmotic solutes are regulated according to the salt concentration in which the cells are found (Galinski and Louis, 1999), and can be rapidly adjusted as required when the outside salinity is changed (by synthesis or uptake from the medium upon salt upshock; by degradation or transformation into osmotically inactive forms; or by excretion following dilution stress) (Trüper and Galinski, 1990). The use of organic osmotic solutes thus bestows a high degree of flexibility and adaptability.

Compatible solutes are strong, water structure formers and as such they are probably excluded from the hydration shell of proteins. This "preferential exclusion" probably explains their function as effective stabilizers of the hydration shell of proteins. This phenomenon of nonspecific exclusion is often described in terms of increased

Table 4. Intracellular ionic concentrations of selected aerobic halophilic Bacteria.

	Medium concentration			Intracellular concentration		
Species	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Halomonas elongata	1.38	0.02		0.31	0.02	
	3.4	0.01		0.63	0.02	
Halomonas canadensis	4.4	0.04		0.62	0.58	
Halomonas halodenitrificans	1.0	0.04	1.0	0.31	0.47	0.055
	3.0		3.0	1.07	0.12	
"Pseudomonas halosaccharolytica"	2.0	0.006	2.0	1.15	0.89	0.98
	3.0	0.006	3.0	1.04	0.67	0.70
Salinivibrio costicola	2.0	0.008		0.90	0.57	

All data relate to exponentially growing cells. Data were derived from Christian and Waltho (1962), Masui and Wada (1973), Matheson et al. (1976), Shindler et al. (1977), and Vreeland et al. (1983). For more extensive data, see Table 5 in Ventosa et al. (1998).

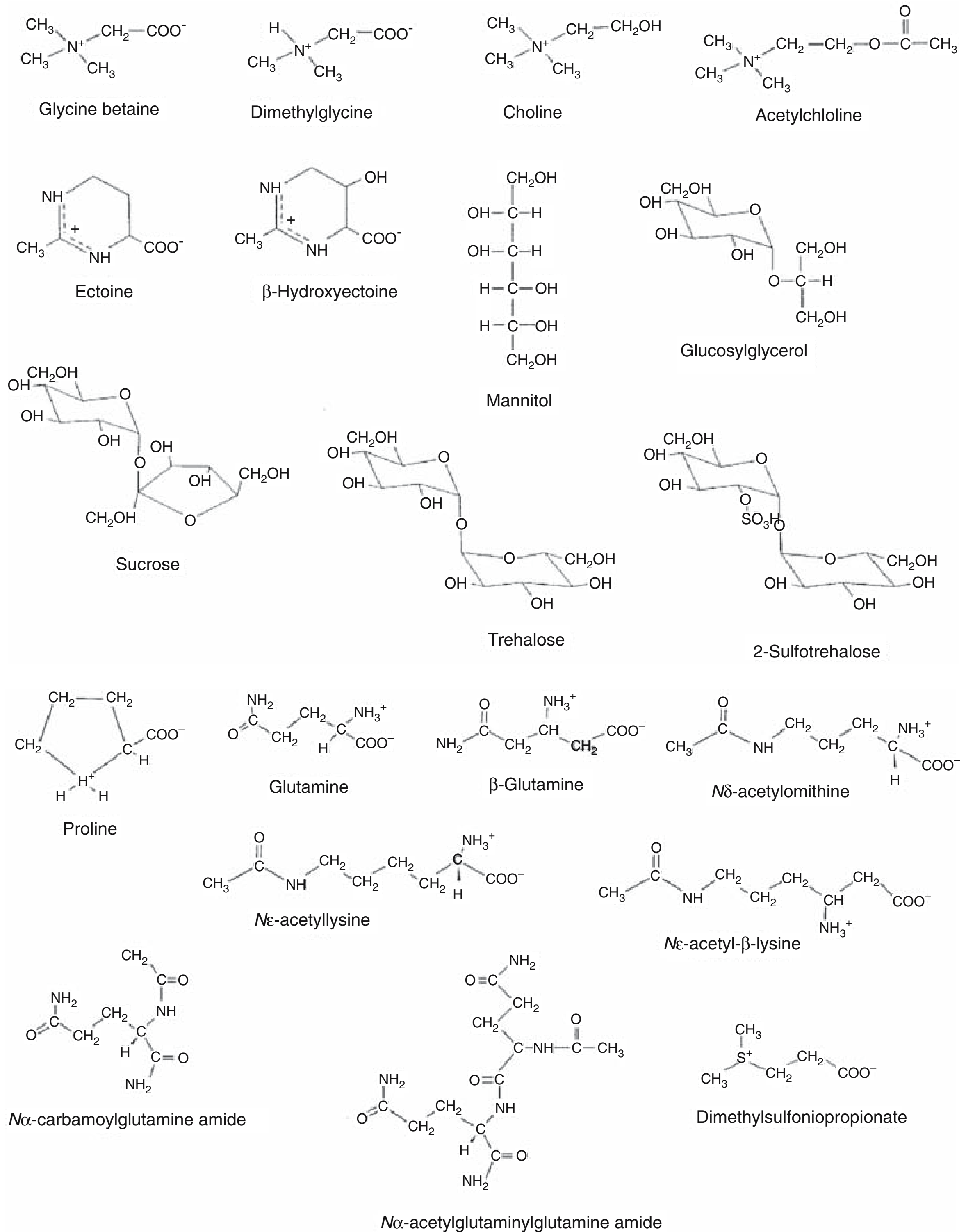

Fig. 10. Organic osmotic solutes documented to occur in halophilic and halotolerant Bacteria and Archaea.

surface tension of water, with the presence of solutes affecting the forces of cohesion between water molecules, minimization of entropy, and reinforcement of the hydrophobic effect. Compatible solutes display a general stabilizing effect by preventing the unfolding and denaturation of proteins caused by heating, freezing, and drying (Galinski, 1993, 1995).

Table 5. Distribution of selected organic osmotic solutes within the bacterial and the archaeal domains.

Solute	Distribution
Glycine betaine	Cyanobacteria, Anoxygenic phototrophic bacteria; Methanogenic bacteria; *Actinopolyspora halophila*; Is taken up by many heterotrophic bacteria and used as osmotic solute.
Dimethylglycine	Methanogenic bacteria
Choline, Acetylcholine	*Lactobacillus plantarum*
Ectoine, Hydroxyectoine	Heterotrophic Proteobacteria of the γ-subdivision; *Halorhodospira* spp., *Rhodobacter sulfidophilus; Micrococcus* spp., many bacilli; *Marinococcus* spp., *Sporosarcina halophila; Brevibacterium*
Proline	Bacilli; *Planococcus citreus*; *Salinicoccus* sp.
Glutamine	Corynebacteria
β-Glutamine	Methanogenic bacteria
Nε-acetyllysine	*Halobacillus halophilus*, other bacilli
Nδ-acetylornithine	*Halobacillus halophilus*, other bacilli
Nε-acetyl-β-lysine	Methanogenic bacteria
Nα-carbamoyl-glutamine amide	*Ectothiorhodospira marismortui*
Nα-acetylglutaminyl-glutamine amide	*Halochromatium; Thiohalocapsa; Rhodopseudomonas* sp.; *Azospirillum brasilense; Rhizobium meliloti; Pseudomonas aeruginosa*
Sucrose	Cyanobacteria
Trehalose	Cyanobacteria, *Halorhodospira* spp.
2-Sulfotrehalose	Alkaliphilic members of the Halobacteriales
Mannitol	*Pseudomonas putida*
Glucosylglycerol	Cyanobacteria; *Rhodobacter sulfidophilus*; *Pseudomonas mendocina*
Dimethylsulfonio-propionate	Marine cyanobacteria

For additional information see e.g., Desmarais et al. (1997); Galinski (1993, 1995); Hagemann et al. (1999); Imhoff (1993); Oren (1999); Trüper et al. (1991); Ventosa et al. (1998); and Wohlfarth et al. (1990).

Concluding Remarks

A comparison of the two strategies of adaptation to high salt concentrations ("salt-in" versus use of organic osmotic solutes) shows that the salt-in strategy is energetically much less costly than the synthesis of organic, compatible solutes (Oren, unpublished data). However, it requires a far-going adaptation of the whole intracellular machinery to the presence of high ionic concentrations. This energetically relatively cheap solution of balancing "salt-out" with "salt-in" is not widely used in nature. Evolutionary processes toward such adaptation, as described by Dennis and Shimmin (1997), have led to the establishment of two specialized groups, the aerobic extremely halophilic Archaea (Tindall, 1992) and the fermentative obligatory anaerobic Bacteria (Oren, 1992).

The use of organic compatible solutes allows much more flexibility with respect to the range of salt concentrations tolerated, and does not require a high degree of adaptation of the intracellular enzymes. The enzymes do not greatly differ from those of non-halophilic prokaryotes, although they may have a somewhat increased content of acidic amino acids (Gandbhir et al., 1995). Many taxonomic groups, displaying a great metabolic diversity, use this strategy. Thus, many of the dissimilatory processes identified in freshwater environments can also take place at high salinity. Certain metabolic types, however, such as methanogenesis from $H_2 + CO_2$ or from acetate, autotrophic nitrification, and others, seem to be absent above 10–15% salt. It is tempting to speculate that it is the too high energetic cost connected with adaptation to life at the highest salt concentrations that has prevented the evolution of halophiles performing these reactions. In any case, also in hypersaline environments, the prokaryotes display an amazing diversity that is well worth being studied in-depth on the level of community structure and metabolism, phylogenetic diversity, and the molecular mechanisms of their adaptation to high salt.

Acknowledgments. I thank Carol D. Litchfield (George Mason University, Fairfax, VA) for her helpful comments on the manuscript.

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Prokaryotes (2006) 2:283–308
DOI: 10.1007/0-387-30742-7_10

CHAPTER 1.10

Alkaliphilic Prokaryotes

TERRY ANN KRULWICH

Introduction and Definitions

Alkaliphilic prokaryotes offer a wealth of opportunities for the isolation of natural products that can be advantageously applied to diverse industrial processes (Horikoshi and Akiba, 1982; Horikoshi, 1991; Horikoshi, 1996). Alkaliphiles also offer a wealth of opportunities to understand the mechanisms by which organisms thrive at pH 10–11.5, often present with other environmental challenges, such as high salt and high temperature (Krulwich, 1995; Krulwich et al., 1998). The diversity of alkaliphiles, which occur among aerobic and anaerobic archaea and prokaryotes as well as eukaryotic fungi (Grant et al., 1990; Jones et al., 1994; Horikoshi, 1991), likely broadens their industrial application. All alkaliphiles confront protein structure and function problems that involve catalysis at the external pH and regulation of cytoplasmic pH. However, among different alkaliphile groups, such as anaerobes, aerobes, ammonium-requiring sulfate-reducing thermophiles, halophiles, methylotrophs, methanogens, and photosynthetic bacteria, different approaches or features to the solution of these central as well as other group-specific problems are likely. Alkaliphiles, an extraordinarily diverse group of bacteria that may have evolved over a very long period, provide us with the opportunity to probe their many adaptive strategies (Zavarzin, 1993; Duckworth et al., 1996; Jones et al., 1998). Also, understanding how alkaliphiles meet extreme challenges of pH homeostasis and other challenges can provide insights that are often translatable to the nonextremophile setting.

Various definitions of an alkaliphile have been used. The only common standard is that the organism's optimal pH for growth must be at least 2 units above neutrality (Kroll, 1990). To clearly distinguish between alkaliphiles and the more abundant alkaline-tolerant prokaryotes, most investigators of these organisms propose a higher pH standard and sometimes (Horikoshi, 1998) include an inability to grow well or grow at all at near-neutral pH values such as 6.5. As discussed below, growth at near-neutral pH may be inversely related to how functional an alkaliphile is at the upper pH limit. The lower pH limit and pattern of growth over a broad pH range also depends upon the particular growth substrate (Gilmour and Krulwich, 1997; Krulwich et al., 1997) and on strain differences that are incompletely understood (Guffanti et al., 1986). My laboratory has used the term alkaliphile for bacteria that grow optimally on substrates (suitable for high pH growth) at pH values above 9.5. This value is chosen because it approximates the upper limit of cytoplasmic pH that is compatible with the growth of bacteria so far studied (Sturr et al., 1994; Krulwich et al., 1998). The term facultative alkaliphile has been applied to species and strains that are able to grow at both pH 6.5–7.5 and above 9.5, whereas alkaliphiles that grow only at 9.5 are termed obligate alkaliphiles.

Historical Notes

Koki Horikoshi, a leading investigator of alkaliphiles, was the first to initiate broad-based studies of these bacteria, starting in the late 1960s. He has commented that when he began he found only sixteen prior literature references to alkaliphilic bacteria (Horikoshi, 1998). The earliest reports of bona fide alkaliphiles were those of *Bacillus pasteurii* by Gibson, 1934 and of *Bacillus alcalophilus* by Vedder, 1934. Investigators of physiology and protein structure have returned to these two alkaliphilic *Bacillus* species in recent years. In the early 1960s, Takahara and colleagues (Takahara et al., 1961; Takahara and Tanobe, 1960; Takahara and Tanobe, 1962) demonstrated that indigo dye reduction depends on maintaining sufficient alkalinity, and they improved the fermentation process by adding alkaliphilic *Bacillus* sp strain S-8, which they had isolated from an indigo ball undergoing fermentation at high pH. Over the years, Horikoshi and colleagues (Horikoshi, 1996) and others (e.g., van der Laan et al., 1991; Ito et al., 1998) advanced this tradition of optimizing alkaliphile fermentation processes or their products while

also contributing to alkaliphile taxonomy and characterization.

The diversity of alkaliphiles was subsequently extended by Grant and colleagues (Tindall et al., 1984; Grant et al., 1990; Grant et al., 1999; Jones et al., 1998), Zhilina and Zavarzin, 1994, and others who enumerated and identified bacteria and archaea that thrive in natural, selective environments such as the highly alkaline soda lakes in Africa and Asia. The number of known alkaliphiles and their diversity has vastly increased as a result of such systematic investigations. These studies also extended the earlier finding by (Kurono and Horikoshi, 1973; Horikoshi, 1991) that alkaliphiles require and thrive in added Na^+, by showing that many categories of alkaliphiles are haloalkaliphiles.

During the 1970s, my laboratory began to study energetic dilemmas of alkaliphiles, taking the chemiosmotic view of energy coupling found in bacterial and eukaryotic membrane systems (Mitchell, 1961). We studied the dilemma of how aerobic alkaliphilic bacteria can both extrude protons during respiration and achieve—as initially presumed and subsequently shown to be necessary—net acidification of the cytoplasm relative to the outside (Krulwich, 1995). As also noted by Garland, 1977, there was the further dilemma of what effect a "reversed pH gradient" would have on reducing chemiosmotic gradients (acid and cations out) and in turn on reducing the energetic driving force, which could create a problem with solute transport, flagellar rotation energization, and ATP synthesis. Perhaps some alkaliphiles generate compensatory electrical potentials across the membrane or use Na^+ as a coupling ion, or perhaps some or all alkaliphiles had developed other interesting mechanisms for bioenergetic work under such circumstances. This area of study became and remains active in several laboratories (Krulwich and Guffanti, 1983; Krulwich and Guffanti, 1989; Krulwich and Ivey, 1990; Krulwich et al., 1998; Hirota and Imae, 1983; Sugiyama et al., 1985; Koyama et al., 1986; Koyama and Nosoh, 1995; Aono and Ohtani, 1990; Hamamoto et al., 1994).

More recent investigations of alkaliphilic enzyme pH stability and optimum have drawn upon 3-dimensional crystal structures (Sobek et al., 1990; Sobek et al., 1992; van der Laan et al., 1992; Martin et al., 1997; Shirai et al., 1997) and properties that can be modeled or deduced from molecular characterizations (Teplyakov et al., 1992; van der Laan et al., 1996; Kobayashi et al., 1999). The physiology and study of bioenergetic properties of alkaliphiles also are enhanced by modern molecular biological techniques as well as proteome and genomic insights. Numerous alkaliphile genes have been sequenced, physical maps have been presented for three genes (Southerland et al., 1993; Park et al., 1994; Gronstad et al., 1998; Takami et al., 1999b), and the genome of an alkaliphilic *BacillusM*, *Bacillus halodurans* C-125, is currently being sequenced (Takami et al., 1999c).

By consensus, there has been a shift in nomenclature from the use of the term "alkalophily" to use of the term "alkaliphily" during the 1990s. The more etymologically correct "alkali" has now become the convention.

Distribution and Taxonomic Groups

Distribution

Alkaliphiles are found in natural and industrial (or other man-made enrichments). In addition to the indigo dye process that has already been noted, sodium hydroxide has been used extensively in paper and pulp processing and calcium hydroxide in cement manufacture. Mining operations and certain food-processing activities also are settings for alkaliphile enrichment (Jones et al., 1998). Similarly, natural enrichments are diverse. Many of these, such as alkaline hot springs, are the source of interesting, generally alkaline-tolerant organisms but are insufficiently buffered to support the extraordinarily high pH values that are consistently maintained (Jones et al., 1998). On the other hand, naturally occurring soda lakes are stable, extremely alkaline environments (e.g., pH >11.5) that are widely distributed and typically found inland (e.g., soda lakes of the East African Rift Valley and of Central Asia). Their NaCl concentrations range from about 5% w/v to >15% w/v. The soda lakes have a paucity of calcium and magnesium ions because they are depressions formed from nonsedimentary rocks, and the sodium, chloride, and bicarbonate/carbonate are the dominant ions. The soda lakes often exhibit the pronounced color of organisms (e.g., cyanobacteria) that are the primary, photosynthetic actors in the nutrient cycle, and the hypersaline lakes often are the color of haloalkaliphiles. Both aerobic and anaerobic cycles occur (Jones et al., 1994; Jones et al., 1998). Alkaliphiles found in the soda lakes are diverse; some organisms are unique to these lakes and some (e.g. the fastidious haloalkaliphiles) appear to represent distinct lineages (McGenity and Grant, 1993; Jones et al., 1998; Grant et al., 1999). Also, evidence from fossil soda lakes, which are similar to those found today, suggests that these environments are of great antiquity. Together, these observations have led to suggestion that substantial evolution of many prokaryotesfound in this type of environment occurred in the soda lakes, i.e., these

communities are very ancient sources of new species of bacteria (Zavarzin, 1993; Jones et al., 1998).

If indeed many alkaliphiles evolved in natural soda lake enrichments, they must have spread beyond those boundaries. Some of the same alkaliphiles that are found in the ancient enrichments of the soda lakes are almost ubiquitous. They are present in garden soils (Guffanti et al., 1980, 1986) and in deep-sea trenches (Takami et al., 1999a) where the pH is not conducive to a thriving alkaliphile presence. Horikoshi and Akiba, 1982 note a substantial presence of alkaliphiles in soils of various pH values, albeit greater in alkaline soils.

Taxonomic Groups

A summary of the taxonomic groups of prokaryotes isolated from soda lakes has been reproduced from a review by Jones et al., 1998 and presented at Table 1. The enormous taxonomic diversity of extreme alkaliphiles is evident and further reflected in the diversity of characteristics. Alkaliphilic cyanobacteria are among the primary photosynthetic organisms that produce oxygen; such organisms include *Spirulina*, *Cyanospira*, *Synechococcus* and *Chorococcus*. Anoxygenic phototrophic bacteria, such as *Ectothiorhodospira*, that use reduced sulfur compounds participate in the primary production via photosynthesis and also are part of the sulfur oxidizing limb of the sulfur cycle of the soda lakes. The sulfur cycle also includes aerobic sulfur oxidizing organisms (Sorokin et al., 1996) and anaerobic sulfate-reducing organisms such as *Spirochaetes* and *Desulfonatronovibrio* (Zhilina et al., 1996a; Zhilina et al., 1997).

Bacillus species are among the most commonly found aerobic, eubacterial alkaliphiles both in soda lakes and in less selective environments (Horikoshi and Akiba, 1982; Krulwich and Guffanti, 1983; Guffanti et al., 1980; Guffanti et al., 1986; Takami et al., 1999a). Fritze et al., 1990, using DNA-DNA hybridization, and Nielsen et al. (Nielsen et al., 1994; Nielsen et al., 1995) using forty-seven physiological and biochemical characteristics as well as DNA base composition, hybridization, and 16S rDNA analyses, proposed clusters of alkaliphiles and alkaline-tolerant *Bacillus*. The 16S rDNA structure indicated two distinct groups within the *Bacillus* radiation (RNA groups 6 and 7) in which most alkaliphilic *Bacillus* isolates are found (Nielsen et al., 1994; Jones et al., 1998). Interestingly, the application of this comprehensive analytic approach resulted in grouping of strains that correlated roughly with somewhat distinct regions of the soda lake environment. *B. alcalophilus* and associated strains were mainly found in muds at the shoreline or dry regions of soda soil where organisms are subjected to fluctuating water levels and concomitant fluctuations in pH and salt levels; many of these strains require only low concentrations of Na^+ for growth. The somewhat diverse "group 7" bacilli, related to *Bacillus clarkii* (Nielsen et al., 1994; Nielsen et al., 1995), are thought to be more prevalent in sediments and waters that are subject to less variability and these alkaliphiles typically exhibit requirements for higher Na^+ for growth (Jones et al., 1998). The molecular physiological studies of Na^+-dependent pH homeostasis in alkaliphiles are consistent with such differences among strains (Krulwich et al., 1982; Garcia et al., 1983), and are beginning to identify respiratory chain components, transporters and cell surface molecules that may be of particular importance to extremophiles that face transitions or fluctuations (Hicks et al., 1991; Ito et al., 1997b).

Some of the most intensely studied alkaliphilic Bacillus strains [i.e., *Bacillus* C-125 (Aono, 1995) and *Bacillus firmus* OF4 (Guffanti et al., 1986)] were characterized before the extensive matrix of physiological and molecular biological criteria, including 16S rDNA, were used to categorize them. In fact, their proposed species, *Bacillus lentus* and *Bacillus firmus*, respectively, were not included in the major alkaliphile clusters (Fritze, 1990; Nielsen et al., 1994; Nielsen et al., 1995). It was apparent that correlation of these alkaliphilic strains with their environmental patterns would require more precise placement. Therefore, recent work, including further biochemical tests and 16S rDNA sequencing, has resulted in proposals for reclassifying *Bacillus* C-125 from a probable *Bacillus lentus* strain to Bacillus halodurans C-125 (Takami and Horikoshi, 1999) and *Bacillus firmus* OF4 to *Bacillus pseudofirmus* OF4 (Takami and Krulwich, in press). The newly deduced relationships of these species to other *Bacillus* species are depicted in Figure 1.

Clostridium strains as well as other diverse anaerobes are well represented in the prokaryotes of soda lakes (Table 1), but detailed studies or major applications of anaerobic, eubacterial alkaliphiles lag behind those of aerobic alkaliphiles. Perhaps the first described alkaliphilic anaerobe, a facultatively anaerobic strain that was subsequently classified as a new genus and species—*Amphibacillus xylanus* (Niimura et al., 1987; Niimura et al., 1989)—is the best studied. This interesting alkaliphile evokes possible shared themes with *B. pasteurii*, inasmuch as it depends upon high concentrations of ammonium for optimal growth. The cells possess an unusual glutamate dehydrogenase that may be involved in the assimilation of the ammonium (Jahns, 1996). *A. xylanus* is deficient in cytochromes, quinones and catalase (Niimura et al., 1987;

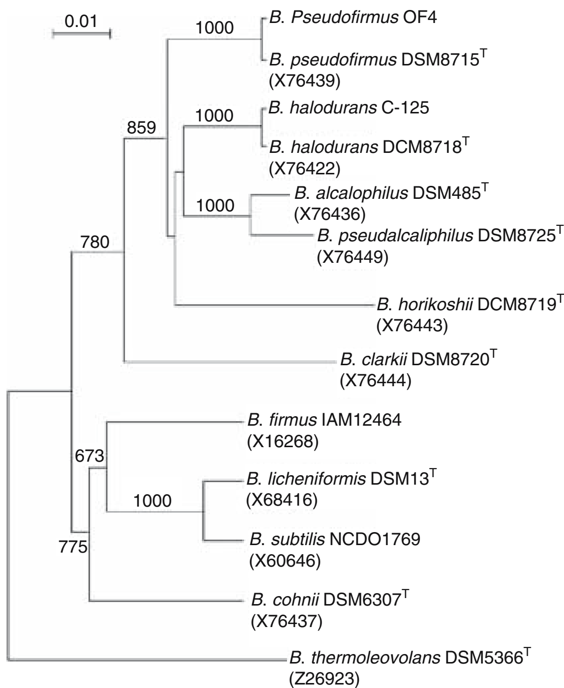

Fig. 1. Unrooted phylogenetic tree showing the relationship of *B. halodurans* C-125 and *B. pseudofirmus* OF4 to other *Bacillus* strains. This figure is a slightly modified version of a figure presented by Takami and Krulwich, in press. The numbers indicate bootstrap samples, from among 1000, that supported the internal branches (Felsenstein, 1985). Bar = 0.01 K_{nuc} unit.

Niimura et al., 1989), and possesses transporters that couple sugar or amino acid transport to Na^+ gradients (i.e., they are Na^+/solute symporters) and are markedly ammonium ion stimulated (Koyama, 1989; Koyama, 1993). Similarly, an apparently V-type NH_4^+- and Na^+-stimulated ATPase has been described (Koyama, 1996; Kaieda et al., 1998). The mechanism and significance of the ammonium effect in this alkaliphile will be of considerable interest as will any special role ammonium might play in pH homeostasis.

Because thermoalkaliphiles were not described in the earliest work on alkaliphiles, this combination of extreme adaptations was considered to be at first incompatible with life. However, during the past decade, major groups of at least moderately thermophilic alkaliphiles began to emerge. These include among others: a novel obligately alkaliphilic *Clostridium* species isolated from sewage (Li et al., 1993; Li et al., 1994; Wiegel, 1998); an asporogenous, Gram-positive ammonifying anaerobe from soda lake deposits, Tindallia magadii (Kevbrin et al., 1998); another xylan-degrading, anaerobic alkalithermophile, strain LB3A (Prowe et al., 1996; Sunna et al., 1997); and an actinomycete, Thermoactinomyces sp. strain HS682 (Tsuchiya et al., 1992). Stetter and colleagues have even described a hyperthermophilic, alkaliphilic archaeum, *Thermococcus alcaliphilus* (Keller et al., 1995), completely laying to rest the notion of incompatibility of thermophily and alkaliphily. Conversely, Kimura and Horikoshi (Kimura and Horikoshi, 1989; Kimura and Horikoshi, 1990) have studied an

Table 1. Taxonomic groups containing prokaryotes isolated from soda lakes (boldface type).

Taxonomic group
Eubacteria
Cyanobacteria
Chroococcales
Oscillatoriales
Spirulina spp.
Firmicutes (Gram-positive bacteria)
Actinomycetes (high G+C Gram-positive bacteria)
Actinomycetales
Micrococcaceae
Nocardiform actinomycetes
Streptomyces
Low G+C Gram-positive bacteria
Bacillaceae
Clostridiaceae
Haloanaerobiales
Proteobacteria
Beta subdivision
Delta subdivision
Gamma subdivision
Ectothiorhodospira
Halomonadaceae
Pseudomonas
Spirochaetales
Spirochaetaceae
Spirochaeta
Thermotogales
Thermopallium
Thermopallium natronophilum
Archaea
Euryarchaeota
Halobacteriales
Halobacteriaceae
Halorubrum
Halorubrum (Natronobacterium vacuolatum)
Natrialba
Natrialba (Natronobacterium) magadii
Natronobacterium
Natronobacterium gregoryi
Unclassified *Natronobacterium* spp.
Natronococcus
Natronococcus amylolyticus
Natronococcus accultus
Unclassified *Natronococcus* spp.
Natronomonas
Natronomonas (Natronobacterium) phamonis
Methanomicrobiales
Methanosarcinaceae
Methanohalophilus
Methanohalophilus oregonensis
Methanohalophilus zhilinaeae
Methanohalophilus sp. Z-7936

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alkalopsychrotrophic Micrococcus that produced an amylase that might be used in food processing.

Finally, the haloalkaliphilic Archaea isolated from hypersaline lakes represent a burgeoning and fascinating group of multiple extremophiles that are now the subjects of broad-based studies. Originally such organisms were classified as *Natronobacterium* or *Natronococcus* (Tindall et al., 1984), halophiles that were most often responsible for the red color of the hypersaline soda lakes (Jones et al., 1998). More recently, the diversity of novel species and lineages has continued to grow (see Kanai et al., 1995; Zhilina et al., 1996b), including many from polymerase chain reaction (PCR)-based analyses of organisms that cannot be cultivated as yet (Grant et al., 1999). Proposals for reorganizing the taxonomy of this group have been made (Kamekura et al., 1997) in what is clearly a "moving target" situation. It is likely that the categories of alkaliphilic methylotrophs and methanogens, which already have been isolated in significant numbers and examples (Boone et al., 1993; Zhilina and Zavarzin, 1994), also will continue to increase in complexity (Kevbrin et al., 1997; Khmelenina et al., 1997).

Adaptation of the Proteins on the Outer Surface and Exoenzymes

An initial set of generalizations describing the basis for protein stability under particular extreme conditions has begun to emerge from the results of experimental and modeling studies carried out on prokaryotic extremophile proteins from thermophiles (Chi et al., 1999; Elcock, 1998; Haney et al., 1999) and halophiles (Eisenberg et al., 1992; Elcock and McCammon, 1998). Generalizations that offer insight into the adaptation of alkaliphile proteins exposed to an extremely alkaline milieu are of similar interest. Many relevant observations have been reported, but broadly, applicable generalizations are not yet clearly in hand. Perhaps the reason lies with the proteins studied, which are often either thermophilic and/or halophilic or both, and therefore special subsets of highly alkaline enzymes or proteins. Or alkaliphily may mandate different responses by different functional categories of proteins. The deduced amino acid sequences, initially made available from genes that were cloned selectively from alkaliphile DNA libraries, revealed a significant number of examples in which the isoelectric point (pI) of the alkaliphile protein or protein domain was much lower than the homologues from other prokaryotes. These sequences had substantially increased aspartate and glutamate and strikingly reduced lysine and arginine. Examples included the prosequence (but not the mature form) of several alkaliphile proteases of the subtilisin type (van der Laan et al., 1991), SecY protein of *B. halodurans* C-125 (Kang et al., 1992), the "periplasmic" cytochrome *c*-binding domain of Cta (cytochrome oxidase) subunit II of *B. pseudofirmus* OF4 (Quirk et al., 1993), and a putative "periplasmic" loop of the FtsH protein from *B. pseudofirmus* OF4 (Ito et al., 1997a). The differences between this latter protein, as deduced from hydropathy analysis and sequence, and the homologue from *Bacillus subtilis* are shown in Fig. 2. Although not shown, there was no pattern of sequence difference in the two putative membrane spanning regions. The extensive pattern of added and substituted acidic residues and loss of basic ones is present throughout the loop, including regions very close to the membrane. In the Cta subunit, the first apparent substitutions of acidic residues for conserved basic ones are within 20 residues of the start of the large hydrophilic domain (Quirk et al., 1993); the net changes in the domain relative to non-alkaliphile homologues is shown in Table 2. The proximity of some of the substitutions to the membrane, in membrane-associated proteins, such as CtaC and FtsH, suggests that the selective pressure that leads to this type of change is exerted very close to the outer surface of the cytoplasmic membrane (Krulwich, 1995).

These differences between alkaliphile and non-alkaliphile proteins or protein domains led to the suggestion that the alkaliphile might globally seek to minimize, in exposed proteins, the content of those basic residues that change charge over the range of external pH values to which the organism was regularly exposed (Krulwich et al., 1998). However, more complete and detailed information from both sequence and structural analyses of extracellular alkaliphile proteins and protein segments suggests that this explanation is unlikely and that the picture is more complex.

A number of laboratories have begun to make chimeras of alkaliphile and non-alkaliphile homologues (Nakamura et al., 1991) to identify domains or residues involved in alkali-tolerance by selective mutagenesis and to design enzymes with increased alkaline-tolerance (Park et al., 1993). These studies give some indication that particular types of enzymes (for example, a particular domain such as a C-terminal domain in cellulases) can be associated with the alkaliphilic property (Nakamura et al., 1991). Moreover, there are now clear-cut examples of "periplasmic" loops and exoenzymes of alkaliphiles in which no evident substitution of acidic for basic residues occurs, relative to non-alkaliphile homologues. Indeed, some exoenzyme proteins,

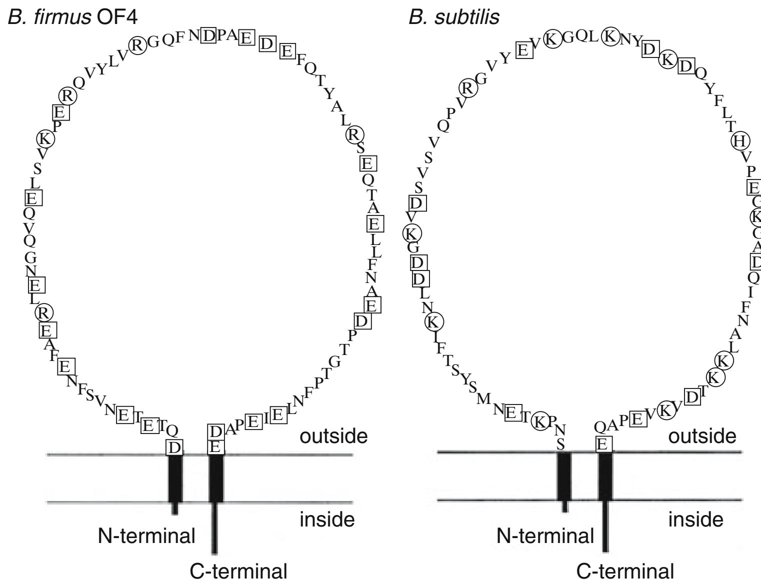

Fig. 2. Comparison of the deduced sequence of the extracytoplasmic domain of the *ftsH* gene products of *B. firmus* OF4 (now *B. pseudofirmus* OF4) and *B. subtilis*. Acidic residues are in squares and basic residues are in circles. Reproduced with permission from the publisher from Ito et al. (1997a).

at least, show the opposite trend as will be discussed in detail in connection with subtilisin type proteases. Then how might the features of FtsH, CtaC, SecY and the prosequence of alkaline proteases be understood as an adaptation to high pH? Ikemura et al., 1987 and Chang et al., 1996 have suggested, based on findings from *B. subtilis* BPN and alkaliphilic *Bacillus* YaB protease studies, respectively, that the prosequence interacts (in trans, outside the cell) with the secreted but inactive protease to facilitate its folding to the active form. The highly charged nature of the prosequence (Ikemura et al., 1987), a feature that is conserved in alkaliphile and non-alkaliphile forms of the prosequence albeit with different sets of residues and vastly different isoelectric points, is proposed to effect this folding. van der Laan et al., 1991 accordingly argue that if acidic residues are not substituted for basic ones, then at the high pH values at which alkaline proteases function, the basic residues largely would be uncharged and protease activity compromised. By analogy the functions of other alkaliphile proteins that differ in the same way from non-alkaliphile homologues may involve interactions that require the protein or domain partner to be highly charged. This suggestion is plausible for at least some of the examples already noted. The hydrophilic domain of CtaC must move in the "periplasm," accepting an electron from complex III and then delivering it to CtaD. The chaperones and secretory proteins also must interact with various partners in specific ways that facilitate protection of conformation or movement. Following the expansion of this group of proteins will permit the adaptation to be more mechanistically elucidated.

What about proteins without a special functional need for high charge density? The most intensive structural and modeling work to date on alkaliphile enzymes has focused on proteases, especially those of the subtilisin, serine protease type. This work has included X-ray crystallographic analysis, modeling and engineering of the active site (Sobek et al., 1990; Sobek et al., 1992; Teplyakov et al., 1992; van der Laan et al., 1992; van der Laan et al., 1996; Martin et al., 1997; Shirai et al., 1997). van der Laan et al., 1992 concluded that the 3-dimensional structure of the alkaline subtilisin-like protease PB92

Table 2. Comparisons of basic and acid amino acid contents of the hydrophilic cytochrome-*c*-binding domains of CtaC from alkaliphilic *Bacillus pseudofirmus* OF4 and non-alkaliphilic *Bacillus cta*-encoded cytochrome oxidases.[1]

Organism	Amino acids (#) in domain	Isoelectric point	Arginime + lysine	Aspartate + glutamate	GenBank Accession #
B. pseudofirmus OF4	234	4.1	14	42	Q04441
B. subtilis	246	7.7	38	38	P24011
B. stearothermophilus	247	5.5	31	36	BAA11111
Bacillus PS3	247	8.6	36	32	Q03438

[1]The hydrophilic domain starts at amino acid residue 109 from the N-terminal methionine or at amino acid residue 87 of the mature protein of the CtaC sequences; the homologous regions were identified from alignments with the domains from other strains.

(also called SBA) revealed no "unique features" that could explain its very high pH optimum and suggested that sequence features could explain this optimum. In contrast to the observations on CtaC, FtsH, alkaline protease prosequences, and others already cited, PB92 and another highly alkaline protease, elastase YaB (Kaneko et al., 1989), had extremely high isoelectric points, as a result of decrease in aspartic acid residues and substitution of arginine for lysine residues, although not in equivalent positions. In addition, the number of tyrosines, which would be negatively charged at the enzyme's pH optimum, is reduced (van der Laan et al., 1992). Interestingly, tyrosines do not always follow the pattern of glutamic and aspartic acid residues, i.e., increasing substantially in alkaliphilic proteins such as FtsH or consistently decreasing in high alkaline proteases (Shirai et al., 1997). It will be of interest, as more correlations become possible, to see whether the number of tyrosine residues, which may be "counted" as a likely source of negative charge predominantly in strongly obligate alkaliphiles, varies with particularly high pH optima for growth. Other alkaliphiles, whose pH range includes lower alkaline values at which tyrosine would be uncharged, might not adapt with a dependence upon this residue.

In a report of the crystal structure of the alkaliphilic *Bacillus* M-protease, another subtilisin-type serine protease, Shirai et al., 1997 further discussed the importance of sequence features in adaptation to alkaline conditions. They noted that of the three alkaliphile proteases of the subtilisin type with very high pH optima (PB92, M-protease, and elastase YaB) all had elevated isoelectric points relative to homologues with lower pH optima. M-protease had a markedly lower aspartic and glutamic acid content, which contributed to raising the isoelectric point. As noted in the other highly alkaline proteases, arginine was increased relative to lysine. The neutral hydrophilic amino acid residues, asparagine, glutamine and histidine, were increased, maintaining the solubility of the protein in water, perhaps by compensating for lost acidic residues and lysine. The increased arginine could contribute to the elevated isoelectric point and since arginine can retain a positive charge under more alkaline conditions than lysine, it is more suitable for ion pair formation. Ion pairs, including a significant number with arginine partners, increased. Hydrogen bonds also increased in the alkaliphile proteases relative to non-alkaliphile homologues. The investigators suggest that these features are important components of alkaline adaptation. Moreover, the "substituted residues" in the 3-dimensional structure had a biased distribution that correlated with peptide shifts

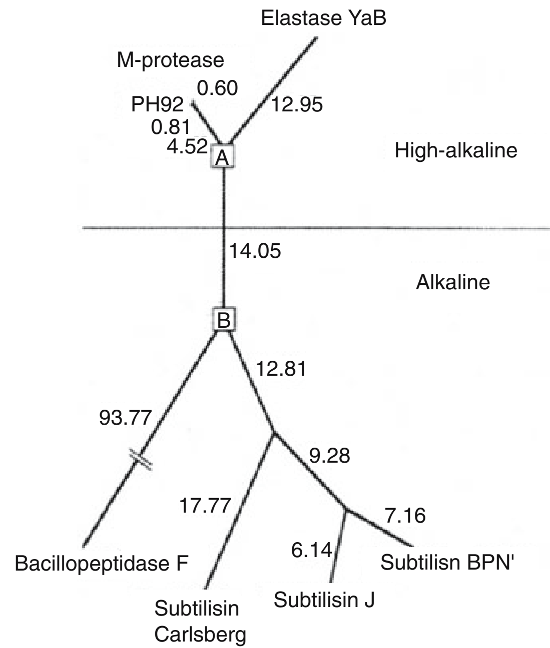

Fig. 3. The phyogenetic tree of M-protease and related proteases showing evolutionary distances. The evolutionary distances are shown on the branches in percent between accepted point mutations. Two boxes indicate positions of hypothetical ancestors A and B (see Fig. 4). All of the branches are more than 95% probable from 1000 bootstrap reconstructions. This figure was reproduced from Shirai et al. (1997), with permission from the publisher.

hypothesized to be responsible for stabilizing conformation under alkaline conditions. Shirai et al., 1997 visualized the high alkaline subtilisin type proteases and the non-alkaliphilic ones as having branched off, respectively, from hypothetical ancestor proteases A and B (see Figs. 3 and 4).

The caveat is that these fascinating proposals may apply to some categories of alkaliphile proteins but not to others. Table 3 shows a small selection of alkaliphile exoenzymes and some examples of enzymes other than proteases (e.g., pectate lyase Pel-7; Kobayashi et al., 1999) that have a highly alkaline pH optimum as well as a high pI. However, other enzymes, including other proteases, have high pH optima but not high pIs. Perhaps diverse sets of adaptations are associated with different catalytic activities and mechanisms, different 3-dimensional structural features, or both. Also important is the conjoining, in many alkaliphiles, of the constraints of thermophily or halophily or both with alkaliphily. Thus, the mature form of the α-amylase from haloalkaliphilic *Natronococcus* sp. Ah-36 was deduced to have a very high acidic amino acid content, but this is a general adaptation to halophily (Lanyi, 1974; Eisenberg et al., 1992).

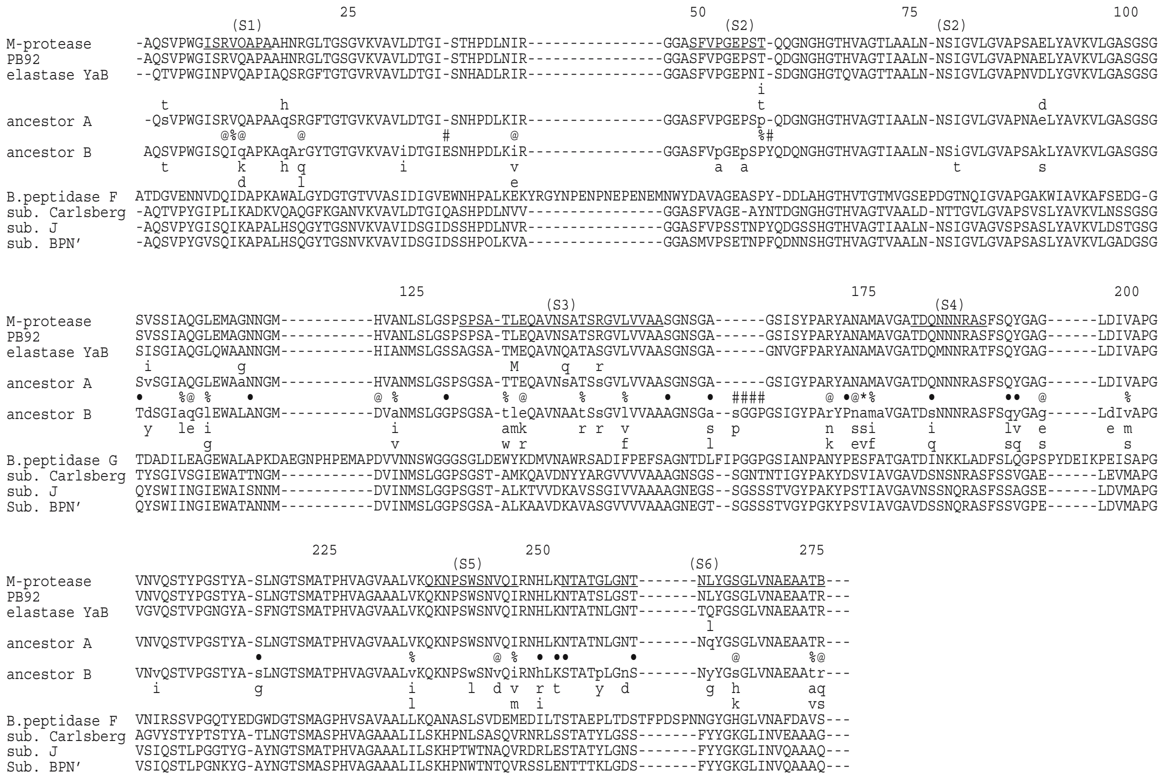

Fig. 4. An alignment of amino acid sequences of M-protease and related proteases showing the sequence of hypothetical ancestors A and B, with the "high-alkaline" protease sequences from Fig. 3 shown above ancestor A and the "alkaline" protease sequences from Fig. 3 shown below ancestor B. Residue numbers of M-protease are indicated. Symbols between the ancestral sequences indicate residues that have been substituted between them. Symbols @, %, and * indicate substitutions of ionizable residues, residues at the interface of shifted segments and others, respectively. Symbol # indicates inserted/deleted residue(s). In the M-protease sequence underlining is used to show positions of shifted sequences in the structural studies. This figure was reproduced, with permission from the publisher, from Shirai et al. (1997).

Table 3. The isoelectric point (pI) and pH optimum for activity of selected exoenzymes from alkaliphilic *Bacillus* strains.

Enzyme	*Bacillus* strain	pI	pH optimum	References
Protease PB92	*B. alcalophilus*	>10	10.5–12	Vander Laan et al., 1991; Zuidweg et al., 1972
Elastase YaB	*Bacillus* YaB	>10	11.7	Tsai et al., 1983; Kaneko et al., 1989
M-protease	*Bacillus* KSM-K16	10.6	12.3	Kobayashi et al., 1995
ALPase II protease[1]	*Bacillus* NKS-21	2.8	10.2	Yamagata and Ichishima, 1989
Protease	*Bacillus* NKS-21	8.2	10–11	Tsuchida et al., 1986
Pectate Lyase Pel-7	*Bacillus* KSMP7	10.5	10.5	Kobayashi et al., 1999
Xylanase J	*Bacillus* sp. str. 41M-1	5.3	9	Nakamura et al., 1993

[1]ALP = alkaline proteinase

Are There Global Adaptations of the Cell Surface Layers?

Cytoplasmic Membrane

Little attention has been focused on the cytoplasmic membrane characteristics of extreme, non-haloalkaliphilic prokaryotes living in alkaline medium—their primary stress. Indications are that studies of membrane lipids will be important in elucidating major adaptations to such environments. Koga et al., 1982 and Nishihara et al., 1982 reported on the lipid composition of alkaliphilic *Bacillus* sp. strain A-007 and several other alkaliphilic *Bacillus* strains, in which they characterized a novel polar lipid identified as *bis*-(monoacylglycero)-phosphate (BMP). This lipid's presence in mammalian lysosomes but not in prokaryotes has been reported (Horikoshi, 1991). Other phospholipids found in *Bacillus* species included phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and often car-

diolipin (CL) with an array of branched-chain and other fatty acids. Cardiolipin concentrations were generally significant, whereas glycolipids and glycophospholipids that often are found in Gram-positive bacteria were absent. The neutral lipid fraction contained diacylglycerol, both squalene and dehydrosqualene, as well as an uncharacterized component that accounted for about 20% of the neutral lipid (Koga et al., 1982).

Subsequently, Clejan et al., 1986 compared the lipid composition of several obligately and facultatively alkaliphilic strains. Again, high levels of CL were reported, i.e., 13% and 25% of the polar lipids in *B. pseudofirmus* RAB and OF4, respectively. The obligately alkaliphilic *B. pseudofirmus* RAB had a much higher neutral/polar lipid ratio than the facultatively alkaliphilic *B. pseudofirmus* OF4; the neutral lipid fraction contained squalene and dehydrosqualene, diacylglycerol and some incompletely characterized long-chain isoprenoid lipids. In a subsequent study (Clejan et al., 1988), the permeability of lipid vesicles prepared with different ratios of the diacylglycerol and isoprenoid fractions was found to be enhanced by diacylglycerol and decreased by the isoprenoid fraction. These neutral lipid components may be part of the balancing of fluidity with barrier functions of the coupling membrane. The obligately alkaliphilic *B. pseudofirmus* RAB also had 90% branched-chain fatty acids as opposed to 72% in the facultatively alkaliphilic *B. pseudofirmus* OF4, and whereas the facultative alkaliphile had no unsaturated fatty acids in its phospholipids, the obligate alkaliphile had a significant amount (Clejan et al., 1986). It was hypothesized that the obligate alkaliphile might have membrane lipid properties that functioned well at highly alkaline pH values but became too fluid and permeable at near neutral pH values. This hypothesis was supported by the finding that the presence of low concentrations of unsaturated fatty acid stops the facultative strain from growing in the low end of its former pH range for growth and upon incorporation of the unsaturated fatty acid into the membrane (Dunkley et al., 1991). Interestingly, a more recent alkaliphile isolate, *Bacillus cohnii* was noted to have an extraordinarily high content of unsaturated fatty acids (Spanka and Fritze, 1993). Aono et al., 1992 have noted the instability of protoplast membranes of *B. halodurans* C-125 at alkaline pH values that are well below the optimum for growth. Similarly, Krulwich et al., 1985a found during studies of cytoplasmic buffering capacities in *Bacillus* species with diverse pH optima for growth, that the alkaliphiles lost stability below pH 7. These phenomena may reflect the same general membrane property that limits the low range of growth pH. As indicated below, increased autolysis of the peptidoglycan also could be involved.

Recently, Gilmour et al. (manuscript submitted) conducted a study of the proteins found in pH 7.5- and pH 10.5-grown *B. pseudofirmus* OF4. Among the proteins that were strongly up-regulated at pH 10.5 were two enzymes that are likely to be involved in branched-chain fatty acid metabolism or production. The upregulation of these enzymes might relate to remodeling involved in adaptation to the high pH.

The membrane lipids of alkaliphilic *Bacillus* species clearly contain novel components whose role is unclear, high levels of CL and of branched-chain fatty acids. The more detailed characterization of the membrane lipid components and mutational analyses of their functions will be important areas for furthering our understanding of the physiology of this group.

Peptidoglycan and Associated Polymers

The alkaliphile peptidoglycan and associated polymers, especially in *Bacillus* species, have received more detailed attention than the membrane lipids. The data that have emerged from studies thus far indicate that both the turnover dynamics of the peptidoglycan itself and pH-dependent changes in associated, negatively charged polymers may be important contributors to alkaliphile physiology. Koyama and Nosoh, 1976 noted that cells of an alkaliphilic *Bacillus* strain were more negatively charged upon growth at pH 10 than at 8.2. Subsequent studies of the composition of a diverse group of alkaliphilic *Bacillus* species at different pH values for growth indicated that these organisms generally have the A1γ type of peptidoglycan in which *meso*-diaminopimelic acid, the third residue in the tetrapeptide of mature peptidoglycan units, is linked via its ε-amino group directly to the terminal D-alanine of another peptide (Aono et al., 1984); some variations have since been found, including the presence of ornithine instead of diaminopimelic acid in *B. cohnii* (Spanka and Fritze, 1993). Some alkaliphiles (e.g. *B. halodurans* C-125) are more susceptible to autolysis at near neutral pH and exhibit a lower (%) crosslinking of the peptidoglycan at lower growth pH than at alkaline pH (Aono and Sanada, 1994). These interesting differences, however, still do not account for the more negative charge of the surface layers at high pH.

Cell wall-associated polymers that are highly acidic appear to be responsible for pH-dependent changes in cell surface charge, and the polymers differ between groups of alkaliphilic *Bacillus* species. *Bacillus halodurans* C-125 was among those species that produced a teichuronic acid composed of *N*-acetyl-D-fucosamine, glucu-

ronic acid and galacturonic acid (Aono, 1985) and a teichuronopeptide composed of a polyglucuronic acid and a polyglutamate polymer (Aono, 1989; Aono et al., 1993). Mutants that were defective in one or both of these polymers were growth defective at high pH (Aono and Ohtani, 1990; Ito et al., 1994), which indicates that these polymers are important in the alkaliphily of this group of *Bacillus* species (Aono and Ohtani, 1990; Aono et al., 1995). Notably, these organisms were cultivated in glucose-containing media where alkaliphiles are often a bit less fastidious than when in a medium of nonfermentative carbon sources (Gilmour and Krulwich, 1997).

The uronic acid polymers are not found in other groups of alkaliphilic *Bacillus* species, e.g., *B. pseudofirmus* OF4 (Aono, 1985; Guffanti and Krulwich, 1994). On the other hand, recent studies have shown that *B. pseudofirmus* OF4 has an acidic S-layer polymer produced from a gene with strong homology to similar genes from *Bacillus anthracis* and *Bacillus licheniformis*. This S-layer polymer was identified in 2-dimensional gel electrophoretic patterns of membrane-associated proteins from pH 10.5- and pH 7.5-grown cells of *B. pseudofirmus* OF4, and characterized as a heterogeneous, apparently processed, protein that is present in greater amounts at high pH. By cloning, sequencing and disrupting the S-layer gene, the S-layer is found to be dispensable for alkaliphily in *B. pseudofirmus* OF4, but confers an advantage to cell growth at pH 10.5 and to cytoplasmic pH homeostasis in a sudden shift from pH 8.5 to 10.5 (Gilmour et al., manuscript submitted). *B. pseudofirmus* OF4 also may have an acidic capsule layer because a partial sequence for genes that are likely to encode enzymes synthetic for a polyglutamate capsule was identified in this species (Ito et al., 1997a). The characterization of the complete sequence and role of this locus will be interesting.

Are There Global Adaptations of Cytoplasmic Components?

Buffering Capacity

A comparison of the cytoplasmic buffering capacity of *Bacillus* species that grow in vastly different pH ranges indicated that alkaliphiles, grown on nonfermentable carbon sources, had higher cytoplasmic buffering capacities at alkaline pH than at lower pH (Krulwich et al., 1985a). In a recent study, Rius and Loren, 1998 reported comparative values for the cytoplasmic buffering capacity of *B. alcalophilus* grown on both fermentative and nonfermentative carbon sources. These investigators used the decay of an acid pulse (Maloney, 1979) to determine both cytoplasmic buffering capacity and membrane H+ conductance, thereby avoiding problems associated with permeabilizing cells. In media with either nonfermentative carbon sources or fermentative carbon sources, the cytoplasmic buffering capacity of *B. alcalophilus* was much higher in pH 10.5-grown than in pH 8.5-grown cells. Strikingly, the alkaliphile cells (and others, such as *Staphylococcus aureus* and *B. subtilis*) had vastly lower cytoplasmic buffering capacity when grown on malate-carbonate media than on media with fermentative carbon sources (Rius and Loren, 1998). Since malate-carbonate media support a more alkaline optimum for the growth pH, there is probably no direct relationship between overall cytoplasmic buffering capacity and the capacity to grow at the upper reaches of pH. On the other hand, it is quite possible that specific compounds play particular roles in connection with alkaliphily. For example, several studies have focused on a shift in the ratio of the major polyamine compounds such as spermidine, which predominates heavily at very alkaline pH values (Chen and Cheng, 1988; Hamana et al., 1989).

Alkali-Stability of Cytoplasmic Components

The general impression is that the cytoplasmic pH, for most alkaliphiles in their optimum pH range, is within about 0.5 pH units of the cytoplasmic pH optimum for most bacteria. Hence there may not be major or even discernible, global adaptations in the protein structure or pH profile (Horikoshi and Akiba, 1982; Horikoshi, 1991). But throughout the literature on alkaliphiles, at least some apparent cytoplasmic enzymes have unusually high pH profiles (Horikoshi and Akiba, 1982). Some reports would seem to beg for an explanation; for example, a putative "intracellular alkaline serine protease" from alkaliphilic *Thermoactinomyces* sp. HS682 was produced from its gene in *Escherichia coli*. The purified enzyme had a pH optimum of 11 (Tsuchiya et al., 1997).

In consideration of such observations and the recognition that some of the soda lake alkaliphiles have evolved over a long period in a consistently high alkaline environment, it may be well worth looking explicitly for exceptions to the expectation that cytoplasmic proteins have no global adaptations to a higher than conventional pH. In a non-alkaliphile that completely lacks active pH homeostatic mechanisms, such as *Clostridium fervidus* (Speelmans et al., 1993), the organism can only grow in a narrow pH range up to about pH 7.7. In the

especially effective pH homeostatic mechanisms of most well studied alkaliphiles (Krulwich et al., 1997), the upper pH limit is generally about pH 9 to 9.5. Even in those alkaliphiles, which have most often been soil isolates originally, subtle but important global adaptations in the cytoplasmic proteins are possible. Perhaps among the soda lake alkaliphiles there are organisms that are less dependent on remarkable pH homeostasis mechanisms because their cytoplasmic enzymes and functional assemblies (e.g., secretion and protein synthetic machinery) are all markedly alkali-adapted relative to those of conventional bacteria. A novel osmolyte has been noted in haloalkaliphilic archaea (Desmarais et al., 1997). Perhaps the cytoplasmic proteins of some of these organisms are not only salt-adapted but also at least unusually alkali-adapted.

It also will be of interest to carefully examine the properties of ribosomal and other proteins that must interact with nucleic acid molecules in a cytoplasm that is generally at least half a pH unit higher than the cytoplasm of conventional bacteria growing at optimal pH (Krulwich, 1995). Horikoshi, 1991 has noted a pH optimum for protein synthesis in an alkaliphilic *Bacillus* was about half a pH unit higher than that of *B. subtilis*. Genes for ribosomal proteins and major polymerases are beginning to be identified so that detailed examinations will be facilitated (Nakasone et al., 1998; Takami et al., 1999d). Thus far, homologous DNA-binding proteins from alkaliphiles and non-alkaliphiles have not been compared in great detail, the two small acid-soluble spore proteins (SASPs) sequenced from alkaliphiles (Quirk, 1993; Wei et al., 1999) have not been examined closely from this perspective.

Proteome Studies

The 2-dimensional gel analysis of proteins from steady-state pH 7.5- and pH 10.5-grown *B. pseudofirmus* OF4 cells, and from cells grown at lower pH and rapidly shifted to pH 10.5, revealed several interesting features that will merit further examination in this and other alkaliphiles (Gilmour et al., manuscript submitted). Significant numbers of proteins were found to be either up-regulated or down-regulated at high pH, but the extent of the change was typically greater for the up-regulated genes. In addition, a substantially greater number of genes were up-regulated transiently with rapid increase of pH to 10.5 than were ultimately up-regulated in the steady-state cells; this find correlates with other data indicating that there are groups of proteins that play a specific role in the initial adjustment to a major alkaline shift.

Genomics

The vastly increasing database on sequences of alkaliphile proteins will help clarify the issues already raised about global adaptations in cytoplasmic proteins or in functional cytoplasmic assemblies (e.g., ribosomes and secretory particles). The completion of the *B. halodurans* C-125 genome will be a major step in this process (Takami et al., 1999b, Takami et al., 1999d). The maps of alkaliphiles and gene order in several large pieces of DNA suggest that interesting features of alkaliphily may emerge (Takami et al., 1999b; Takami et al., 1999d; Gronstad et al., 1998; Wei et al., 1999).

Some, but probably not the majority, of the alkaliphilic *Bacillus* species and strains have been found to harbor endogenous plasmids. Most of these have been relatively small and have not been exciting candidates for development of host-vector systems. Rather, the plasmids that have been extensively developed for use in *B. subtilis* generally have been adapted and applied to molecular manipulations of alkaliphilic *Bacillus* species (Horikoshi, 1991). However, Fish et al., 1999 have recently found that eight alkaliphile halomonads, out of the seventeen they examined, possessed one or more plasmids in the size range of 5.3 to 33 kb; they concluded that some of these would be suitable for vector development. A large endogenous plasmid, approximately 30 kb, also has been found in *B. pseudofirmus* OF4 (Gronstad et al., 1998) and bears the cadmium-resistance locus (Ivey et al., 1992). There is no evidence to date for a role of plasmid-borne genes in alkaliphily.

Active pH Homeostasis and the Involvement of Secondary Na^+/H^+

Antiporters and Secondary Na^+/Solute Symporters

In alkaliphilic *Bacillus* species, active ion transport mechanisms are central to the crucial process of pH homeostasis, and this process appears to limit the upper pH limit for growth (Krulwich, 1995; Krulwich et al., 1997). Sturr et al., 1994 conducted studies of the bioenergetic parameters of *B. pseudofirmus* OF4 in a pH-controlled continuous culture apparatus in which the bacteria were grown aerobically on malate-containing media at various, fixed pH values from pH 7.5 to 11.4. In fact, the upper limit for growth was not found in this range, with a long but certainly viable generation time of 700 min found at pH 11.4. As shown in Fig. 5, *B. pseudofirmus* OF4 grows slightly better at pH 8.5 to 10.5 than at pH 7.5, and throughout this pH range, the cytoplas-

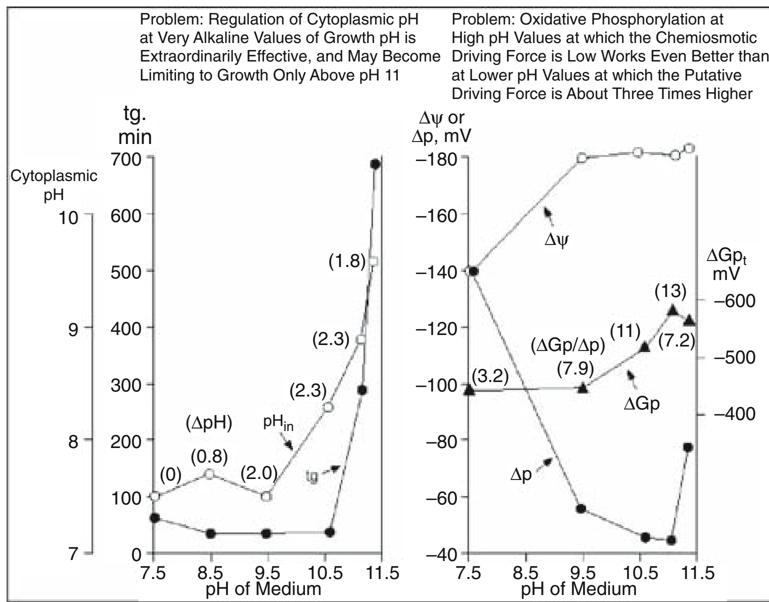

Fig. 5. Bioenergetic parameters of *B. pseudofirmus* OF4 during growth in continuous cultures at various controlled pH values. The data from Sturr et al., 1994 were replotted to highlight particular features of interest. Left: the doubling time (tg, min) and cytoplasmic pH (pH_{in}) are shown as a function of the growth pH. The numbers in parentheses above the points on the cytoplasmic pH curve are the values for δpH. Right: The transmembrane electrical potential, positive out (δ ψ), the total bulk electrochemical proton gradient (δp), and the phosphorylation potential (δgp) are all shown in mV as a function of growth pH. The numbers in parentheses are the δGp/δp, which would reflect the H^+ stoichiometry if coupling were strictly to a bulk gradient. This figure was reproduced with permission from the publisher from Krulwich (1995).

mic pH remains below 8.5; at external pH values of 9.5 and above, the δpH (transmembrane pH gradient) is 2 full pH units or more. At the highest external pH included in the study, pH 11.4, the internal pH was at about 9.6. The growth rate slowed dramatically, and in parallel, with the increasing cytoplasmic pH above a value of 8.2. Thus in this type of alkaliphilic *Bacillus*, pH homeostasis is remarkable: cytoplasmic pH is maintained at about pH 7.5 until the δpH exceeds 2 and thereafter, the rise in cytoplasmic pH is correlated with a decrease in growth rate (i.e., increase in generation time). The finding of optimal growth rates up to about pH 10.5, at which the cytoplasmic pH is maintained below pH 8.5, was corroborated in other chemostat studies (Guffanti and Hicks, 1991) as well as batch culture studies (Hirota and Imae, 1983; Aono et al., 1997). Under conditions in which the cells are well energized, the magnitude of the δpH found among different alkaliphilic *Bacillus* species using several different kinds of probes to assess this parameter has been very consistent. Significantly, the full δpH that the alkaliphiles maintain was not measured in the earliest studies (e.g., Guffanti et al., 1978) because the measurements were carried out in buffers without energy sources. Thus compendia of such measurements often contain citations to a mixture of experimental conditions which determine the extent to which alkaliphiles grow.

There is a large body of evidence for the crucial Na^+ cycle involvement in net acidification of the cytoplasm during growth of alkaliphilic prokaryotes at alkaline pH. That alkaliphiles required Na^+ and used Na^+ as the coupling ion for transport systems (Koyama et al., 1976; Kitada and Horikoshi, 1977; Guffanti et al., 1978) had already been demonstrated when work on both wild type alkaliphiles and pH homeostasis-negative non-alkaliphilic mutants began to implicate Na^+/H^+ antiporters as key mediators of cytoplasmic acidification (Mandel et al., 1980; Koyama et al., 1986; Krulwich and Guffanti, 1983, Krulwich and Guffanti, 1989). That maintenance of a cytoplasmic pH well below the external pH depends upon the availability of Na^+ (Mandel et al., 1980; Krulwich et al., 1982; McLaggan et al., 1984; Krulwich et al., 1985b) was established in several types of alkaliphiles. Diverse mutant strains, non-alkaliphiles that were specifically deficient in their ability to regulate cytoplasmic pH, also were shown to be deficient in Na^+/H^+ antiport (Mandel et al., 1980; Krulwich et al., 1982; Garcia et al., 1983; Hamamoto et al., 1994; Krulwich et al., 1996). Conversely, *B. pseudofirmus* OF4 cells taken from continuous cultures maintained at pH 11.4, contained variants with elevated Na^+/H^+ antiporter activity (Sturr et al., 1994).

Electrogenic Na^+/H^+ antiporters that catalyze exchange of intracellular Na^+ for a stoichiometrically greater number of H^+ from the external milieu transport net positive charge inward during each turnover. Accordingly, these fluxes can be energized by the transmembrane electrical potential component (δ) of the total electrochemical proton gradient (δp) that is established by the proton-extruding respiratory chain (or by H^+-extruding ATPases in anaerobic cells). Components of the respiratory chain and diverse, electrogenic Na^+/H^+ antiporters are depicted in a diagram of an aerobic alkaliphilic *Bacillus* in Fig. 6. Because the δ can energize the electro-

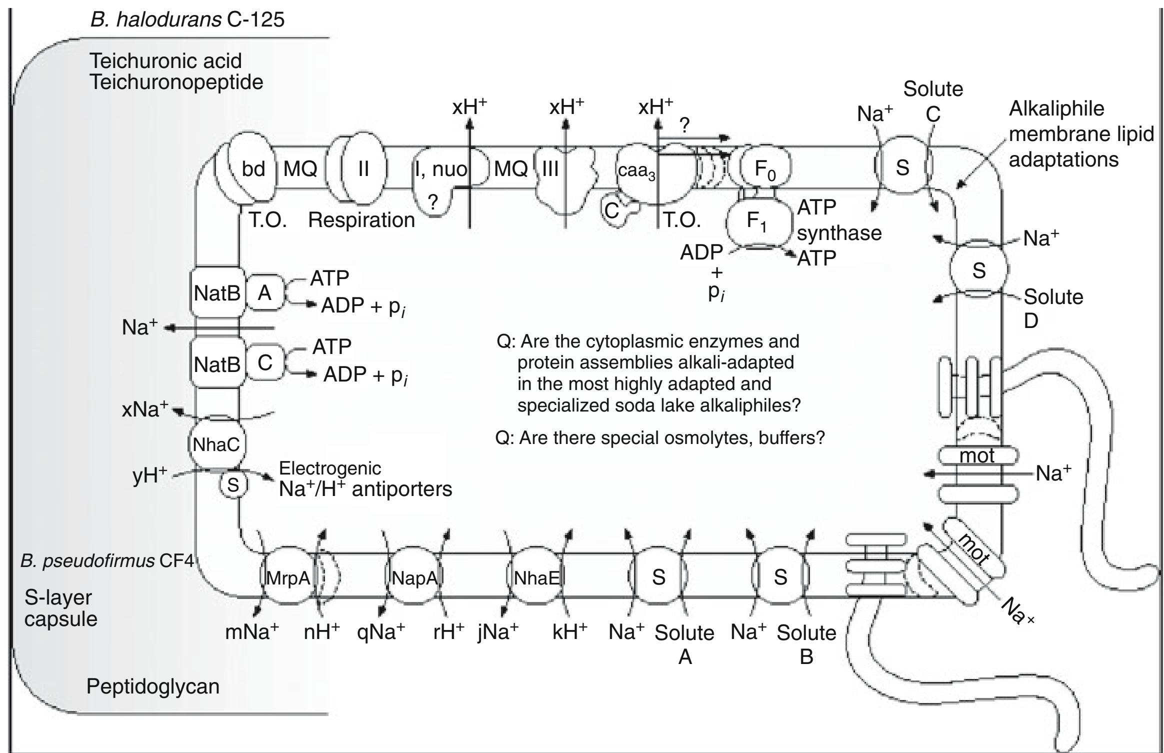

Fig. 6. A schematic of an alkaliphilic *Bacillus*, showing the membrane-associated ion-translocating proteins and complexes involved in the primary generation of a δp as well as the secondary antiporters (Mrp, Nap, Nha) and symporters (S) that together catalyze solute uptake and net proton accumulation to achieve a lower cytoplasmic than medium pH. Also shown are: the flagellar assembly and associated Na^+ channel that may provide another physiologically important Na^+ reentry route in addition to the Na^+-coupled symporters; and the F_1F_O-ATP synthase. This proton-coupled synthase may, at pH > 9.2, accept protons that are somehow sequestered as indicated by arrows parallel to, and either just above or just below, the membrane surface. The dotted outline of the caa_3-type oxidase represents a hypothetical route by which protons are transferred directly from this complex to the synthase in protein-protein interactions. The dotted outline of MrpA indicates that at least under some conditions this antiporter may function as part of a complex. Outlines of a cell-wall-associated layer reflect the finding that in *B. halodurans* C-125 and *B. pseudofirmus* OF4, different negatively charged polymers play at least some role in pH homeostasis. Other possibilities that are yet to be clarified are indicated by the questions (Q:) presented in the cytoplasmic space.

genic antiport, it is possible for such antiporters, working in concert with respiration, to acidify the cytoplasm relative to the medium (McNab and Castle, 1987). For such antiport to support pH homeostasis on a continuous basis, however, Na^+ must be recycled to maintain the source of the cytoplasmic substrate for the Na^+/H^+ antiporters. Compelling evidence has been presented for an important role of the numerous Na^+-coupled solute uptake systems, Na^+/solute symporters, in Na^+ reentry in support of pH homeostasis (Krulwich et al., 1985b). In fact, it was hypothesized, when pleiotropy was observed in some non-alkaliphilic mutant strains, that the alkaliphile Na^+/H^+ antiporter(s) and Na^+/solute symporter(s) playing critical roles in pH homeostasis might share a common subunit (Guffanti et al., 1981). However, both the ion specificity of the transporters (Sugiyama et al., 1985) and the many genes that now have been identified fail to support such a common structure. Rather, the pleiotropy of many non-alkaliphilic mutants likely relates to the complexity of the physiological networks surrounding the important cell functions of pH homeostasis, solute transport and Na^+-resistance (Krulwich et al., 1996, Krulwich et al., 1997, Krulwich et al., 1998) and the likely complexity of one of the major antipor/t systems (Hamamoto et al., 1994; Hashimoto et al., 1994; Ito et al., 1999). In addition to the symporters, some pH-sensitive mechanism for Na^+ reentry must complete the Na^+ cycle that supports pH homeostasis even when solutes are not present and Na^+ is not abundant (Booth, 1985; Krulwich and Guffanti, 1989; Krulwich et al., 1997). A good candidate for a pH-dependent Na^+ entry route of this sort, as depicted in Fig. 6, is the Na^+-translocating channel associated with flagellar rotation in the alkaliphilic *Bacillus* species (Sugiyama, 1995). Although much less complete pictures of the pH homeostasis cycle of alkaliphiles outside the genus *Bacillus* have been

presented, parameters of the cycle (Cook et al., 1996) and involvement of Na^+ or Na^+/H^+ antiporters or both in the central cell energetics of other alkaliphiles have been described (Buck and Smith, 1995; Prowe et al., 1996).

The cycle outlined above for pH homeostasis has been the model of this function in many non-alkaliphilic bacteria, but the alkaliphilic *Bacillus* species have an extraordinary capacity for pH homeostasis as well as some specificity that is not shared by non-alkaliphiles. *B. pseudofirmus* OF4 has at least 10 times the aggregate Na^+/H^+ antiporter activity as *B. subtilis* and has far more capacity for pH homeostasis than the non-alkaliphile. In addition, pH homeostasis in *B. subtilis* can draw upon K^+/H^+ antiport as well as well as Na^+/H^+ antiport, which is carried out by antiporters that can use either cation. On the other hand in the alkaliphilic *Bacillus* species, the process depends specifically on Na^+ (Krulwich et al., 1994, Krulwich et al., 1999). Although there is evidence for K^+/H^+ antiporters in alkaliphiles (Mandel et al., 1980; Kitada et al., 1997), and the first Na+(K+)/H^+ antiporter-encoding gene has now been identified in *B. pseudofirmus* OF4 (Wei et al., manuscript submitted), it is unclear what the role of the K^+/H^+ antiport is and why it does not come into play in any obvious way in pH homeostasis. If *B. subtilis* is subjected to a sudden shift in pH from 7.5 to 8.5, for example, the initial cytoplasmic pH of about 7.5 is maintained as long as either K^+ or Na^+ is present. Whereas in the alkaliphile, only Na^+ can support this homeostasis or comparable homeostasis during a pH 8.5 to 10.5 shift in the external pH (Krulwich et al., 1994; Krulwich et al., 1999; Ivey et al., 1998).

Most work on pH homeostasis of alkaliphilic *Bacillus* species has lately been carried out on *B. halodurans* C-125 and *B. pseudofirmus* OF4 and has focused upon identification of the antiporter-encoding genes that have roles in pH homeostasis. It has been clear for several years that, as in other bacteria (Padan and Schuldiner, 1996; Krulwich et al., 1999), alkaliphilic *Bacillus* species have more than one Na^+/H^+ antiporter system with a role in pH homeostasis (Kitada et al., 1994; Krulwich, 1995; Ito et al., 1997b). Currently, more Na^+/H^+ antiporters have been identified in *B. pseudofirmus* OF4 than in any other single organism; these are indicated in Fig. 6 as mrp, nhaC, napA, and nhaE. It is likely that one of these antiporters has the dominant role in pH homeostasis. This antiporter is encoded by a locus first identified and partially characterized by Horikoshi, Kudo and colleagues in *B. halodurans* C-125 (Kudo et al., 1990; Hamamoto et al., 1994). It is also found in *B. pseudofirmus* OF4 (Krulwich et al., 1998; Krulwich et al., 1999) and other bacteria (Putnoky et al., 1998; Hiramatsu et al., 1998) and has been named *mrp* (for multiple resistances and pH) in *B. subtilis* (Ito et al., 1999). The locus encoding all of these homologues is an intriguing 7-gene operon in which all of the deduced products are hydrophobic and several of them have sequence similarity to membrane-embedded subunits of the NDH-1 type of NADH dehydrogenase complexes (Yagi, 1993). A point mutation in the *mrpA* gene of *B. halodurans* C-125 renders the bacteria non-alkaliphilic and extremely defective in pH homeostasis (Hamamoto et al., 1994). Deletion of the mrpA of *B. subtilis* makes this strain extremely sensitive to Na^+ and modestly deficient in pH homeostasis in certain concentration ranges of Na^+ or K^+ (Ito et al., 1999). An unusual dependence of MrpA function on other genes in the operon (Hiramatsu et al., 1998; Ito et al. 1999) has led to the speculation that *mrp* might be an obligatory complex (Hiramatsu et al., 1998). Because it appears that MrpA can function as a secondary antiporter system in stoichiometric excess but not in the absence of the other gene products, another complex-involved mode of transport is still possible (Ito et al., 1999); this is indicated by the dotted lines in Fig. 6. The dissection of the structural form(s) of the active complex, their mechanism and roles in the alkaliphile, and their differences from homologues in non-alkaliphiles, are important areas of current investigation.

The *nhaC* gene of *B. pseudofirmus* OF4 was the first alkaliphile Na^+/H^+ antiporter-encoding gene to be cloned, which was achieved by functional complementation of an antiporter-deficient *Escherichia coli* mutant (Ivey et al., 1991). Subsequent deletion of the gene showed that the antiporter played a role in high affinity Na^+/H^+ antiport at both pH 7.5 and 10.5. It was the major antiporter with such affinity in pH 7.5-grown cells, whereas another high affinity system was induced during growth at pH 10.5 and the constitutive higher affinity antiport (MrpA-associated?) was preserved (Ito et al., 1997a). An antiporter that confers both Na^+/H^+ and K^+/H^+ antiporter activities upon E. coli has been designated *nhaE*; this antiporter also has a modest role at both pH 7.5 and 10.5 and is in fact expressed more at the lower pH as evidenced by northern analyses (Wei et al., manuscript submitted). Finally, a homologue of the napA antiporter, first described in *Enterococcus hirae* by Waser et al., 1992 and for which a homologue was subsequently reported in *Bacillus megaterium* (Tani et al., 1996), also has been found in *B. pseudofirmus* OF4 and shown to restore Na^+-resistance to antiporter-deficient *E. coli* strains (Wei, Y. and Ito, M. unpublished data).

One of the key tasks in further clarifying the basis for the alkaliphile's capacity for pH homeostasis is the enumeration of all the antiporters involved in a single species and the determination of their roles, mechanisms, and interplay. Another area of interest will be the development of information at a similar molecular level about the reentry routes; do secondary Na^+/solute symporters that play a role in pH homeostasis as well as simple solute uptake have particular properties associated with this dual role? Also, it will be important to further examine aerobes growing on fermentative substrates, where some sparing of Na^+-dependent, respiration-driven pH homeostasis has been observed (Gilmour and Krulwich, 1997) and to characterize the process in a broader spectrum of extreme alkaliphiles.

Primary Membrane Transport and Motility

Primary Active Membrane Transport Systems

Secondary active transport systems are centrally important both to Na^+-coupled uptake of many solutes and to a Na^+ cycle that functions in pH homeostasis. Increasingly, primary active transport systems also are being characterized in diverse alkaliphiles. Among the ATP-driven systems are: Na^+-translocating ATPases, at least one of which is a V-type system (Koyama, 1996; Kaieda et al., 1998; Prowe et al., 1996); a Na^+-translocating ABC-type transport system (*natCAB*; Wei et al., 1999) that is homologous to a similar system in *B. subtilis* (Cheng et al., 1997), and P-type ATPases that confer Cd^+- (Ivey et al., 1992) and Na^+-resistance (Koyoma, 1999). Other apparent ABC-type systems have been noted in sequences already presented, but studies that might indicate whether alkaliphile transporters have any common features have not been reported as yet. Among the haloalkaliphiles, retinal-based, light-driven primary transport systems have been found and characterized. Although the absence of bacteriorhodopsin-like pigments from these organisms was reported (Bivin and Stoeckenius, 1986), the chloride pump, halorhodopsin, has been characterized in considerable detail (Lanyi et al., 1990; Scharf et al., 1994; Varo et al., 1995; Varo et al., 1996).

Motility

Aono et al. (1992) noted that *B. halodurans* C-125 produced flagella only in the more alkaline part of its pH range for growth, and Sturr et al., 1994 made comparable observations on *B. pseudofirmus* OF4. The *hag* gene of the former alkaliphile has been sequenced (Sakamoto et al., 1992), whereas the amino acid composition of the flagellin from the latter alkaliphile was determined (Guffanti and Eisenstein, 1983). In both instances, a rather low calculated pI was observed relative to homologues from non-alkaliphiles. The identification of the remaining motility and flagellar assembly genes awaits completion of more genomic studies of alkaliphiles, but the *mot* assemblies were visualized by rapid freeze electron microscopy (Khan et al., 1992).

Imae and his colleagues (Hirota et al., 1981; Hirota and Imae, 1983; Sugiyama et al., 1986) first showed that motility in alkaliphilic *Bacillus* species was energized by an electrochemical gradient of Na^+ as opposed to the δp-driven systems of most non-alkaliphiles. Indeed, amiloride and some of its analogues, inhibitors of various Na^+ translocation pathways in eukaryotes, were found to inhibit alkaliphile flagellar rotation (Sugiyama et al., 1988; Atsumi et al., 1990). Because, however, the molecular characterization of the motility-related genes in alkaline-tolerant marine bacteria that also use an electrochemical Na^+ gradient is incomplete, extensive recent progress on specific properties of the Na^+-dependent motility mechanism has occurred in those systems rather than in alkaliphiles (McCarter, 1995; Yorimitsu et al., 1999).

Oxidative Phosphorylation

Respiratory Chain

Although diverse marine bacteria have been shown to have respiration-coupled, primary Na^+ extrusion systems (Tokuda and Unemoto, 1984; Tomb et al., 1993; Beattie et al., 1994; Skulachev, 1994; Pfenninger-Li et al., 1996; Park et al., 1996), thus far the non-marine, non-halophilic alkaliphiles—including the best studied *Bacillus* species—have been found to have H^+-translocating respiratory chains (Lewis et al., 1983; Krulwich and Guffanti, 1989). These respiratory chains are often branched, with multiple terminal oxidases, and the component cytochrome and iron sulfur protein components are characteristically present at high levels in the membranes (Hicks and Krulwich, 1995; Krulwich et al., 1998). The H^+-translocating respiratory chains may facilitate the support of pH homeostasis and the extra costs of oxidative phosphorylation at high pH; but, notably, the alkaliphilic *Bacillus* species have high molar growth yields on malate (Guffanti and Hicks, 1991; Sturr et al., 1994). In addition to the high concentration of membrane cytochromes and other respiratory chain components, especially at high pH (Lewis et al., 1981; Quirk et al., 1993;

Hicks and Krulwich, 1995; Aono et al., 1996), the respiratory chain components of alkaliphiles have characteristically low midpoint potentials (Lewis et al., 1981; Kitada et al., 1983; Yumoto et al., 1991) as shown for *c*-type cytochromes in Fig. 7. Yumoto et al., 1991 have suggested that the low midpoint potentials may facilitate electron movement in the inward direction in membranes that maintain a rather high (positive out) transmembrane electrical potential.

The respiratory chain components that have been characterized in alkaliphilic *Bacillus* species have been summarized (Hicks and Krulwich, 1995). Although there is some indirect indication a proton-translocating NADH dehydrogenase (complex I) is present (Hicks and Krulwich, 1995), this is far from established and awaits compelling biochemical and genomic evidence one way or the other. On the other hand, succinate dehydrogenases have been purified and characterized (Gilmour and Krulwich, 1996; Qureshi et al., 1996) and an incompletely characterized *bc* complex is evident (Lewis et al., 1981; Reidel et al., 1993). Also evident are diverse terminal oxidases, including a pH-regulated *caa*$_3$-type oxidase in *B. pseudofirmus* OF4 (Quirk et al., 1993), a comparable enzyme recently purified from *Bacillus* YN-1 (Higashibata et al., 1998), a *bd*-type cytochrome oxidase in the same species (Gilmour and Krulwich, 1997), and an aco3-type oxidase in *Bacillus* YN2000 (Qureshi et al., 1990; Yumoto et al., 1993). Engelhard and colleagues (Scharf et al., 1997) have begun to dissect the respiratory chain of the haloalkaliphilic archaeon *Natronobacterium pharaonis* and note the high respiratory-chain component content and possible presence of *bc* and *ba*$_3$ complexes; these investigations should help establish what may be more general properties of respiratory complexes in diverse alkaliphiles.

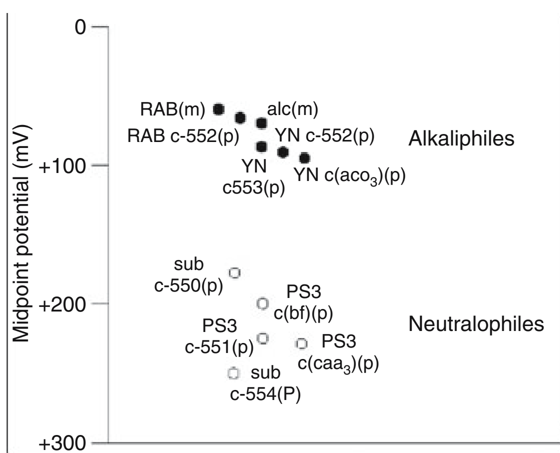

Fig. 7. Midpoint potentials from various alkaliphile c-cytochromes [RAB, *B. pseudofirmus* RAB; alc, *B. alcalophilus*; YN, *Bacillus* YN-2000] in comparison to those from non-alkaliphilic *Bacillus* species [sub, *B. subtilis*; PS3, *Bacillus* PS3]; m (composite membrane data); and p (purified protein). This figure was reproduced with permission from the publisher from Hicks and Krulwich, 1995.

While the status of energy-transducing NDH-1 type NADH dehydrogenases is still equivocal in alkaliphiles, the status of NDH-2 type NADH dehydrogenases are not (Xu et al., 1989; Niimura et al., 1995; Aono et al., 1996; Koyama et al., 1998). An interesting set of findings and proposals is emerging from recent studies that bring together the issue of peroxide toxicity, catalase, and the alkaliphile respiratory chain. Yumoto et al., 1990 first purified a protoheme-containing catalase from alkaliphilic *Bacillus* YN-2000 and reported that it was present in higher activity at elevated pH.

In *B. pseudofirmus* OF4, a complicated profile of three different catalase isozymes has been presented by Hicks, 1995 who also showed that this alkaliphile was more sensitive to killing by hydrogen peroxide at pH 10.5 than at pH 7.5, even though the aggregate catalase activity was about two-fold higher at pH 10.5. Niimura et al., 1995 observed that the NADH oxidase from *A. xylanus*, which, as described above, is cytochrome-deficient, reduces oxygen to hydrogen peroxide. When the 22-kDa AhpC disulfide-containing protein from *Salmonella typhimurium* was added to the reactions, the hydrogen peroxide was reduced to water. Under those circumstances, the net reaction is the oxygen-dependent oxidation of NADH with the production of NAD^+ and water. The NADH-2 from *Bacillus* YN-1 has now been shown to have similar properties to the *A. xylanus* enzyme, and there is an AhpC candidate upstream (Koyama et al., 1998). Thus NDH-2, as well as catalases, may be part of a detoxification system.

Studies of cyanide-sensitivity of oxygen reduction by obligately alkaliphilic *Bacillus* YN-1 (Higashibata et al., 1998) have indicated that the cyanide-sensitive component that is attributed to the *caa*$_3$-type terminal oxidase represents only 10% of the total. The majority, a cyanide-insensitive component, was associated with a low-molecular-weight nonproteinaceous material that has not been completely characterized. The investigators propose a model of alkaliphile respiration in which this cyanide-insensitive terminal respiratory component, in concert with catalase, is of major importance in the respiratory mechanisms.

Respiration-Dependent ATP Synthesis

Perhaps in the haloalkaliphilic Archaea, with primary light-driven pumps and unusual mem-

brane lipids, very high transmembrane electrical potentials are generated to offset the δpH (acid in) that alkaliphiles maintain. In the alkaliphilic aerobic *Bacillus* species, however, oxidative phosphorylation presents a clear conundrum, which relates to the higher total chemiosmotic driving force for a proton-coupled process, the electrochemical proton gradient or δp, at pH 7.5 than at pH 10.5. Most measurements (Hirota and Imae, 1983; Sturr et al., 1994) indicate that at the lower pH, the δp is about 3-times the magnitude of that at pH 10.5. Yet the phosphorylation potential, proportional to [ATP]/[ADP][Pi], which reflects the ATP sustained at equilibrium, is greater at pH 10.5 than at pH 7.5 (Fig. 5, right). While Hoffmann and Dimroth, 1991b have calculated somewhat lower discrepancies, their data on ATP synthesis by *B. alcalophilus* nonetheless show clearly better synthesis at higher pH values where the δp is lower than at near neutral pH values. Moreover, unlike motility and solute symport systems, ATP synthesis by alkaliphilic *Bacillus* species such as *B. alcalophilus* and *B. pseudofirmus* OF4 does not "avoid" the problem of the low electrochemical proton gradient, δp, by using a larger electrochemical Na^+ gradient instead. The F1FO-ATP synthases of these organisms were purified and functionally reconstituted into proteoliposomes (Hicks and Krulwich, 1990; Hoffmann and Dimroth, 1991) and shown to be H^+- and not Na^+-coupled. Thus while there do exist Na^+-coupled ATP synthases in several anaerobic marine organisms (Kluge et al., 1992; Forster et al., 1995), the alkaliphilic *Bacillus* species have not elected that solution. Yet they grow at high molar growth yields on non-fermentative substrates. This suggests that ATP synthesis either operates with a variable coupling stoichiometry, or that it operates well out of equilibrium with the bulk δp because some sort of proton sequestration or other basis for disequilibrium exists. The possibility of a variable stoichiometry merits ongoing consideration and has been proposed for H^+-coupled synthesis of ATP in cyanobacteria (Krenn et al., 1993; Van Walraven et al., 1997). In the alkaliphilic *Bacillus* species, though, the conundrum is further delineated by the observation that respiration-dependent ATP synthesis proceeds well at pH values above about 9, only when respiration itself is the energy source (i.e., artificially imposed gradients of the same magnitude are not efficacious; Guffanti et al., 1984; Guffanti and Krulwich, 1992; Guffanti and Krulwich, 1994; Ivey et al., 1998). This would not be expected if a variable stoichiometry could be employed. Moreover, Ivey et al., 1994 presented data that supportthe actuality of one ATP synthase assembly with an invariant subunit stoichiometry in *B. pseudofirmus* OF4.

A variety of sequestration models also merit consideration. Although discrete organelles of various sorts were suggested (Skulachev, 1991), fine structural evidence does not support the presence of pronounced organelles (Rhode et al., 1989; Sturr et al., 1994). Alternatives that are schematically suggested in Fig. 6, include some help from trapping of protons by cell-wall-associated polymers; however, the parameters of oxidative phosphorylation are present in right-side-out membrane vesicles that lack peptidoglycan assemblies (Guffanti and Krulwich, 1994) and oxidative phosphorylation is not restricted at least in *B. pseudofirmus* OF4, mutants lacking the S-layer (Gilmour et al., manuscript submitted). Protons might also be sequestered by being transferred from a proton-pumping respiratory chain complex, such as the caa3-type oxidase shown in Fig. 6, during a direct protein-protein interaction in the near membrane region of the phospholipid headgroups (Krulwich, 1995). Alternatively, protons might move rapidly along the surface as has been observed in some experimental systems (reviewed by Gutman and Nachliel, 1995). Such translocation, however, should be sensitive to ionic strength, which was not the case for oxidative phosphorylation by ADP and phosphate (Pi)-loaded right-side-out vesicles of *B. pseudofirmus* OF4 (Guffanti and Krulwich, 1994). It is notable that features, referred to as "alkaliphile-specific sequence motifs," have been found in important membrane-associated regions of FO subunits of several alkaliphilic *Bacillus* species (Ivey and Krulwich, 1991, Ivey and Krulwich, 1992; Krulwich et al., 1998). Thus one of the interesting experimental approaches that can be used to test various hypotheses in connection with the energization of alkaliphile oxidative phosphorylation is to alter these motifs and determine whether synthesis is particularly affected at highly alkaline pH.

Why Do Alkaliphiles Generally Grow Poorly or Fail to Grow at Near Neutral pH?

There are several indications that the alkaliphiles with the very highest upper edge or highest pH optimum for growth also may be obligate alkaliphiles that cannot grow at pH values much below 9 or 9.5. However, this conjecture has not been examined rigorously in carefully pH-controlled continuous culture conditions. In batch cultures, for example, Dunkley et al., 1991 noted that an obligate alkaliphile "outcompeted" a closely related facultative alkaliphile at pH 10.5. The putative inverse

relationship between maximally effective alkaliphily and the ability to thrive at conventional pH values can be viewed as adaptations that foster growth at the extreme but are disabling at lower pH values ("inverse adaptiveness"). Alternatively, the adaptation to high pH may not be directly injurious at lower pH values but constitute an energy cost that compromises growth ("irrelevant cost"). Or, growth at near-neutral pH may elicit essentially a "stress response" among those alkaliphiles that are maximally adapted to optimal growth at highly alkaline pH; some of the consequent shutdown, which is then manifest as a poorer growth rate, could be seen as a stress adaptation ("neutral pH as stress"). Possible examples of each of these may be found in connection with the cell surface. An example of the "inverse adaptiveness" scenario would be the hypothesis (Clejan et al., 1986; Clejan et al., 1988; Dunkley et al., 1991) that obligate alkaliphilic *Bacillus* strains have bulky branched-chain and unsaturated fatty acids that are adaptive for growth at the upper edges of their pH range but which render the membrane unstable at neutral pH. A possible example of the "irrelevant cost" hypothesis is the finding that the deletion of the S-layer gene from *B. pseudofirmus* OF4 causes a modest growth rate reduction and a correspondingly moderate compromise of pH homeostasis at pH 10.5 but increases the growth rate at pH 7.5 (Gilmour et al. manuscript submitted). A possible example of the "neutral pH as stress" scenario is autolysin activation in *B. halodurans* C-125 at the low end of its pH range for growth (Aono and Sanada, 1994).

Industrial Applications

Enzymes

Horikoshi (1991), (1996) has surveyed the applications of alkaliphiles in industrial processes, and Ito et al., 1998 have reviewed the alkaliphile enzymes that have been specifically used in laundry and dishwashing detergents. A major application of alkaliphile hydrolases, especially those that are also thermotolerant, is in laundry mixes that have alkaline pH. The recognition that these enzymes are useful fostered the rapid growth of information about alkaliphile proteases, in particular, but also of numerous additional hydrolases, including lipases, cellulases and pullulanases. Each of these classes of enzymes have other uses, such as the proteases in dehairing processes (Horikoshi, 1996) and in the degradation of gelatin-containing X-ray films for silver recapture (Fujiwara et al., 1991).

Pullulanases and amylases also have had applications in other settings (e.g., the food industry). Alkaliphilic amylases and pullulanases have been isolated from numerous alkaliphiles, even including a haloalkaliphilic amylase from *Natronococcus* (Kobayashi et al., 1994). Alkaliphilic *Bacillus* sp. strain KSM-1378 was found to produce an alkaline amylopullulanase that has two independent active sites for the individual reactions (Ara et al., 1995). In some of the food industry applications, cold-adapted rather than heat-adapted enzymes are more useful, and such enzymes have accordingly been sought (Kimura and Horikoshi, 1990).

Environmental concerns have created pressure to minimize the chlorine-intense processes used to bleach alkali-treated wood pulp. This, in turn, has encouraged the search for thermostable, alkaline xylanases that could substitute for the chemical process. Numerous xylanases have been characterized from alkaliphiles some of which were produced as exoenzymes and had desirable properties vis à vis the bleaching process, such as no cellulase activity (Nakamura et al., 1993; Nakamura et al., 1994; Blanco et al., 1995). Recent xylanase-producing alkaliphilic or alkalitolerant prokaryotes have been isolated from geothermal enrichments (Dimitrov et al., 1997; Lopez et al., 1998; Sunna et al., 1997). Horikoshi, 1991 also has noted reports of alkaliphilic enzymes that were being developed for possible use in the lignin-degradation processes that are important in pulping.

Pectinases have been isolated from alkaliphiles and evaluated for use in fruit and vegetable processing industries, including the degradation of pectin in wastewater from such industries; some efficacy of these enzymes has been reported for these uses as well as for the retting process involved in production of Japanese paper (see Horikoshi, 1991). Recently, a pectate lyase, designated Pel-7, was purified from alkaliphilic *Bacillus* sp. strain KSM-P7 and characterized as a thermostable, highly alkaline enzyme (Kobayashi et al., 1999).

Cyclomaltodextrin glucanotransferases (CGTases) produce cyclodextrins, which are homogeneous cyclic oligosaccharides, from starch. Cyclodextrins are used in industrial preparations of foods, pharmaceuticals and other chemicals. The application of CGTases from alkaliphiles has been an important application of alkaliphile enzymes (Horikoshi, 1991).

Antibiotic Production or Screening, Biotransformations

Many conventional antibiotics are unstable at very alkaline pH values, but it is nonetheless possible that the alkaliphiles themselves (e.g., alka-

liphilic *Bacillus* or actinomycetes) will produce alkali-stable antimicrobials. Among the published reports of such work are compounds isolated from alkaliphilic soil isolates (Sato et al., 1980; Tsujibo et al., 1992). It might be of particular interest to examine whether soda lake organisms, whose environment is more consistently and extremely alkaline, produce antibiotics. Since biological productivity is high, the capacity to produce antibiotics may well confer competitive advantage, and those antibiotics might have novel features of interest.

As noted by Hsieh et al., 1998 in connection with Staphylococcus aureus mutants that lack a major multidrug efflux pump, even wild type cells will exhibit a 15- to 60-fold increase in sensitivity to antimicrobials at an alkaline pH that favors accumulation of cations and weak bases. Thus alkaliphiles are particularly susceptible to inhibition by toxic cations and weak bases and might offer a way to detect small quantities of such antibiotic substances in impure test samples unless the organisms are equipped with correspondingly high activity, multidrug efflux protection. The completion of the *Bacillus halodurans* C-125 sequence may clarify whether the latter adaptation is likely.

Paavilainen and colleagues (Paavilainen et al., 1994; Paavilainen et al., 1995) have studied the dynamics of growth-induced changes in the medium pH and organic acid production by different alkaliphilic *Bacillus* species and have begun to characterize properties associated with alkaliphile catabolic patterns. When complete, the sequence of the *B. halodurans* C-125 genome may well provide clues for biotransformative and/or bioremediation capacities that will be of interest. Some heavy metal resistance determinants have been identified, for example, in alkaliphilic *B. pseudofirmus* OF4 (Ivey et al., 1992). Kimura et al., 1994 used a newly isolated alkaliphilic *Bacillus* to achieve conversions of hydroxyls of cholic acid to keto groups, in various combinations and at high yield.

Conclusions

Fossil evidence suggests that extremely alkaliphilic bacteria probably were part of an ancient group of prokaryotes that evolved in natural enrichments. An enormous diversity exists among alkaliphiles. Thus, there may be a corresponding diversity in the alkaline adaptations both in presently existing soda lakes alkaliphiles and in the widespread alkaliphiles that may now be found even in apparently nonselective settings. Much of the exploitation of a considerable industrial potential and most of the studies of fundamental physiological adaptations have focused on alkaliphilic *Bacillus* species. The current trend of increasing inclusion of haloalkaliphilic archaea and different anaerobic alkaliphiles in these efforts is salutory. Much has been learned about the major features of pH homeostasis, motility, membrane transport, cell structure, protein adaptation to high pH, and conundrums such as oxidative phosphorylation; but each finding has also provoked new and interesting questions for which the contemporary explosion of genomics, and novel ecological, molecular, structural and biophysical approaches will provide the basis for new understanding and applications for alkaliphilic prokaryotes.

Acknowledgments. Work from the author's laboratory was supported in part by research grants GM28454 from the National Institutes of Health and DE-FG0286ER13559 from the Department of Energy. I am grateful to many colleagues in my laboratory and from other laboratories for sharing ideas and questions. I especially wish to express my appreciation to Arthur Guffanti, David Hicks, and Yi Wei, of Mount Sinai School of Medicine, and Masahiro Ito, of Tokyo University.

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Prokaryotes (2006) 2:309–335
DOI: 10.1007/0-387-30742-7_11

CHAPTER 1.11

Syntrophism among Prokaryotes

BERNHARD SCHINK AND ALFONS J. M. STAMS

Introduction: Concepts of Cooperation in Microbial Communities, Terminology

The study of pure cultures in the laboratory has provided an amazingly diverse diorama of metabolic capacities among microorganisms, and has established the basis for our understanding of key transformation processes in nature. Pure culture studies are also prerequisites for research in microbial biochemistry and molecular biology. However, desire to understand how microorganisms act in natural systems requires the realization that microorganisms don't usually occur as pure cultures out there, but that every single cell has to cooperate or compete with other micro- or macroorganisms. The pure culture is, with some exceptions such as certain microbes in direct cooperation with higher organisms, a laboratory artifact. Information gained from the study of pure cultures can be transferred only with great caution to an understanding of the behavior of microbes in natural communities. Rather, a detailed analysis of the abiotic and biotic life conditions at the microscale is needed for a correct assessment of the metabolic activities and requirements of a microbe in its natural habitat.

In many cases, relationships of bacteria with other organisms may be relatively unimportant, as appears to be the case with most aerobes: they can usually degrade even fairly complex substrates to water and carbon dioxide without any significant cooperation with other organisms. Nutritional cooperation may exist, but may be restricted to the transfer of minor growth factors, such as vitamins, from one organism to the other. However, we have to realize that this assumption is based on experience gained from pure cultures that were typically enriched and isolated in simple media, and the selection aimed at organisms that were easy to handle, independent of possible interactions with others. Estimations assume that we know only a small fraction of the microorganisms present in nature, perhaps 0.1–1.0%. Thus, we cannot exclude that other bacteria out there might depend to a large extent on cooperation with partner microbes, and perhaps this is just one of the reasons why we failed so far to isolate them.

Anaerobic microorganisms, on the other hand, depend to a great extent on the cooperation of several metabolic types of bacteria in feeding chains. The complete conversion of complex organic matter, e.g., cellulose, to methane and carbon dioxide in a lake sediment is catalyzed by the concerted action of at least four different metabolic groups of bacteria, including primary fermenters, secondary fermenters, and at least two types of methanogenic archaeobacteria (Bryant, 1979; McInerney, 1988; Stams, 1994; Schink, 1991; Schink, 1997). The degree of mutual dependence among these different metabolic groups ("functional guilds") can vary considerably; whereas the latter members in the feeding line always depend on the former ones for substrate supply, they may also influence significantly the former chain members by removal of metabolic products. In an extreme case, this can mean that the fermenting bacterium depends entirely on cooperation with a methanogen to fulfill its function in, e.g., methanogenic fatty acid oxidation. This type of cooperation is called "syntrophic."

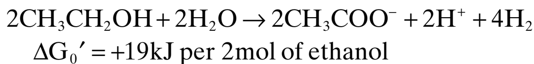
Mutual metabolic dependencies also can emerge from the cooperation of phototrophs with sulfur- or sulfate-reducing bacteria. Sulfur-reducing, acetate-oxidizing, chemotrophic bacteria such as *Desulfuromonas acetoxidans* and phototrophic green sulfide-oxidizing bacteria like *Chlorobium* sp. can cooperate closely in a phototrophic conversion of acetate plus CO_2 to bacterial cell mass, using a sulfide/sulfur cycle as an electron shuttle system between both. The two partners cooperate very closely also in this system for which the term "syntrophy" was originally coined (Biebl and Pfennig, 1978).

Syntrophy is a special case of symbiotic cooperation between two metabolically different types of bacteria which depend on each other for degradation of a certain substrate, typically

through transfer of one or more metabolic intermediate(s) between the partners. The pool size of the shuttling intermediate has to be kept low to allow efficient cooperation.

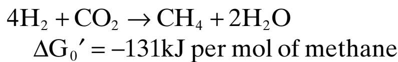
The term "syntrophy" should be restricted to those cooperations in which partners depend on each other to perform the metabolic activity observed and in which the mutual dependence cannot be overcome by simply adding a cosubstrate or any type of nutrient. A classical example is the "*Methanobacillus omelianskii*" culture (Barker, 1940), which was later shown to be a coculture of two partner organisms, the S strain and the strain M.o.H. (Bryant et al., 1967). Both strains cooperate in the conversion of ethanol to acetate and methane by interspecies hydrogen transfer, as follows:

Strain S:



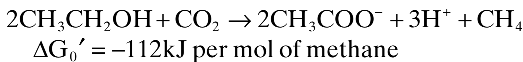
$$2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 4H_2$$
$$\Delta G_0{}' = +19 \text{kJ per 2mol of ethanol}$$

Strain M.o.H.



$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$
$$\Delta G_0{}' = -131 \text{kJ per mol of methane}$$

Corulture:



$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COO^- + 3H^+ + CH_4$$
$$\Delta G_0{}' = -112 \text{kJ per mol of methane}$$

Thus, the fermenting bacterium cannot be grown with ethanol in the absence of the hydrogen-scavenging partner organism because it carries out a reaction that is endergonic under standard conditions. The first reaction can occur and provide energy for the first strain only if the hydrogen partial pressure is kept low enough ($<10^{-3}$ bar) by the methanogen. Therefore, neither partner can grow with ethanol alone, and the degradation of ethanol depends on the cooperating activities of both.

We avoid in this article the term "consortium" which is quite often used to describe any kind of enrichment cultures cooperating in whatever way. This term was originally coined for the structured phototrophic aggregates *Pelochromatium* and *Chlorochromatium* etc. and should be restricted to such spatially well-organized systems (Pfennig, 1980; Overmann, 2001).

Electron Flow in Methanogenic and Sulfate-Dependent Degradation

The degradation of complex organic matter to methane and CO_2 is a process widespread in anoxic environments which receive only a limited supply of oxygen, nitrate, sulfate, or oxidized iron or manganese species. Methanogenesis is the typical terminal electron-accepting process in freshwater sediments rich in organic matter, in swamps or water-logged soils such as rice paddies, or in anaerobic wastewater and sewage treatment plants. It is also an important process in fermentations occurring in the intestinal tract of animals, especially of ruminants. Methanogenic degradation is the least exergonic process in comparison to aerobic degradation or the alternative anaerobic respiration. Conversion of hexose to methane and carbon dioxide releases only 15% of the energy that would be available in aerobic degradation, and this small energy yield of methanogenic degradation may be the reason why methanogenensis is the last one to occur, after the other electron acceptors have been reduced.

The carbon and electron flow in methanogenic degradation of complex organic matter follows a rather simple pattern. Polymers (polysaccharides, proteins, nucleic acids, and also lipids) are first converted to oligo- and monomers (sugars, amino acids, purines, pyrimidines, fatty acids, and glycerol), typically through the action of extracellular hydrolytic enzymes. These enzymes are produced by the "classical" primary fermenting bacteria which ferment the monomers further to fatty acids, branched-chain fatty acids, succinate, lactate, alcohols, aromatic acids, etc. (group 1; Fig. 1). Some of these fermentation products, such as acetate, H_2, CO_2 and other one-carbon compounds, can be used directly by methanogens which convert them to methane and carbon dioxide (groups 2 and 3; Fig. 1). For methanogenic degradation of other fermentation products, e.g., fatty acids longer than two carbon atoms, alcohols longer than one carbon atom, branched-chain and aromatic fatty acids, a further group of fermenting bacteria, the so-called "secondary fermenters" or "obligate proton reducers" (group 4; Fig. 1) is needed. These bacteria convert their substrates to acetate, carbon dioxide, hydrogen, perhaps also formate, which are subsequently used by the methanogens.

The situation is slightly different in sulfate-rich anoxic habitats such as marine sediments. There, the primary processes of polymer degradation are carried out by primary fermenting bacteria which form the classical fermentation products. Different from methanogens, sulfate-reducing bacteria are metabolically versatile, and a broad community of sulfate reducers can use all products of primary fermentations, and oxidize them to carbon dioxide, simultaneously reducing sulfate to sulfide (Widdel, 1988). As a consequence, the complete oxidation of complex organic matter to carbon dioxide with simultaneous sulfate reduction proceeds in a two-step process and does not depend on syntrophic fermentations. One might add that this two-step scheme might be augmented also by cooperative sidepaths taken by different types of sulfate-reducing bac-

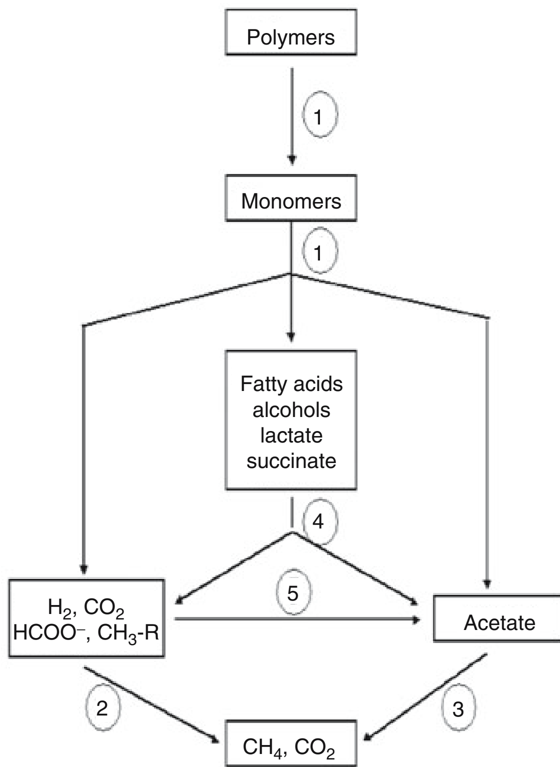


Fig. 1. Carbon and electron flow in the methanogenic degradation of complex organic matter. Groups of prokaryotes involved: 1) primary fermentative bacteria; 2) hydrogen-oxidizing methanogens; 3) acetate-cleaving methanogens; 4) secondary fermenting bacteria (syntrophs); and 5) homoacetogenic bacteria.

teria, e.g., completely and incompletely oxidizing ones (Küver and Widdel, 2001), but such cooperative activities are not required for complete sulfate-dependent oxidation of organic matter.

In methanogenic and sulfate-rich environments, the primary fermenting bacteria (group 1) profit from the activities of the hydrogen-oxidizing partners at the end of the degradation chain as well. A low hydrogen partial pressure ($<10^{-4}$ bar) allows electrons at the redox potential of NADH (–320 mV) to be released as molecular hydrogen, and fermentation patterns can shift to more acetate, CO_2, and hydrogen production rather than to ethanol or butyrate formation, thus allowing additional ATP synthesis via substrate-level phosphorylation, as opposed to production of reduced fermentation products (ethanol, lactate and butyrate). Thus, such fermenting bacteria may profit from hydrogen-oxidizing partners, but they do not depend on such cooperation.

In a well-balanced anoxic sediment in which an active hydrogen-utilizing community maintains a low hydrogen partial pressure, the flux of carbon and electrons goes nearly exclusively through the "outer" paths of the electron flow scheme (Fig. 1), and therefore reduced fermentation intermediates play only a minor role. Nonetheless, the flux through the "central" paths will never become zero because long-chain and branched-chain fatty acids and others are always produced in the fermentation of lipids, and amino acids as well. The reduced intermediates of the central path become more important if the hydrogen pool increases for any reason, e.g., excess supply of fermentable substrate, inhibition of hydrogenotrophic methanogens due to a drop in pH (<6.0), or to the presence of toxic compounds, etc. Under such conditions, the pools of fatty acids increase and might even shift the pH further downwards, thus inhibiting the hydrogenotrophic methanogens even further. The consequence may be that the whole system "turns over," meaning that methanogenesis ceases entirely and the fermentation stops with accumulation of huge amounts of foul-smelling fatty acids, as this is encountered with ill-balanced anaerobic sewage digestors. Obviously, the hydrogen/formate-utilizing methanogens act as the primary regulators in the total methanogenic conversion process (Bryant, 1979; Zehnder, 1978; Zehnder et al., 1982), and the syntrophically fatty acid-oxidizing bacteria are affected most severely by a failure in methanogenic hydrogen or formate removal.

The function of homoacetogenic bacteria (group 5; Fig. 1) in the overall process is less well understood. They connect the pool of one-carbon compounds and hydrogen with that of acetate. Owing to their metabolic versatility, they can participate also in sugar fermentation and degradation of special substrates such as *N*-methyl compounds or methoxylated phenols (Schink, 1994). In certain environments, e.g., at lower pH or low temperature, they may even successfully compete with hydrogenotrophic methanogens and take over their function to a varying extent (see below).

Energetic Aspects

Anaerobes grow with small amounts of energy, and syntrophically cooperating anaerobes are extremely skilled in the exploitation of minimal energy spans. Synthesis of ATP as the general currency of metabolic energy in living cells requires +32 kJ per mol at equilibrium under standard conditions; under the conditions assumed to prevail in an actively growing cell ([ATP] = 10 mM; [ADP] = 1 mM; and [P_i] = 10 mM), +49 kJ per mol is required (Thauer et al., 1977). In addition, part of the total energy

budget is always lost in irreversible reaction steps as heat, thus rendering the overall metabolic process irreversible. This heat loss (on average about 20 kJ per mol ATP) has to be added to the above value, which gives a total of about 70 kJ per mol ATP synthesized irreversibly in the living cell. This is the minimum amount of energy required for the synthesis of one mol of ATP in all known metabolic systems (Schink, 1990). One may argue that (especially under conditions of energy limitation) an organism may waste less energy in heat production, or that it may operate at an energy charge considerably lower than that quoted above for well-growing *Escherichia coli* cells. Nonetheless, one cannot expect the energy requirement for irreversible ATP synthesis to go substantially below about +60 kJ per mol.

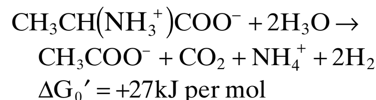
The key postulate of the Mitchell theory of respirative ATP synthesis is that ATP formation is coupled to a vectorial transport of charged groups, typically protons, across a semipermeable membrane (Mitchell, 1966). For several years, it was widely accepted that three protons cross the membrane (either of bacteria or mitochondria) per ATP hydrolyzed. As a consequence, the smallest quantum of metabolically convertible energy is that of an ion transported across the cytoplasmic membrane, equivalent to one third of an ATP unit. Combined with the calculations above, this means that a bacterium needs a minimum of about –20 kJ per mol reaction to exploit a reaction's free energy change (Schink and Thauer, 1988; Schink, 1990).

On the basis of studies on the structure and function of F_1-F_0 ATPases in recent years, the stoichiometry of ATP synthesis versus proton translocation appears not to be as strictly fixed as suggested above. Rather, the system may operate like a sliding clutch, meaning that at very low energy input, the energy transfer into ATP synthesis may be substoichiometric. Moreover, the stoichiometry is not necessarily three protons per one ATP, but is governed by the number of subunits arranged in the F_0 versus the F_1 complex. This concept would allow also stoichiometries of 4 to 1, perhaps even 5 to 1 (Engelbrecht and Junge, 1997; Cherepanov et al., 1999; Stock et al., 1999; Dimroth, 2000; Seelert et al., 2000). As a consequence, the minimum energy increment that can still be used for ATP synthesis may be as low as −15 or −12 kJ per mol reaction. In some cases, to make their living, bacteria cooperating in syntrophic fermentations are limited to this range of energy; Hoehler et al. (2001) calculated from metabolite concentrations in natural habitats for the partner bacteria cooperating in syntrophic conversions minimum amounts of exploitable energy in the range of −10 to −19 kJ per mol reaction.

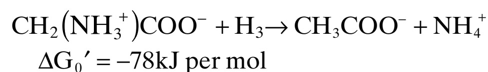
Degradation of Amino Acids

During protein hydrolysis, a complex mixture of amino acids and small peptides is produced. These amino acids and peptides can serve as energy substrates for anaerobic microorganisms. Detailed information on the anaerobic fermentation of amino acids can be found in Barker (1981) and McInerney (1988). Many anaerobic amino acid-degrading bacteria require complex mixtures of amino acids, perhaps because a one-sided diet causes a serious imbalance in the internal amino acid metabolism of these cells, but growth by fermentation of single amino acids is also common. Mixtures of amino acids are often degraded by coupled fermentation of pairs of amino acids via the Stickland reaction. Table 1 summarizes some oxidative conversions of amino acids. In the classical Stickland fermentation, these oxidation reactions are coupled within the same organism to reduction of other amino acids such as glycine or proline. Also phenylalanine, leucine, or compounds like sarcosine and betaine (Naumann et al., 1983; Gottschalk, 1986) can act as electron acceptors, and oxidation of leucine coupled to the reduction of acetate to butyrate has been described as well (Girbal et al., 1997).

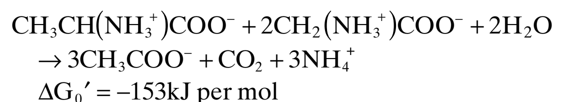
These pure culture fermentations can also be catalyzed by cooperation of two different bacteria via interspecies hydrogen transfer, according to the following reactions with a mixture of alanine and glycine:



$$CH_3CH(NH_3^+)COO^- + 2H_3O \rightarrow CH_3COO^- + CO_2 + NH_4^+ + 2H_2 \quad \Delta G_0' = +27 \text{kJ per mol}$$



$$CH_2(NH_3^+)COO^- + H_3 \rightarrow CH_3COO^- + NH_4^+ \quad \Delta G_0' = -78 \text{kJ per mol}$$



$$CH_3CH(NH_3^+)COO^- + 2CH_2(NH_3^+)COO^- + 2H_2O \rightarrow 3CH_3COO^- + CO_2 + 3NH_4^+ \quad \Delta G_0' = -153 \text{kJ per mol}$$

Thus, the electrons derived in amino acid degradation by a fermenting bacterium can be used in glycine reduction as shown, but can be transferred as well in the form of molecular hydrogen to sulfate-reducing, homoacetogenic or methanogenic partner bacteria, depending on the availability of such partner bacteria and their respective electron acceptors. That amino acid oxidation and glycine reduction can be uncoupled from each other has been shown in detail with *Eubacterium acidaminophilum* (Zindel et al., 1988). This bacterium can run either one of the first two reactions separately or combine them on its own, according to the third reaction, depending on the partner bacteria which act as hydrogen sources or sinks, and on the availability

Table 1. Changes of Gibbs free energies under standard conditions in hydrogen-releasing reactions during fermentation of amino acids.

Fermentation reaction	$\Delta G_0'$ (kJ per mol rct.)
Alanine + $2H_2O \rightarrow$ acetate$^-$ + CO_2 + NH_4^+ + $2H_2$	+2.7
Glycine + $2H_2O$ + H^+ $\rightarrow$ $2CO_2$ + NH_4^+ + $3H_2$	+17.8
Serine + $H_2O \rightarrow$ acetate$^-$ + CO_2 + NH_4^+ + H_2	–85.3[a]
Threonine + $H_2O \rightarrow$ propionate$^-$ + CO_2 + NH_4^+ + H_2	–83.0[a]
Histidine + $4H_2O$ + H^+ $\rightarrow$ glutamate$^-$ + CO_2 + $2NH_4^+$ + H_2	[b]
Proline + $2H_2O \rightarrow$ glutamate$^-$ + H^+ + $2H_2$	[b]
Glutamate$^-$ + $2H_2O$ + H^+ $\rightarrow$ propionate$^-$ + $2CO_2$ + NH_4^+ + $2H_2$	–16.6
Glutamate$^-$ + $2H_2O \rightarrow$ 2 acetate$^-$ + CO_2 + NH_4^+ + H_2	–38.6[a]
Aspartate$^-$ + $2H_2O$ + H^+ $\rightarrow$ acetate$^-$ + $2CO_2$ + NH_4^+ + $2H_2$	–24.1

[a]These fermentations may also allow growth in pure culture.
[b]These reactions are always coupled to further fermentation of glutamate.
All calculations are based on published tables (see Thauer et al., 1977; Dimroth, 1983). For H_2S and CO_2, values for the gaseous state were used.

of selenium in the medium which is required for expression of the active glycine reductase complex.

In methanogenic environments, methanogens can act as scavengers of reducing equivalents in the oxidation of amino acids, thus taking over the role of the reductive part of the Stickland reaction. Nagase and Matsuo (1982) observed that in mixed methanogenic communities, the degradation of alanine, valine and leucine was inhibited when methanogens were inhibited. Nanninga and Gottschal (1985) could stimulate the degradation of these amino acids by addition of hydrogen-scavenging sulfate-reducers. These early observations indicated a functional role for hydrogen consumption also in the degradation of amino acids.

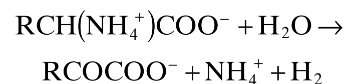
Influence of Methanogens

Methanogens can affect anaerobic oxidation in various ways. Fermentative oxidation of some amino acids can be coupled to hydrogen formation only if the hydrogen partial pressure is kept low, similar to the "*Methanobacillus omelianskii*" example described above. This results in an obligately syntrophic relationship between the fermenting microorganism and the methanogen. Some amino acids are degraded by the same fermenting bacterium either in pure culture or in mixed culture with methanogens; however, methanogens can cause a shift in metabolism resulting in a changed product formation pattern.

Clostridium sporogenes (Wildenauer and Winter, 1986; Winter et al., 1987), *Eubacterium acidaminophilum* (Zindel et al., 1988), *Acidaminobacter hydrogenoformans* (Stams and Hansen, 1984), *Aminomonas paucivorans* (Baena et al., 1999a), *Aminobacterium colombiense* and *Aminobacterium mobile* (Baena et al., 1998; Baena et al., 2000), as well as strain PA-1 (Barik et al., 1985) are mesophilic bacteria which degrade one or more amino acids in syntrophic association with hydrogen-consuming anaerobes. Moderately thermophilic bacteria include *Caloramator coolhaasii* (Plugge et al., 2000), *Caloramator proteoclasticus* (Tarlera et al., 1997; Tarlera and Stams, 1999), *Thermanaerovibrio acidaminovorans* (previously named *Selenomonas acidaminovorans*; Cheng et al., 1992; Baena et al., 1999b), *Gelria glutamica* (Plugge et al., 2002) and *Clostridium* P2 (Øygsson et al., 1993; Øygsson, 1994). There are probably many other fermenting bacteria with the ability to grow syntrophically with certain amino acids and to release reducing equivalents as molecular hydrogen; however, such capacities are only rarely checked.

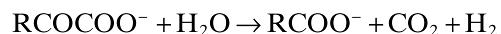
Obligately Syntrophic Amino Acid Deamination

The initial step in the oxidative degradation of alanine, valine, leucine and isoleucine is an NAD(P)-dependent deamination to the corresponding α-keto acid, and the reaction, if coupled to reoxidation of NAD(P)H by proton reduction, would read as follows:



$$RCH(NH_4^+)COO^- + H_2O \rightarrow RCOCOO^- + NH_4^+ + H_2$$

The $\Delta G_0'$ of this reaction is about +55 kJ per mol; thus, very efficient hydrogen consumption would be required to pull this reaction. The α-keto acid is converted further via oxidative decarboxylation to a fatty acid:



$$RCOCOO^- + H_2O \rightarrow RCOO^- + CO_2 + H_2$$

a reaction which is much more favorable ($\Delta G_0'$ about –52 kJ per mol). Therefore, it is not surprising that one does not observe bacteria catalyzing the first reaction step only, and that the organisms involved—as far as they have been tested—can grow in pure culture with α-keto

acids. Such α-keto acids were used successfully for the isolation of amino acid-degrading proton-reducing bacteria (Stams and Plugge, 1990). Conversion of the keto acids to the corresponding fatty acids likely proceeds through energy-rich CoA and phosphate derivatives, thus allowing ATP synthesis via substrate-level phosphorylation.

Eubacterium acidaminophilum degrades aspartate to acetate in syntrophic association with methanogens (Zindel et al., 1988). The degradation pathway is not known. Aspartate is either oxidized directly to oxaloacetate by means of an aspartate dehydrogenase or is degraded first to fumarate by aspartase activity. Fumarate is then converted to malate which is subsequently oxidized to oxaloacetate. In this pathway, the conversion of malate to oxaloacetate plus H_2 is most unfavorable; the $\Delta G_0'$ is +48 kJ per mol. Recently, a moderately thermophilic sugar-fermenting bacterium, *Gelria glutamica*, was isolated which can grow also by the analogous conversion of glutamate to propionate, provided that the hydrogen partial pressure is kept low by a methanogen (Plugge et al., 2002). In this bacterium, glutamate is first oxidatively deaminated to α-ketoglutarate, which is subsequently oxidatively decarboxylated to succinyl-CoA and further to propionate (Plugge et al., 2001). In coculture with methanogens, it is also able to oxidize proline to propionate. This is remarkable because proline is generally thought to be reductively degraded to aminovalerate. Similar to the above-mentioned amino acids, the aromatic amino acids phenylalanine, tryptophan and tyrosine might also support growth by oxidative deamination leading to phenylacetate, indolylacetate, *p*-hydroxyphenylacetate, respectively, as products. This, however, has not yet been demonstrated for syntrophic amino acid-degrading bacteria.

Syntrophic Arginine, Threonine and Lysine Fermentation

Acidaminobacter hydrogenoformans, *Aminomonas paucivorans* and *T. acidaminovorans* can grow with histidine, ornithine, arginine, lysine and threonine when cocultured with hydrogenotrophic bacteria (Stams and Hansen, 1984; Cheng et al., 1992; Baena et al., 1999a). Remarkably, *T. acidaminovorans* and *A. paucivorans* grow in pure culture with arginine, forming citrulline and/or ornithine as products. In this conversion, carbamyl phosphate is formed as an intermediate, which is further converted to yield ATP (Plugge and Stams, 2001). Ornithine and citrulline could be degraded when these bacteria were cocultured with a hydrogenotrophic methanogen. It is not clear which pathway is used for ornithine degradation, and which metabolic step is hampering growth of these bacteria in pure culture.

Acidaminobacter hydrogenoformans degrades threonine and lysine in syntrophic association with a hydrogen-consuming anaerobe. Acetate is the main product of the degradation of these two amino acids, and large amounts of hydrogen are formed. In pure culture, these compounds do not support growth. The stoichiometry of the fermentation is not exactly known, and the pathways involved in these fermentations have not been resolved as yet.

Facultatively Syntrophic Growth with Amino Acids

The effect of methanogens on the metabolism of amino acid-fermenting anaerobes has been studied most extensively with glutamate. Glutamate fermentation is carried out by a variety of fastidious anaerobes, including a number of *Clostridium* species, *Peptostreptococcus asaccharolyticus*, and *Acidaminococcus fermentans* (Gottschalk, 1986). These microorganisms ferment glutamate to acetate and butyrate by two different pathways, the β-methylaspartate or the hydroxyglutarate pathway (Buckel and Barker, 1974). In this fermentation, reducing equivalents formed in the oxidation of glutamate to acetate are disposed of either partly or completely by reductive formation of butyrate from acetate (acetyl-CoA).

Anaeromusa acidaminophila ("*Selenomonas acidaminophila*") ferments glutamate to acetate plus propionate (Nanninga et al., 1987; Baena et al., 1999b). In this bacterium, reducing equivalents are disposed of by reduction of pyruvate to propionate. However, in the last decade, several bacteria have been isolated which during growth on glutamate release reducing equivalents exclusively as hydrogen, both in the formation of acetate and the formation of propionate (Table 1). These microorganisms ferment glutamate to acetate only (*Caloramator coolhaasii* [Plugge et al., 2000] and *Caloramator proteoclasticus* [Tarlera and Stams, 1999]), propionate only (*Aminobacterium colombiense* [Baena et al. 1998] and *Gelria glutamica* [Plugge et al., 2002]) or acetate plus propionate (*A. hydrogenoformans* [Stams and Hansen, 1984; Meijer et al., 1999], *T. acidaminovorans* [Cheng et al., 1992; Baena et al., 1999b], and *Aminomonas paucivorans* [Baena et al., 1999a]), with CO_2 as coproduct (Table 1).

Acidaminobacter hydrogenoformans ferments glutamate to 2 acetate, 1 CO_2, NH_3 and 1 H_2, or to 1 propionate, 2 CO_2, NH_3 and 2 H_2 (Table 1; Fig. 2). In pure culture, *A. hydrogenoformans* can also form formate. However, in the presence of the hydrogen-utilizing *Methanobrevibacter*

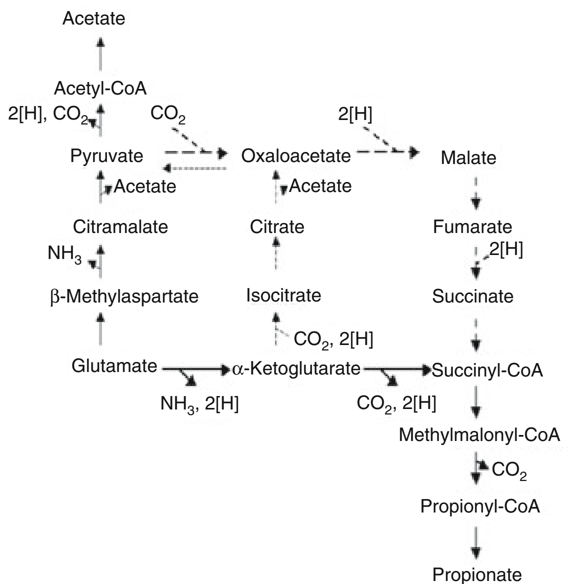


Fig. 2. Pathways involved in the fermentation of glutamate to propionate and acetate in anaerobic bacteria. Thin solid line (left part): acetate formation by the commonly used β-methylaspartate pathway. Thick solid line: oxidative formation of propionate as catalyzed by, e.g., *Gelria glutamica*. Dashed line: reductive formation of propionate as performed by *Anaeromusa acidaminophila*. Dotted line: acetate formation via enzymes of the reductive tricarboxylic acid (TCA) cycle as used by *Acidaminobacter hydrogenoformans* when cocultured with hydrogenotrophic methanogens.

arboriphilus, formate is not formed (Stams and Hansen, 1984). The ratio at which acetate and propionate are formed depends on the hydrogen partial pressure (Stams and Hansen, 1984). Propionate formation is favored at low hydrogen partial pressure, whereas at high hydrogen pressure mainly acetate is formed. Enzyme measurements revealed that the strain when grown in pure culture uses the β-methylaspartate pathway for acetate formation. However, if the bacterium is grown in coculture with a methanogen, both acetate and propionate appear to be formed via α-ketoglutarate (Stams et al., 1998). Reductive carboxylation of α-ketogluratate leads via isocitrate to citrate which is cleaved to acetate and pyruvate, and pyruvate is converted further to acetate. Propionate formation occurs via oxidative decarboxylation of α-ketoglutarate to succinyl-CoA, which is further converted to propionate. Apparently, a crucial step that determines the degradation pathway is the oxidative deamination of glutamate to α-ketoglutarate which is highly endergonic if coupled to hydrogen formation. Under standard conditions, this conversion is energetically impossible ($\Delta G_0' = +60$ kJ per mol). However, at a hydrogen partial pressure of 10^{-5} atm, the $\Delta G_0'$ of this reaction would be +30 kJ per mol, and if the intracellular NH^{4+} concentration would be 10 mM and the glutamate/α-ketoglutarate ratio about 500, the $\Delta G'$ of this reaction would become about zero. A similar shift in product formation was described for *Aminomonas paucivorans* (Baena et al., 1999a).

Thermanaerovibrio acidaminovorans also forms acetate and propionate as products. Unlike the mesophilic organisms, this thermophile forms propionate already in pure culture. This bacterium uses the β-methylaspartate pathway for acetate formation both in pure culture and in coculture with a methanogen (Plugge et al., 2002) Some of the glutamate-fermenting bacteria also are able to grow with histidine, forming similar products and shifts in product formation as with glutamate. Histidine is supposed to be degraded through glutamate as an intermediate (Gottschalk, 1986).

Stickland Reaction versus Methanogenesis

Some of the bacteria which can degrade amino acids syntropically, including *Clostridium sporogenes*, *Eubacterium acidaminophilum*, *Acidaminobacter hydrogenoformans* and *Caloramator proteoclasticus*, are able to perform a Stickland reaction. The environmental conditions under which the Stickland reaction is favored and conditions under which reducing equivalents are disposed of as molecular hydrogen remains to be determined. Energetically, the reductive conversion of glycine is more favorable than methanogenesis, homoacetogenesis, or sulfate reduction (Table 2). It is likely that in environments rich in amino acids, the Stickland reaction will dominate, whereas in environments with low amino acid supply and high methanogenic activity, reducing equivalents may be preferentially channeled to methanogenesis. Unfortunately, up to now little information was available on such comparative ecological aspects.

During growth of a coculture of *Caloramator proteoclasticus* and *Methanobacterium thermoautotrophium* Z245 in a medium with alanine plus glycine, about 60% of the reducing equivalents were channeled to methanogenesis when a dense culture of the methanogen was inoculated with 0.2% of a culture of *C. proteoclasticus*. However, if 1% inoculum was applied, all the reducing equivalents were used to reduce glycine.

Syntrophic Degradation of Fermentation Intermediates

Syntrophic Ethanol Oxidation

The case of "*Methanobacillus omelianskii*" is the classical example of interspecies hydrogen

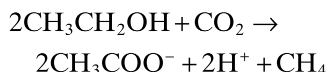
Table 2. Changes of Gibbs free energies under standard conditions in hydrogen-consuming reactions involved in interspecies hydrogen transfer.

	G_0' (kJ per mol rct.)	G_0' (kJ per electron pair)
$4H_2 + 2\,CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	–94.9	–23.8
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	–131.0	–32.7
$H_2 + S^0 \rightarrow H_2S$	–33.9	–33.9
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	–151.0	–37.6
$H_2C(NH_3^+)COO^- + H_2 \rightarrow CH_3COO^- + NH_4^+$	–78.0	–78.0
$Fumarate^{2-} + H_2 \rightarrow succinate^{2-}$	–86.0	–86.0

All calculations are based on published tables (see Thauer et al., 1977; Dimroth, 1983). For H_2S and CO_2, values for the gaseous state were used.

transfer. Both partners operate in an overall reaction process which becomes exergonic for the first partner only through maintenance of a low hydrogen partial pressure by the second partner. After description of the cooperative nature of this process, the original S-strain was lost, but other syntrophically ethanol-oxidizing bacteria have been isolated, such as *Thermoanaerobium brockii* (Ben-Bassat et al., 1981) and various *Pelobacter* strains (Schink, 1984; Schink, 1985a; Eichler and Schink, 1986). Also certain ethanol-oxidizing sulfate reducers such as *Desulfovibrio vulgaris* are able to oxidize ethanol in the absence of sulfate by hydrogen transfer to a hydrogen-oxidizing methanogenic partner.

Unfortunately, the energetics of this syntrophic cooperation are still unclear. The total reaction



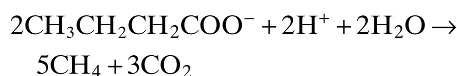
$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COO^- + 2H^+ + CH_4$$

yields –112 kJ per 2 mol ethanol under standard conditions. On the side of the ethanol oxidizer, e.g., the "S-strain" of "*Methanobacillus omelianskii*" (Bryant et al., 1967) or other fermenting anaerobes with similar metabolic capacities such as *Pelobacter acetylenicus* (Schink, 1985a), ethanol dehydrogenase, acetaldehyde ferredoxin oxidoreductase (acetyl CoA-forming), phosphotransacetylase, and acetate kinase have been shown to be involved, forming one ATP per ethanol through substrate-level phosphorylation. Since the methanogenic hydrogen oxidizer requires at least one third of an ATP unit for growth (–20 kJ per reaction run, see above), only about –45 kJ is available to the ethanol oxidizer per mol ethanol oxidized, which is too little energy to form one full ATP. It has to be postulated, therefore, that part of the energy bound in ATP has to be reinvested somewhere to push the overall reaction and balance the energy budget, but this reverse electron transport system has not yet been identified. In syntrophically ethanol-oxidizing *Desulfovibrio* strains, the pathway leads from acetaldehyde directly to acetate, without a phosphorylation step, which explains why *Desulfovibrio* cannot grow in this syntrophic association (Kremer et al., 1988).

Syntrophic Butyrate Oxidation

Similar cooperations have been described with syntrophic cultures degrading fatty acids. An overview of the reactions catalyzed is presented in Table 3; a list of described strains of syntrophically fermenting bacteria follows in Table 4. In general, degradation of fatty acids to acetate and hydrogen or, in the case of propionate, to acetate, hydrogen and CO_2, are reactions far more endergonic under standard conditions than ethanol oxidation. Consequently, for fatty acid degradation, the hydrogen partial pressure has to be decreased to substantially lower values ($<10^{-4}$ bar) than with ethanol ($<10^{-3}$ bar).

The energetic situation of the partner bacteria involved in butyrate conversion to methane and CO_2 has been discussed in detail in earlier publications (Wallrabenstein and Schink, 1994a; Schink, 1997). The overall reaction



$$2CH_3CH_2CH_2COO^- + 2H^+ + 2H_2O \rightarrow 5CH_4 + 3CO_2$$

yields under standard conditions a $\Delta G_0'$ of –177 kJ per 2 mol of butyrate. With concentrations better comparable to those prevailing in a natural habitat, e.g., a freshwater sediment or a sewage sludge digestor (butyrate: 10 μM, CH_4: 0.7 bar, and CO_2: 0.3 bar), the free energy of this process changes to –140 kJ per 2 mol of butyrate. Since the overall process is shared by seven partial reactions (two are involved in the butyrate oxidation, one in CO_2 reduction to methane, and four in acetate cleavage) the free energy change is about –20 kJ per mol for every partial reaction, if the energy is shared by all reactions at equal rates. Measured partial pressures of hydrogen and acetate concentrations in active sewage sludge and various sediments (Zehnder et al., 1982) are in a range of 10^{-4}–10^{-5} bar and 10–100 μM, respectively, which is in good agreement with the assumption of equal energy sharing in

Table 3. Changes of Gibbs free energies under standard conditions in hydrogen-releasing reactions during oxidation of fermentation intermediates.

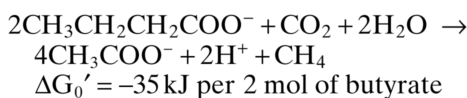
	G_0' (kJ per mol rct.)	No. of electron pairs
Primary alcohols		
$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.6	2
Fatty acids		
$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2$	+48.3	2
$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	+76.0	3
$CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$	+94.9	4
$CH_3CH(CH_3)CH_2COO^- + CO_2 + 2H_2O \rightarrow 3CH_3COO^- + 2H^+ + H_2$	+25.2	1
Glycolic acid		
$CH_2OHCOO^- + H^+ + H_2O \rightarrow 2CO_2 + 3H_2$	+19.3	3
Aromatic compounds		
$C_6H_5COO^- + 6H_2O \rightarrow 3CH_3COO^- + 2H^+ + CO_2 + 3H_2$	+49.5	3
$C_6H_5OH + 5H_2O \rightarrow 3CH_3COO^- + 3H^+ + 2H_2$	+10.2	2
Amino acids		
$CH_3CH(NH_3^+)COO^- + 2H_2O \rightarrow CH_3COO^- + NH_4^+ + CO_2 + 2H_2$	+2.7	2

All calculations are based on published tables (see Thauer et al., 1977; Dimroth 1983). For H_2S and CO_2, values for the gaseous state were used.

such a cooperative community. The corresponding energy yields of about –20 kJ per mol reaction are confirmed by growth yield determinations with pure cultures of methanogens (Schönheit et al., 1980; Zehnder et al., 1982).

On the side of the syntrophic fermenting partner, 1 ATP is synthesized by substrate-level phosphorylation through thiolytic acetoacetyl-CoA cleavage (Wofford et al., 1986), but part of this energy has to be reinvested in reverse electron transport to allow proton reduction with electrons from the butyryl CoA dehydrogenase reaction at a hydrogen partial pressure of 10^{-4}–10^{-5} bar (Thauer and Morris, 1984). Experimental evidence of a reverse electron transport system between the crotonyl-CoA/butyryl-CoA couple ($E^{\circ\prime}$ = –125 mV) and the H^+/H_2 couple has been provided with *Syntrophomonas wolfei* (Wallrabenstein and Schink, 1994a). If two protons are transferred in this reverse electron transport system, one third of the ATP synthesized by substrate-level phosphorylation (equivalent to –20 kJ per mol) would remain for growth and maintenance of the fatty acid-oxidizing bacterium, in accordance with the above assumptions.

The energetic situation of a binary mixed culture degrading butyrate to acetate and methane is considerably more difficult:



$$2CH_3CH_2CH_2COO^- + CO_2 + 2H_2O \rightarrow 4CH_3COO^- + 2H^+ + CH_4$$
$$\Delta G_0' = -35\,\text{kJ per 2 mol of butyrate}$$

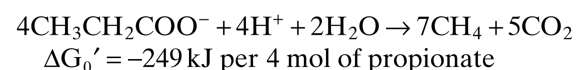
This overall reaction has to feed two organisms in three partial reactions, so each step has only –12 kJ available under standard conditions, and the total changes to –46 kJ at butyrate and acetate concentrations in the range of 10 mM as used in laboratory cultures. Under these conditions, the energetic situation for the partners gets tough (–15 kJ per mol reaction), especially at the end of the substrate conversion process. Very slow, often nonexponential growth and substrate turnover as usually observed with such binary mixed cultures (Dwyer et al., 1988) indicates that the energy supply is insufficient. We have often observed, as did other authors, that accumulating acetate (>10 mM) inhibits butyrate degradation in such cultures substantially.

The energetic difference between the ternary mixed culture and an artificial binary mixed culture demonstrates that the acetate-cleaving methanogens fill an important function in removal of acetate, and with this, "pull" the butyrate oxidation reaction. The above calculations also explain why addition of an acetate-cleaving methanogen to a defined binary mixed culture enhances growth and substrate turnover considerably (Ahring and Westermann, 1988; Beaty and McInerney, 1989).

Syntrophic oxidation of long-chain fatty acids from lipid hydrolysis probably proceeds via β-oxidation with concomitant release of electrons as hydrogen via reverse electron transport, analogous to the process described above for butyrate oxidation. Long-chain dicarboxylic acids are degraded stepwise by β-oxidation, analogous to fatty acids, and decarboxylation occurs at the C-5 or C-4 state (glutarate or succinate; Matthies and Schink, 1993).

Syntrophic Propionate Oxidation

For syntrophic propionate oxidation according to the equation



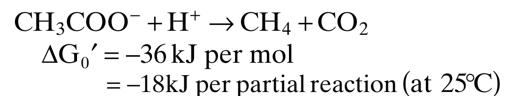
$$4CH_3CH_2COO^- + 4H^+ + 2H_2O \rightarrow 7CH_4 + 5CO_2$$
$$\Delta G_0' = -249\,\text{kJ per 4 mol of propionate}$$

a metabolic flow scheme can be drawn, leaving a free energy change in the range of –22 to –23 kJ per mol reaction (11 partial reactions) to all partners involved (Stams et al., 1989; Schink, 1991). Studies in defined mixed cultures and in undefined communities in rice field soil have basically confirmed this assumption, although the amount of energy available to the propionate oxidizer may be sometimes substantially lower (Scholten and Conrad, 2000; Fey and Conrad, 2000). The pathway of propionate oxidation in such bacteria is basically a reversal of fermentative propionate formation, including methylmalonyl CoA, succinate, malate, pyruvate, and acetyl CoA as intermediates (Koch et al., 1983; Schink, 1985b; Schink, 1991; Houwen et al. 1987; Houwen et al., 1990). The initial substrate activation is accomplished by CoA transfer from acetyl CoA (Houwen et al., 1990; Plugge et al., 1993) or succinyl CoA. Of the redox reactions involved, succinate oxidation and malate oxidation are the most difficult ones to couple to proton reduction: hydrogen partial pressures of 10^{-15} or 10^{-8} bar would be required, respectively, which are far lower concentrations than a methanogen can maintain. The enzymes and electron transfer components involved in propionate oxidation were studied with *Syntrophobacter wolinii* (Houwen et al., 1990; Plugge et al., 1993) and *Syntrophobacter pfennigii* (Wallrabenstein et al., 1995b). Experiments on hydrogen formation in the presence and absence of protonophores (carbonyl cyanide *m*-chlorophenyl hydrazone [CCCP] or dicyclohexyl carbodiimide [DCCD]) indicated again that an intact proton or sodium motive force maintained by ATP hydrolysis is required for hydrogen release, probably in the first oxidation step (Dörner, 1992). The situation is complicated further by the fact that all syntrophically propionate-oxidizing bacteria known so far have turned out to be able also to reduce sulfate (Harmsen et al., 1993; Harmsen et al., 1995; Wallrabenstein et al., 1994b; Wallrabenstein et al., 1995b). Thus, electron transport components detected in these bacteria (even after growth in syntrophic mixed culture) do not necessarily refer to a function in syntrophic oxidation but may be involved in the sulfate-reducing apparatus.

Studies with *Syntrophobacter fumaroxidans* have shown that not only hydrogen but also formate could contribute to interspecies electron transport in this system. Syntrophic propionate oxidation was possible only in cooperation with formate- and hydrogen-oxidizing methanogens, not with *Methanobrevibacter* strains that are unable to oxidize formate (Dong et al., 1994b). This view was further supported by enzyme measurements: cells grown syntrophically with propionate contained tenfold higher formate dehydrogenase activity in comparison with cells grown in pure culture with fumarate; the hydrogenase activity was unchanged (F. A. M.de Bok, unpublished observation). An alternative for syntrophic propionate degradation was discovered in the late 1990s. *Smithella propionica* (Liu et al., 1999) converts propionate to a mixture of acetate and butyrate in the presence of a hydrogen- and formate-oxidizing partner bacterium. The labelling patterns of products formed from specifically labelled propionate indicate that propionate degradation by this bacterium proceeds neither through methylmalonyl CoA nor through the acrylyl CoA pathway but through dimerization to a six-carbon intermediate that is subsequently cleaved to an acetyl and a butyryl moiety, with partial further oxidation (de Bok et al., 2001). This new pathway could also explain the results of labelling experiments with an enrichment culture which were first interpreted as indicative of a reductive carboxylation of propionate to a butyryl residue (Tholozan et al., 1988; Tholozan et al., 1990).

Syntrophic Acetate Oxidation

A special case is the syntrophic conversion of acetate to $2CO_2$ and $4H_2$ which was described first for a moderately thermophilic (58°C) bacterium, strain AOR (Zinder and Koch, 1984). This syntrophic acetate oxidizer could be grown in pure culture like a homoacetogen by hydrogen-dependent reduction of CO_2 to acetate, thus reversing syntrophic acetate oxidation (Lee and Zinder, 1988b; Lee and Zinder, 1988c). Biochemical studies revealed that it uses the carbon monoxide dehydrogenase pathway (“Wood pathway”), as do other homoacetogens (Lee and Zinder, 1988a). The small energy span available in acetate conversion to methane and CO_2 is hardly sufficient to feed two bacteria:



$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$
$$\Delta G_0' = -36\,\text{kJ per mol} = -18\,\text{kJ per partial reaction (at 25°C)}$$

The free energy change is slightly higher at 58°C (–42 kJ per mol) than under standard conditions (25°C). However, a syntrophic acetate-oxidizing culture has been described as well which operates at 35°C (Schnürer et al., 1994; Schnürer et al., 1996) and thus proves that this free-energy change (–38°C) is sufficient for acetate degradation and (very little) growth at this temperature. Another interesting feature of this type of metabolism is that these bacteria can run acetate formation and acetate degradation, in both directions, with probably the same biochemical reaction apparatus, just depending on the prevailing concentrations of substrates and

products, and even can synthesize ATP both ways. This example shows how close to the thermodynamic equilibrium the energy metabolism of an anaerobic bacterium can operate.

The higher energy yield at elevated temperature may explain why at 35°C and lower this reaction is typically carried out by one single bacterium, e.g., *Methanosarcina barkeri* or *Methanosaeta soehngenii*, and why cooperations of syntrophic associations of the above-mentioned type are found nearly exclusively at elevated temperatures. Nonetheless, acetate conversion to methane at higher temperatures can as well be catalyzed by a single aceticlastic methanogen, e.g., *Methanosarcina thermophila*. Syntrophic acetate oxidation at lower temperatures is found only if further stress factors such as high ammonium concentrations inhibit aceticlastic methanogens (Schnürer et al., 1994).

Unfortunately, strain AOR was lost only few years after its description. Another thermophilic strain with similar properties, *Thermoacetogenium phaeum*, was isolated recently (Hattori et al., 2000), and was found to use the CO dehydrogenase pathway (the Wood pathway) for acetate oxidation, too (S. Hattori et al., unpublished observation), and the same is true for the mesophilic acetate-oxidizing *Clostridium ultunense* (Schnürer et al., 1997). However, we do not know yet to what extent and in which steps energy is conserved by these bacteria: either in the acetate synthesis or the acetate oxidation reaction chain.

Syntrophic acetate oxidation was observed also in a coculture of the iron-reducing bacterium *Geobacter sulfurreducens* together with the nitrate-reducing anaerobe *Wolinella succinogenes* (Cord-Ruwisch et al., 1998). In this coculture, the hydrogen partial pressures were far below the levels observed in the cultures mentioned above, and it was hypothesized that perhaps an extracellular cytochrome could act as electron shuttling vector in these cultures. We found out recently that not the cytochrome but cysteine added to the cultures as reducing agent actually mediates interspecies electron transfer in this artificially composed syntrophic coculture (J. Kaden and B. Schink, in preparation).

Syntrophic Glycolate Oxidation

Glycolate is not a fermentation intermediate but an important excretion product of algal cells. It is syntrophically oxidized by homoacetogenic and methanogenic cocultures to two molecules of CO_2, and hydrogen is the electron carrier between the fermenting bacterium (*Syntrophobotulus glycolicus*) and a hydrogen-oxidizing partner bacterium (Friedrich et al., 1991; Friedrich et al., 1996). The primary fermentation is an endergonic process under standard conditions (Table 3) and needs coupling to, e.g., a methanogenic partner. The degradation pathway includes oxidation of glycolate to glyoxylate, condensation of glyoxylate with acetyl CoA to form malyl CoA, ATP formation in a malyl CoA synthetase reaction, malate oxidation and decarboxylation by the malic enzyme, and oxidative decarboxylation of pyruvate to acetyl CoA through pyruvate synthase, thus closing the chain for a new reaction cycle (Friedrich et al., 1991). In this cycle, the oxidation of glycolate to glyoxylate is the critical oxidation step ($E^{\circ\prime} = -92$ mV) which, if coupled to proton reduction, requires a reverse electron transport which has to be fueled by partial hydrolysis of the ATP formed in substrate-level phosphorylation. Proof of proton gradient-dependent hydrogen release was provided in experiments with membrane vesicles: Such vesicles converted glycolate stoichiometrically to glyoxylate and hydrogen in the presence of ATP. This hydrogen formation was abolished entirely by addition of CCCP and other protonophores, as well as by DCCD. Monensin and other sodium ionophores had no specific effect (Friedrich and Schink, 1993). The process is even reversible: Membrane vesicles incubated in the presence of glyoxylate and hydrogen catalyzed a substrate-dependent net synthesis of ATP from ADP and P_i. The ratio of hydrogen-dependent glyoxylate reduction over ATP formation in isolated membrane vesicle preparations (0.2–0.5 mol per mol; Friedrich and Schink, 1995) indicates that probably two thirds of an ATP unit can be formed this way per reaction run. Thus, at least with this system, we have rather reliable data on the reaction stoichiometry.

Syntrophic Oxidation of Aromatic Compounds

So far, defined syntrophic cocultures for methanogenic degradation of aromatic substrates exist only for benzoate, gentisate, and hydroquinone (Table 3). The biochemistry of anaerobic degradation of aromatic compounds has been studied in most detail so far with benzoate, and a degradation pathway has been elaborated in the last years mainly with phototrophic and nitrate-reducing bacteria (for reviews of this subject, see Fuchs et al., 1994; Heider and Fuchs, 1997; Schink et al., 2000). The basic concept is an initial activation to benzoyl-CoA by an acyl CoA synthetase reaction requiring two ATP equivalents, followed by partial ring saturation and subsequent ring opening by a mechanism analogous to β-oxidation of fatty acids. The resulting C_7-dicarboxylic acid undergoes further β-oxidation to form three acetate residues and one CO_2.

The reductive dearomatization of benzoyl CoA in nitrate reducers requires two ATP equivalents and leads to cyclohexa-2,6-diene carboxyl CoA which undergoes further hydration and oxidation (Heider and Fuchs, 1997). This makes the initial steps in the total process rather ATP consuming (4 ATP equivalents consumed before ring cleavage). Nitrate reducers will gain these ATP investments back during subsequent oxidation of the acetyl residues. Syntrophically benzoate-oxidizing bacteria (Auburger and Winter, 1992; Schöcke and Schink, 1997; Elshahed et al., 2001) activate benzoate through a benzoyl-CoA ligase reaction, too, which consumes two ATP units and forms pyrophosphate as side product. Part of the energy invested can be regained through a membrane-bound, proton-translocating pyrophosphatase (Schöcke and Schink, 1998), and another fraction of an ATP can be conserved by the action of a membrane-bound, sodium ion-translocating glutaconyl-CoA decarboxylase which stores the energy of the C_5 dicarboxylic acid decarboxylation to crotonate in a transmembrane sodium ion gradient (Schöcke and Schink, 1999).

The overall energy budget of these fermenting bacteria can be balanced to a net gain of 1/3 to 2/3 ATP (about –45 kJ per reaction run in the ternary coculture) per benzoate oxidized, only if the ring dearomatization consumes significantly less ATP than observed with the nitrate reducers. Product patterns obtained from incubation experiments with labeled benzoate indicate that indeed a different primary reduction reaction is involved (Schöcke and Schink, 1999; Elshahed et al., 2001), but these results are not conclusive as yet. If degradation of benzoate and phthalates (carboxy-benzoates) by granular sludge was inhibited by bromoethane sulfonate, substantial amounts of cyclohexane carboxylate were formed (Kleerebezem et al., 2000), indicating again that such reduced derivatives may be side products of benzoyl CoA reduction in fermentative benzoate degraders.

Although the energetic situation of syntrophic benzoate oxidizers in methanogenic cocultures is substantially better than that of butyrate oxidizers, it appears that these bacteria depend on efficient acetate removal as well. Benzoate was nearly completely converted to acetate, methane and CO_2 in binary mixed cultures with *Methanospirillum hungatei* as hydrogen scavenger. Remnant benzoate concentrations at apparent equilibrium (in the range of 20–70 μM) increased in the presence of added acetate or propionate, and decreased in the presence of a more efficient hydrogen consumer, e.g., a sulfate-reducing partner. The corresponding hydrogen concentrations measured in such cultures were in the range of $0.5–5 \times 10^{-5}$ bar, leaving a total ΔG of –30 to –45 kJ per mol reaction for the benzoate degrader. Similar results were obtained during studies on the kinetics of benzoate degradation with *Syntrophus aciditrophicus* in the presence of a sulfate-reducing partner bacterium (Warikoo et al., 1996).

Syntrophic Oxidation of Branched-Chain Fatty Acids

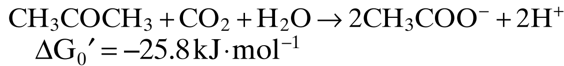
Branched-chain fatty acids are formed during fermentative degradation of the corresponding amino acids by oxidative deamination and decarboxylation, or reductive deamination (see above). The further degradation is not difficult with 2-methyl butyrate (neovalerate) because it can be β-oxidized, analogous to the degradation of butyrate to acetate and propionate by, e.g., *Syntrophospora bryantii* (Stieb and Schink, 1985). Methanogenic degradation of isobutyrate proceeds via isomerization to butyrate and further β-oxidation (Stieb and Schink, 1989); the isobutyrate/butyrate isomerization has been described in a defined culture as a coenzyme B_{12}-dependent rearrangement of the carbon skeleton (Matthies and Schink, 1992). A similar isomerization may also occur as a side reaction with valerate, leading to formation of 2-methylbutyrate (Wu et al., 1994a). Whether a similar isomerization occurs also during isobutyrate degradation by the thermophilic *Syntrophothermus lipocalidus* (Sekiguchi et al., 2000) still remains to be examined.

So far, only one defined culture has been described as capable of fermentative degradation of 3-methyl butyrate (isovalerate; Stieb and Schink, 1986), and this culture specializes in degradation of this substrate, converting it with CO_2 as cosubstrate to three molecules of acetate and one molecule of hydrogen (Tables 3 and 4). The pathway is rather complicated and involves a carboxylation, a dehydrogenation of a saturated fatty acid residue, and one substrate-level phosphorylation step (Stieb and Schink, 1986). The whole conversion is endergonic under standard conditions and depends on syntrophic hydrogen removal. Since three acetate residues are released per substrate molecule oxidized, acetate should have a far more pronounced influence on the total energetics. Details of the energetics of metabolism of this bacterium have been discussed earlier (Schink, 1991).

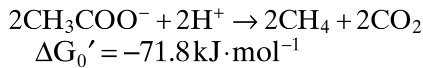
Fermentation of Acetone

A special situation is the fermentative conversion of acetone to methane and CO_2, which is catalyzed by syntrophically cooperating bacteria as well. In this case, acetate is the only interme-

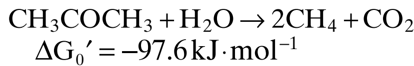
diate between both partners, as illustrated by the following equations:



$$CH_3COCH_3 + CO_2 + H_2O \rightarrow 2CH_3COO^- + 2H^+ \quad \Delta G_0' = -25.8\,kJ \cdot mol^{-1}$$



$$2CH_3COO^- + 2H^+ \rightarrow 2CH_4 + 2CO_2 \quad \Delta G_0' = -71.8\,kJ \cdot mol^{-1}$$



$$CH_3COCH_3 + H_2O \rightarrow 2CH_4 + CO_2 \quad \Delta G_0' = -97.6\,kJ \cdot mol^{-1}$$

Although in this case all partial reactions are exergonic under standard conditons, the primary fermenting bacterium depends on the methanogenic partner, and acetone degradation in the mixed culture is substantially impaired in the presence of acetylene as an inhibitor of methanogens (Platen and Schink, 1987). Experiments with the primary acetone-fermenting bacterium in dialysis cultures revealed that acetate accumulation at concentrations higher than 10 mM inhibited growth and acetone degradation (Platen et al., 1994). Under these conditions, the free energy available to the acetone fermenter is still in the range of –40 kJ per mol. Since acetone metabolism by these bacteria starts with an endergonic carboxylation reaction, this might be the amount of energy that they need to invest into this primary substrate activation reaction, perhaps through a membrane-associated enzyme system (Dimroth, 1987). Unfortunately, the acetone-fermenting bacterium was never obtained in pure culture and hence detailed studies on its biochemistry and energetics were never performed.

Interspecies Metabolite Transfer

Although hydrogen due to its small size and fast diffusion appears to be an ideal carrier for electrons between bacteria of different metabolic types, formate also could act in a similar manner. A possible alternative involvement of formate in such electron transfer processes had been considered from the very beginning (Bryant et al., 1967; McInerney and Wofford, 1992) because the original partner bacteria used could oxidize both hydrogen and formate. The standard redox potential of the CO_2/formate couple is nearly identical with that of H^+/H_2 at pH 7.0 (–420 versus –414 mV) and hence the energetic problems are the same with both. Because both electron carrier systems couple inside the cell with similar, if not the same electron transfer components, e.g., ferredoxins, most bacteria involved in interspecies electron transfer exchange hydrogen against formate and vice versa (e.g., Wu et al., 1993; Bleicher and Winter, 1994). This renders a differentiation between both electron transfer systems rather difficult, and in many cases, both carriers may even be used simultaneously (Schink, 1991). Thus, a formate/CO_2 shuttle could replace hydrogen transfer, and this idea has been brought up again on the basis of experiments with undefined floc cultures from methanogenic fermenters (Thiele and Zeikus, 1988) and with pure cultures (Thiele and Zeikus, 1988; Zindel et al., 1988), as well as on the basis of calculations of diffusion kinetics (Boone et al., 1989a; Boone et al., 1989b).

Exclusive action of hydrogen as electron carrier has been proven so far only for the butyrate-oxidizing coculture *Syntrophomonas wolfei* (Wofford et al., 1986), the glycolate-oxidizing *Syntrophobotulus glycolicus* (Friedrich and Schink, 1993) and the thermophilic, syntrophically acetate-oxidizing strain AOR (Lee and Zinder, 1988a; Lee and Zinder, 1988b; Lee and Zinder, 1988c); all these strains exhibit in coculture high hydrogenase and very little formate dehydrogenase activity. Syntrophic oxidation of propionate by *Syntrophobacter fumaroxidans* or of butyrate by *Syntrophospora bryantii* require partner bacteria that are able to use both hydrogen and formate, indicating that both carriers are involved in interspecies electron transfer (Dörner, 1992; Dong et al., 1994a; Dong et al., 1994b; Dong and Stams, 1995a; Dong and Stams, 1995b). Nonetheless, hydrogenase activities in these cultures exceeded formate dehydrogenase activities substantially indicating that hydrogen played a dominant role as well. Similar conclusions were drawn from hydrogen and formate transfer experiments with thermophilic granular sludge preparations and different partner bacteria (Schnürer et al., 1994). In isobutyrate-degrading cocultures, formate appeared to play a role besides hydrogen as an electron carrier (Wu et al., 1996). On the basis of calculations of diffusion kinetics (Boone et al., 1989a; Boone et al., 1989b), one can speculate that formate/CO_2 would be the preferred electron transfer system in suspended cultures of single cells where the carrier molecule has to diffuse over long distances through an aqueous phase, whereas hydrogen would be more efficient in densely packed aggregates which dominate in anaerobic digestors and probably also in sediments.

A key problem in an assessment of the relative importance of formate as electron shuttle is a reliable measurement of formate at low concentrations: at 0.3 bar CO_2 as typical of e.g., sewage sludge or sediments, a hydrogen partial pressure of 10^{-4}–10^{-5} bar (as required for the redox reactions discussed) is equivalent to formate concentrations of about 1–10 μM (Schink, 1994). Whereas hydrogen partial pressures can today be measured reliably down to 10^{-7} bar with mercury oxide based detectors, there is hardly an

efficient method available which allows measurement of formate in concentrations of few micromolar and less. Moreover, most indications of formate formation were obtained in the presence of inhibitors that caused accumulation of measurable amounts of formate, but it remains doubtful whether such experiments describe adequately the electron flow in the undisturbed system.

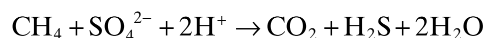
Beyond hydrogen and formate, acetate is also excreted by syntrophically fermenting bacteria and is further metabolized by methanogens. The model used above shows that also acetate removal can have a profound influence on the total energetics of syntrophic degradation of fatty acids or benzoate. Its importance may be even higher with, e.g., isovalerate degradation, since three molecules of acetate and only one hydrogen molecule are formed (Table 3). Indeed, inhibition by acetate accumulations or by addition of acetate has been reported for syntrophic degradation of fatty acids and for benzoate (Ahring and Westermann, 1988; Fukuzaki et al., 1990; van Lier et al., 1993; Warikoo et al., 1996; Schöcke and Schink, 1997).

In the recently reported acetate-oxidizing coculture of *Geobacter sulfurreducens* with *Wolinella succinogenes*, we observed that cysteine acted as electron carrier between both partners (J. Kaden and B. Schink, unpublished observation). The use of this carrier system of higher redox potential ($E°' = -270$ mV) than the hydrogen or formate system makes sense in the coupling to the comparably positive electron acceptor used by this coculture. Perhaps the same carrier is active also in other syntrophic associations which exhibit fast electron transfer to an acceptor system of comparably high redox potential (Daniel et al., 1999; Meckenstock, 1999). In the natural environment, also exogenous electron carriers such as humic substances may play a role as interspecies electron carriers, as suggested for electron transfer from iron-reducing bacteria to insoluble iron minerals (Lovley et al., 1996). Cervantes et al. (2000) observed recently that the oxidation of phenolic compounds in methanogenic sewage sludge can be coupled to the reduction of the humic compound analogue anthraquinone-2,6-disulfonate, indicating that this acceptor could perhaps be reduced also by syntrophic phenol oxidizers.

Anaerobic Methane Oxidation

Anaerobic, sulfate-dependent methane oxidation is an important reaction in anoxic marine sediments, as documented on the basis of measurements of sulfate and methane gradients in such sediments (Reeburgh, 1980; Iversen and Jørgensen, 1985). The process could also be demonstrated with radiolabelled methane in anoxic incubations of sediment samples. Zehnder and Brock (1979) showed by similar experiments that methanogenic bacteria were responsible for this oxidation of labelled methane and that methane oxidation and formation were simultaneous. However, in their assays, methane production always exceeded methane oxidation by two to three orders of magnitude, and thus this process could hardly explain the net methane oxidation observed in sediments. For a long time, no cultures were available to study this process in detail because all efforts to isolate anaerobic methane oxidizers failed, and all reports on putative methane-oxidizing anaerobes in the literature have turned out to be unsubstantiated due to lack of sufficient controls.

From a thermodynamic point of view, sulfate-dependent methane oxidation is an exergonic reaction



$$CH_4 + SO_4^{2-} + 2H^+ \rightarrow CO_2 + H_2S + 2H_2O$$

which yields under standard conditions a $\Delta G_0'$ of –18 kJ per mol. Concentrations of the reaction partners in situ in the active sediment layers are in the range of 10^{-2} bar methane, and 1–3 mM of both sulfate and free hydrogen sulfide. Thus, the overall energetics become only slightly more favorable if in situ conditions are taken into consideration. This amount of energy can feed only one bacterium, provided that the bacterium is able to exploit this biological minimum energy quantum. Based on the observation that methanogens can catalyze an oxygen-independent methane oxidation (Zehnder and Brock, 1979) and the description of a reversal of homoacetogenic fermentation by strain AOR and others (see above; Table 3), it was speculated that "reversed methanogenesis" may be the key to an understanding of this process (Hoehler et al., 1994; Schink, 1997). If the overall reaction is actually a syntrophic cooperation involving a methanogen running methane formation backwards and a sulfate-reducing bacterium, it is obvious that only one of the partners can gain metabolic energy from the reaction, and the other one has to run this process only as a cometabolic activity. This would explain at least why scientists have always failed to enrich for methane-oxidizing sulfate reducers in the past, simply because one cannot enrich for a bacterium on the basis of a cometabolic activity.

The last three years have advanced our view of anaerobic methane oxidation dramatically (Valentine and Reeburgh, 2000a). Analysis of lipids of marine archaea and sulfate-reducing bacteria in anoxic sediment layers indicated that these organisms fed on (^{13}C-depleted) methane

(Pancost et al., 2000), and similar findings, combined with molecular population analysis, were reported for archaeal/bacterial communities in marine sediments and close to submarine methane seeps and gas hydrates (Hinrichs et al., 1999; Orphan et al., 2001; Thomsen et al., 2001). In sediments overlying methane hydrates off the coast of Oregon, United States, active anaerobic methane oxidation was found to be associated with discrete, spherical microbial aggregates which consisted, according to fluorecent in-situ hybridization analysis (FISH), of *Methanosarcina*-like archaea in the center, surrounded by *Desulfosarcina*-related sulfate-reducing bacteria (Boetius et al., 2000). The energetics of sulfate-dependent methane oxidation at these gas hydrate sites (with methane pressures of about 80 bar) are considerably more favorable than in deeplying marine sediments, and the overall free energy change of the reaction in situ (–40 kJ per mol) may really allow energy conservation and growth for both partners in this cooperation. Thus, these aggregates represent a first model system to understand sulfate-dependent methane oxidation as a syntrophic cooperation phenomenon, but it still needs to be proven whether this model can also be applied to methane oxidation in deeplying, methane-poor marine sediments.

It is also still open whether methane activation proceeds through a reversal of the methyl-coenzyme M reductase reaction, and which metabolites are transferred between the two partners. Representatives of both genera, *Methanosarcina* sp. and *Desulfosarcina* sp., are metabolically rather versatile, and could metabolize either hydrogen, formate, methanol or acetate. Various cultures of methanogens failed to produce significant amounts of hydrogen from methane (Valentine et al., 2000b), indicating that "reverse methanogenesis" is not a widespread capacity among methanogens. From an energetic point of view, a transfer of acetate between both partners appears the easiest solution, but this is still a matter of speculation.

"Obligately Syntrophic" Bacteria: Cultivation and Biochemical Studies

The mutual dependence of partner bacteria in syntrophic associations has caused severe difficulties in the cultivation of such organisms, and defined cocultures have been obtained only recently. For isolation, pure cultures of known methanogenic or sulfate-reducing partner bacteria are usually provided in excess as a background "lawn" during the cultivation and dilution process to isolate the syntrophically fermenting bacterium in defined binary or ternary mixed culture. Today, all well-described syntrophically fermenting bacteria can be cultivated also in pure culture with different substrate combinations (see below). Hence, we should no longer talk about "obligately syntrophic bacteria" (because they are not obligately syntrophic) but only about syntrophic relationships or syntrophic conversion processes.

Many efforts have been made to grow syntrophically fermenting bacteria in the absence of partner bacteria. Removal of hydrogen by nonbiological procedures (low pressure, and gas diffusion through thin membranes) had only little success with ethanol oxidation, and no success at all with fatty acid oxidation. In other cases, hydrogen removal by palladium catalysts spread on either charcoal or $CaCO_3$ surfaces, with alkenes or alkynes as oxidant have shown some success (Mountfort et al., 1986), as did efforts to couple hydrogen release to reoxidation by electrochemically controlled platinum electrodes. Whether a new cultivation apparatus which maintains the hydrogen partial pressure at subnanomolar concentrations (Valentine et al., 2000c) will hold more promise has still to be proven. More successful was the use of fumarate as external electron acceptor in cultivation of syntrophic propionate degraders (Stams et al., 1993). Today, pure cultures of syntrophically fermenting bacteria of all known metabolic types have been isolated. Typically, this has been accomplished with substrates that are more oxidized than the original one, and can be fermented by dismutation. As an example, ethanol-oxidizing syntrophs can be grown in pure culture with acetaldehyde analogues such as acetoin or acetylene (Schink, 1985a; Eichler and Schink, 1986), butyrate- or benzoate-degrading syntrophs with crotonate (Beaty and McInerney, 1987a; Zhao et al., 1989; Wallrabenstein et al., 1995a) or with pentenoate as external electron acceptor (Dong et al., 1994a), and syntrophically propionate-degrading bacteria with pyruvate (Wallrabenstein et al., 1994b) or propionate plus fumarate (Stams et al., 1993). Beyond that, all syntrophic propionate oxidizers (with the exception of *Smithella propionica*) have been shown to be able also to reduce sulfate, and can be isolated in pure culture with propionate plus sulfate, although they grow only very slowly with this substrate combination (Harmsen et al., 1993; Harmsen et al., 1995; Wallrabenstein et al., 1994b; Wallrabenstein et al., 1995b).

Biochemical studies with defined cocultures of syntrophically fermenting bacteria have been carried out successfully with cell-free extracts prepared by, e.g., lysozyme (Wofford et al., 1986) or mutanolysin (Wallrabenstein and Schink,

1994a) treatment, which opens selectively only the fermenting bacterium and leaves the methanogenic partner intact, owing to its archaeal cell wall chemistry. In another approach, the partner organisms were separated by centrifugation in Percoll gradients before cell disruption and enzyme assays (Beaty et al., 1987b).

Homoacetogenic Conversions and the Effect of Temperature

Under standard conditions, methanogenic hydrogen oxidation yields more energy than homoacetogenic hydrogen oxidation (Table 2), and one would therefore assume that homoacetogens have little chance to compete successfully against methanogens for hydrogen at limiting concentrations. The function of homoacetogens in the complex electron flow scheme depicted in Fig. 1 remains unclear, therefore, and the general assumption is that they take advantage of their metabolic versatility which allows them to compete with several partners of various metabolic types, and consume two or more substrates simultaneously (Schink, 1994). Such simultaneous utilization of more than one substrate may increase the effective affinity for every single substrate, as studies with *Escherichia coli* in continuous cultures with multiple substrate supply have proven (Egli, 1995; Lendenmann et al., 1996). However, there are exceptional situations in which homoacetogens may definitively outcompete methanogens in their function as hydrogen consumers in sulfate-poor anoxic environments. One such situation may be slightly acidic lake sediments such as that found in Knaack Lake, Wisconsin, United States, where at pH 6.1 the total electron flow goes through the acetate pool, and no methane is formed by direct CO_2 reduction (Phelps and Zeikus, 1984). Obviously, hydrogen-oxidizing methanogens do not perform sufficiently well under these conditions. Thus, homoacetogens take over their function, however, only at low acetate concentration (at 10^{-4} bar H_2 and 10 μM acetate), homoacetogenic hydrogen oxidation yields a ΔG of –26 kJ per mol of acetate and has to be maintained by aceticlastic methanogens.

Temperature is a further effector which improves the ability of homoacetogens to compete successfully against methanogens for hydrogen. At temperatures lower than 20°C, homoacetogens appear to take over significant parts of hydrogen oxidation in paddy soil and lake sediments (Conrad et al., 1989; Conrad and Wetter, 1990). The known species of hydrogen-oxidizing methanogens are not significantly active at such temperatures (Zeikus and Winfrey, 1976), and homoacetogens appear to be less restricted in this respect. Dominance of homoacetogenesis in the total electron flow is even more expressed in tundra wetland soils at temperatures lower than 10°C (Kotsyurbenko et al., 1996). This effect becomes understandable from a look at the temperature dependence of hydrogen-dependent methanogenesis and homoacetogenesis. At hydrogen partial pressures lower than 10^{-4} bar and acetate concentrations at 10 mM, homoacetogenesis reaches at 5°C the same energy gain as hydrogen-dependent methanogenesis does at 35°C. Thus, the general scheme of electron flow in methanogenic environments (Fig. 1) has to be modified for slightly acidic or for low temperature habitats (Fig. 3a). Under these conditions, there is no significant hydrogen-dependent methanogenesis (group 2), and the electrons flow nearly exclusively via acetogenesis and aceticlastic methanogenesis (groups 5 and 3).

The opposite situation emerges at high temperature habitats as discussed already above in the context of syntrophic acetate oxidation. Under these conditions, homoacetogenesis can operate in the opposite direction, and the electron flow goes from acetate through the C-1 pool and hydrogen towards methane (Fig. 3b). Thus, the general flow scheme in Fig. 1 represents kind of an intermediate situation that probably describes the situation of a sewage sludge digestor correctly, but has to be modified for high and low temperature situations in the way indicated.

Structure and Growth Dynamics of Syntrophic Associations

The separation of metabolic functions and their distribution within metabolically different microorganisms, i.e., substrate oxidation and hydrogen formation in the one and hydrogen oxidation and CO_2 reduction in the other organism, is a rather unusual strategy that we do not observe, at least not to this extent, in oxic environments. It may have its advantages because it allows a high degree of metabolic specialization to the single organisms concerned, which requires very little effort into refined regulation of energy metabolism. However, the metabolic efficiency of such cooperating communities depends on an efficient metabolite transfer between the partners involved: the flux of, e.g., hydrogen between the hydrogen-forming fermenter and the hydrogen-consuming methanogen is inversely proportional to the distance between both (Schink and Thauer, 1988). Optimal metabolite transfer can be achieved

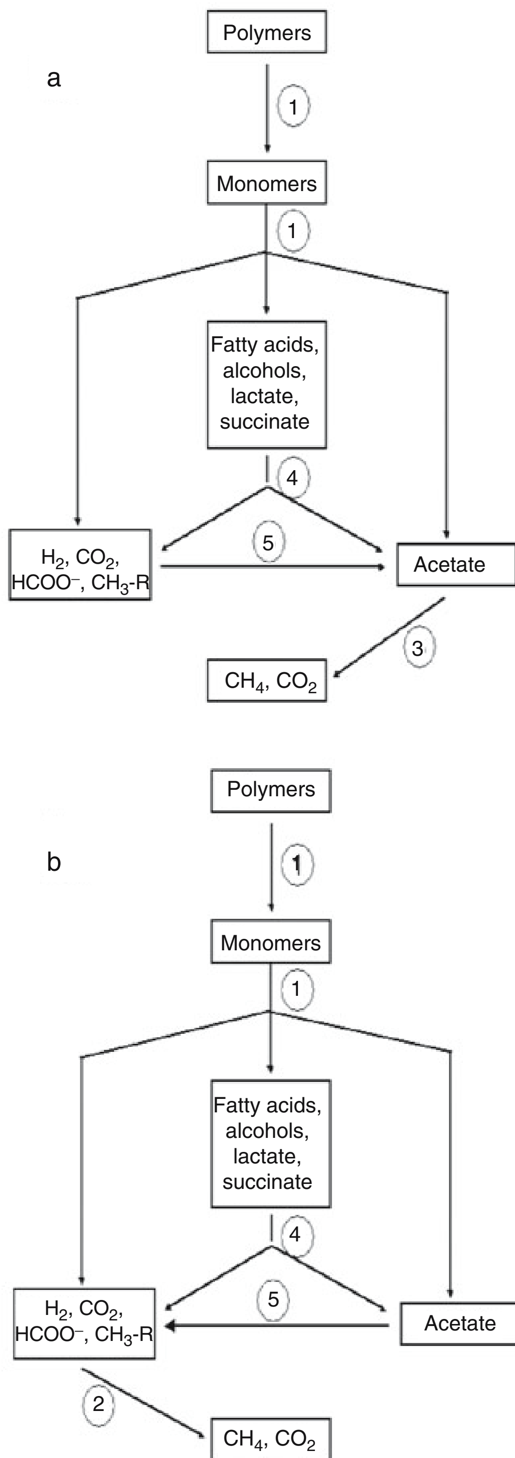


Fig. 3. Carbon and electron flow in methanogenic degradation of complex organic matter. Groups of prokaryotes involved: 1) primary fermentative bacteria; 2) hydrogen-oxidizing methanogens; 3) acetate-cleaving methanogens; 4) secondary fermenting bacteria (syntrophs); and 5) homoacetogenic bacteria. Carbon and electron flow a) at low temperature; and b) at high temperature.

best when both partners are in close contact (i.e., directly attached to each other, forming an aggregate or floc). Such flocs form preferentially in anaerobic digestors in which fatty acids are degraded, although the establishment of stable floc formation may require substantial amounts of time, sometimes even several months after start-up (Lettinga et al., 1988). Indirect evidence of aggregate formation in sediments and sludges has been obtained on the basis of hydrogen exchange measurements (Conrad et al., 1985; Conrad et al., 1986), and the recently discovered aggregates involved in sulfate-dependent methane formation (Boetius et al., 2000) exhibit even a high degree of spatial organization.

Efforts to synthesize stable aggregates (granules) of microbiologically defined composition from pure cultures have been made, and the success depended very specifically on the type of partners used. Butyrate-degrading aggregates with only two partners were stable only with *Methanobacterium formicicum*, not with *Methanospirillum hungatei*, whereas propionate-degrading granules depended on the additional presence of an acetate-degrading partner (Wu et al., 1996). In suspended cultures, *M. hungatei* is often the dominant hydrogen utilizer, but it is obviously not the ideal partner for formation of efficient granules. This experiment demonstrates again that our enrichment and cultivation techniques influence to a high degree the results obtained, and may give a rather incorrect picture of the situation prevailing in the natural or seminatural system. The composition and stability of the formed granules is further influenced by the fact that methanogenic granules in waste-degrading reactors have to deal with mixed substrate supplies that add further microbial constituents into the architecture of the resulting granule, including, e.g., sulfate-reducing bacteria (Wu et al., 1991). In any case, optimal cooperation will be secured in granules in which the partner organisms are randomly mixed to near homogeneity, rather than in situations in which the partners form “nests” of identical subpopulations.

A basic problem remains in that each partner bacterium multiplies and produces offspring only of its own kind. Thus, the situation given in Fig. 4a will change after several generations into one similar to that depicted in Fig. 4b, and the efficiency of metabolite transfer will decrease with age of the aggregates unless there are ways of internal mixing of the aggregates. Thin sections of methanogenic granules have shown examples of fully mixed as well as of nest structures (Dubourguier et al., 1988), and similar pictures were recently obtained with thin sections in which the partner bacteria were identified by specifically RNA-directed probes (Harmsen et

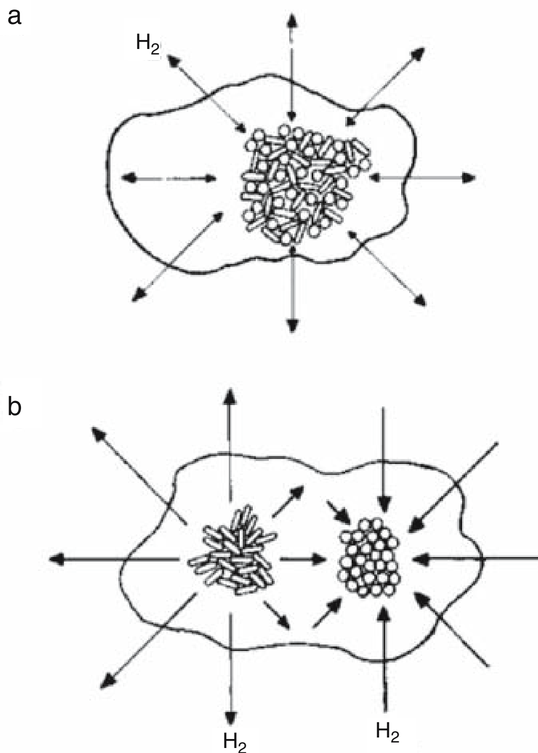


Fig. 4. Exchange of hydrogen (or other intermediates) in anaerobic bacterial flocs containing a) a homogenously mixed community of hydrogen formers and hydrogen consumers or b) hydrogen producers and hydrogen consumers in spatially separated nests.

al., 1996). The largely segregated arrangement of the two partners in the spherical aggregates active in syntrophic sulfate-dependent methane oxidation (Boetius et al., 2000) probably represents a terminal stage in the growth of these partners, and the rather regular size of these aggregates indicates that it is limited by metabolite diffusion kinetics. The dynamics of growth and internal structure development in syntrophic aggregates appears to be an interesting object of research now that gene probes provide excellent tools for direct in situ identification of the various microbial components involved. First efforts in this respect have shown that there are clear functional separations between the subpopulations in granular sludge, especially if also some sulfate is available for fatty acid oxidation (Oude Elferink et al., 1998; Santegoeds et al., 1999). A similar study on syntrophic propionate oxidizers in thermophilic granular sludge revealed that these organisms were localized mainly in the centers of the granules, closely associated with hydrogen-scavenging methanogens (Imachi et al., 2000).

It may be worth mentioning in this context that the observed maximum specific growth rates of syntrophic amino acid-degrading associations are much lower than those of other amino acid-fermenting bacteria. For example, the μ_{max} of *Clostridium* sp. growing on glutamate is 0.3–0.6 h^{-1} (Laanbroek et al., 1979) and that of *S. acidaminophila* is 0.13 h^{-1} (Nanninga et al., 1987). These bacteria use a butyric acid and propionic acid fermentation for the degradation of glutamate, respectively. However, the μ_{max} of *A. hydrogenoformans* growing on glutamate in syntrophic association with a hydrogenotrophic anaerobe is only 0.10 h^{-1}. The μ_{max} of a *Campylobacter* sp. growing on aspartate is about 0.17 h^{-1} (Laanbroek et al., 1978), whereas the μ_{max} of a coculture of *E. acidaminophilum* and a methanogen is below 0.1 h^{-1} (Zindel et al., 1988). Nevertheless, in environments with a high methanogenic activity, e.g., granular sludge from anaerobic bioreactors, high numbers of bacteria can be counted which grow syntrophically with methanogens. Three explanations can be given for this: 1) the growth rates of the syntrophic associations at low substrate concentrations are higher than those of other amino acid-fermenting bacteria, i.e., the syntrophic associations have a higher affinity for the substrate; 2) the syntrophic associations grow on mixtures of substrates rather than on single substrates; and 3) the growth rates of the syntrophic associations are higher than the ones that have been measured. The reported growth rates of the cocultures refer to suspended growth, whereas in methanogenic biofilms and aggregates growth rates might be much higher because of the shorter interbacterial distances. These observations give further support to the assumption that the growth rates of syntrophic associations are limited by the rate of interspecies metabolite transfer.

Cooperation with Protozoa, Hydrogenosomes

The function of the primary fermenting bacteria (group 1) in conversion of complex organic matter to methane and CO_2 (Fig. 1) may be taken over also by eukaryotic organisms. Anaerobic fungi, ciliates and flagellates, are known which thrive in entirely anoxic environments under reducing conditions (Finlay and Fenchel, 1992; Fenchel and Finlay, 1995), and some of them are extremely oxygen-sensitive. Since aerobic respiration is not possible in such habitats, anaerobic protozoa do not contain mitochondria. Instead, intracellular organelles are present which release hydrogen and have been called

"hydrogenosomes." The metabolism of these protozoa is fermentative; particles, especially bacterial cells, are ingested into food vacuoles and digested by hydrolysis and further fermentation, and acetate is probably the most important fermentation product.

Anaerobic protozoa can be associated with symbiotic methanogens, either extracellularly or intracellularly. Ciliates living in strictly anoxic, eutrophic sediments carry methanogenic partner bacteria inside the cell (van Bruggen et al., 1983; van Bruggen et al., 1985), often closely associated with the hydrogenosomes. The advantage of this cooperation with hydrogenotrophic methanogens for the protozoan host is obvious: removal of hydrogen and maintenance of a low hydrogen/formate concentration in the cell allows fermentation of complex organic matter mainly to acetate and CO_2. Thus, waste of organic precursors into reduced endproducts such as ethanol, fatty acids, etc. can be avoided, and the fermenting protozoon obtains a maximum ATP yield. The symbiotic methanogen takes over part of the function that mitochondria have in aerobic higher cells. Reducing equivalents are removed by the symbiotic partner, and the eukaryotic host cell runs a fermentative metabolism with maximum ATP yield.

It is assumed that the hydrogen released by hydrogenosomes stems mainly from pyruvate oxidation to acetyl CoA (pyruvate synthase reaction; Müller, 1988). Hydrogenosomes contain this enzyme, as well as ferredoxin and hydrogenase. In some cases, especially with the larger types of anaerobic protozoa, close associations of methanogenic endosymbionts with hydrogenosomes have been observed. Smaller protozoa may achieve the same effect of hydrogen release also with an extracellular partner bacteria because the diffusion distance to the surface may be short enough. The same applies to the rumen ciliates which in their comparably rich habitat cooperate only occasionally with symbiotic partners on their cell surface (Stumm et al., 1982).

Hydrogenosome and methanogenic endosymbiont together form a functional entity. In some cases, especially with the comparably big ciliates such as *Plagiopyla frontata*, hydrogenosomes and methanogens are organized in an alternating sandwich arrangement that allows optimal hydrogen transfer in highly refined structures (Finlay and Fenchel, 1992). It has been speculated that hydrogenosomes of strictly anaerobic protozoa have evolved from mitochondria of their aerobic predecessors; other speculations assume a relationship of hydrogenosomes to clostridia. The high structural development of hydrogenosomes in some protozoa may suggest that such arrangements could operate also in transfer of hydrogen from less easily available electron donors than the pyruvate synthase system represents. Unfortunately, detailed studies on the cooperation of methanogenic endosymbionts with their protozoan hosts have been hampered so far by extreme difficulties in handling defined cultures of strictly anaerobic protozoa.

Taxonomy of Syntrophs

The survey of described bacteria active in syntrophic oxidation of alcohols, fatty acids, and aromatic compounds (Table 4) documents that these bacteria are found only in two groups within the taxonomic system based on sequence similarities of the 16S rRNA, namely, the Gram-positive bacteria with low G+C content, and the Δ-Proteobacteria. Some metabolic specializations are clustered in certain taxonomic groups, e.g., ethanol, propionate and benzoate oxidation in the Δ-Proteobacteria (with the exception of *Thermoanaerobium brockii*), or fatty acid β-oxidation and acetate oxidation in the Gram-positive bacteria with low G+C content. Nonetheless, these groups are not really homogenous but encompass representatives of many other metabolic types, e.g., sulfate reducers that do not exhibit any tendency to transfer electrons to partner organisms. Finally, as pointed out above, the fermentative degradation of amino acids includes so many different expressions of obligate and facultative syntrophy within taxonomically extremely different groups of organisms that syntrophy can hardly be assumed to be associated with a single evolutionary trait. Rather, syntrophy appears to be kind of a lifestyle that is experienced and perfected by many different organisms to varying extents.

Conclusions

The energetics of syntrophic fatty acid and alcohol-oxidizing processes represent exciting examples of energy metabolism based on the smallest energy quantum that, to our present understanding, can be exploited by living cells. This minimum amount of energy which can be converted into ATP in the living cell is in the range of –20 kJ per reaction run or even lower, and this is the amount of energy available to the respective partners in most of the degradation processes discussed here. Models of metabolic cooperation and energy sharing between syntrophic partners can be based on this assumption, and experimental evidence of reverse electron transport systems to balance the energy

Table 4. Pure or defined mixed cultures of bacteria catalyzing syntrophic substrate oxidations via interspecies hydrogen transfer.

Isolate	Substrate range	Gram type	Phylogenetic position	Reference (s)
a) Oxidation of primary alcohols				
S-strain	Ethanol	–	Unknown	Bryant et al., 1967
Desulfovibrio vulgaris	Ethanol + sulfate	–	δ-Proteobacteria	Bryant et al., 1977
Thermoanaerobacter brockii	Ethanol, sugars etc.	+	Low G+C Gram positives	Ben-Bassat et al., 1981
Pelobacter venetianus	Ethanol, propanol		δ-Proteobacteria	Schink and Stieb, 1983
Pelobacter acetylenicus	Ethanol, acetylene	–	δ-Proteobacteria	Schink, 1985
Pelobacter carbinolicus	Ethanol, 2,3-butanediol		δ-Proteobacteria	Schink, 1984
b) Oxidation of butyrate and higher homologues				
Syntrophomonas wolfei	C_4–C_8	–	Low G+C Gram positives	McInerney et al., 1979, 1981
Syntrophomonas sapovorans	C_4–C_{18}	–	Low G+C Gram positives	Roy et al., 1986
Syntrophospora bryantii	C_4–C_{11}, 2-methylvalerate	+	Low G+C Gram positives	Stieb and Schink, 1985; Zhao et al., 1989
Thermosyntropha lipolytica	C_4–C_{18}, crotonate, and betaine	+	Low G+C Gram positives	Svetlichnyi et al., 1996
Syntrophothermus lipocalidus	C_4–C_{10}, isobutyrate, and crotonate	+	Low G+C Gram positives	Sekiguchi et al., 2000
c) Oxidation of propionate				
Syntrophobacter wolinii	Propionate,	–	δ-Proteobacteria	Boone and Bryant, 1980
Syntrophobacter pfennigii	Pyruvate	–	δ-Proteobacteria	Wallrabenstein et al., 1995a
Syntrophobacter fumaroxidans	Propionate + fumarate	–	δ-Proteobacteria	Harmsen et al., 1998
Smithella propionica	Propionate	–	δ-Proteobacteria	Liu et al., 1999
d) Oxidation of acetate				
Thermoacetogenium phaeum	Acetate, pyruvate, glycine, cysteine, formate, and H_2/CO_2	+	Low G+C Gram positives	Hattori et al., 2000
Clostridium ultunense	Acetate, formate, and cysteine	+	Low G+C Gram positives	Schnürer et al., 1996
e) Oxidation of isovalerate				
Strain GraIva1	Isovalerate only	+	Unknown	Stieb and Schink, 1986
f) Oxidation of glycolate				
Syntrophobotulus glycolicus	Glycolate and glyoxylate	+	Low G+C Gram positives	Friedrich et al., 1991, 1996
g) Oxidation of aromatic compounds				
Syntrophus buswellii	Benzoate and crotonate	–	δ-Proteobacteria	Mountfort and Bryant, 1982
Syntrophus gentianae	Benzoate, gentisate, and hydroquinone	–	δ-Proteobacteria	Wallrabenstein et al., 1995b
Syntrophus aciditrophicus	Benzoate and crotonate	–	δ-Proteobacteria	Jackson et al., 1999

requirements for hydrogen release have been obtained in several instances.

Recent studies on the biochemistry of syntrophic fatty acid oxidizers revealed that these bacteria are by no means "primitive" but actually admirable creatures from the point of view of energy conservation and efficient energy utilization. Most of these bacteria grow in plain mineral media and synthesize all their cellular components on the basis of only the minimum quantum of energy which can be exploited by living cells at all. They are spectacular examples of how diligently nature has organized the components of global energy flux down to those environments where very little energy is available to their living inhabitants.

Acknowledgments. This article is dedicated to the late Marvin Bryant who made fundamental contributions to our present understanding of cooperations in methanogenic microbial communities. The text is based on other reviews written earlier on the same or related subjects (Stams, 1994; Schink, 1991; Schink, 1994; Schink, 1997). The authors express their gratitude to Marvin P. Bryant, Ralph Wolfe, Norbert Pfennig, Rudolf Thauer, Fritz Widdel, Alex Zehnder, and Ralf Conrad for numerous fruitful discussions about the biology and energetics of syntrophic anaerobes, and to their former and present coworkers for their dedicated work on these extremely fastidious bacteria.

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Prokaryotes (2006) 2:336–353
DOI: 10.1007/0-387-30742-7_12

CHAPTER 1.12

Quorum Sensing

BONNIE L. BASSLER AND MELISSA B. MILLER

Introduction

Bacteria have an exquisite ability to sense and adapt to a constantly fluctuating environment. They have evolved the capacity to detect a variety of temporal and spatial cues, and in response to such stimuli, bacteria initiate signal transduction cascades that culminate in changes in gene expression. The ability to rapidly alter gene expression, and consequently behavior, in response to a dynamic environment presumably gives bacteria the plasticity to survive in rich, neutral and hostile situations.

One changing parameter that bacteria encounter is cell population density. Bacteria experience situations in which they exist essentially alone (low cell density) and also situations in which they exist in a community (high cell density). Furthermore, in the high cell density situation, bacteria can be in either a mono-culture or in a mixed species consortium. Bacteria sense and respond to fluctuations in cell population density, as well as changes in the species composition of the community, using a cell-cell communication system that is called "quorum sensing."

Quorum-sensing bacteria produce, release, detect and respond to small hormone-like molecules called "autoinducers." As a population of autoinducer-producing bacteria grows, the concentration of released autoinducer increases. When a critical threshold concentration of the signal molecule is achieved, the bacteria are able to detect its presence and initiate a signaling cascade that results in changes in target gene expression. Therefore, regulation of gene expression by quorum sensing allows bacteria to behave differently when they exist alone versus when they exist in a community. Furthermore, communication via quorum sensing enables bacteria to coordinate the gene expression of the entire community, thereby allowing the bacteria to behave as a multicellular organism.

Bacteria use quorum sensing to communicate both within and between species. Both species-specific and species-nonspecific autoinducers exist. As mentioned, these signals enable bacteria to distinguish low from high cell population density, but, further, independent responses to the species-specific and species-nonspecific signaling molecules allow the bacteria to behave differently when they exist in a pure culture versus when they exist in a consortium. Presumably this facet of quorum sensing allows mixed-populations to act synergistically to take advantage of metabolic or other processes that are not common to all the species in the mixture. Therefore, quorum sensing could allow species in the mixed-population to succeed better than each species could in isolation. Conversely, interspecies quorum sensing could also allow bacteria to measure and respond appropriately to increases in numbers of competitor bacteria. Detection of the presence of competitors coupled with the initiation of defensive behaviors could allow a population of quorum-sensing bacteria to slow or stop the growth of competing species.

This chapter describes several different model bacterial quorum-sensing signaling circuits and their uses. The first quorum-sensing circuit, that of the bioluminescent marine bacterium *Vibrio fischeri*, was identified and reported in 1983 (Engebrecht et al., 1983). At that time, cell-cell communication in bacteria was assumed to be a very limited phenomenon. Therefore, intercellular communication in *V. fischeri* was considered an interesting anomaly of no real significance. However, in the last decade, dozens of other species of Gram-negative bacteria have been identified that use a very similar quorum-sensing circuit to that of *V. fischeri* (De Kievit and Iglewski, 2000). Quorum sensing has also now been described in numerous Gram-positive bacterial species, and an interspecies quorum-sensing system has also been discovered that is shared by both Gram-negative and Gram-positive bacteria. In these latter cases, the signal molecules and the detection machinery are different from that of *V. fischeri* and other Gram-negative bacteria (Bassler, 1999b; Kleerebezem et al., 1997). However, all quorum-sensing systems allow bacteria to accomplish the same task, i.e., to count one another and regulate gene expression in response to cell number. The findings of the last ten years indicate that quorum

sensing is a widespread, fundamental signaling process that is critical for bacterial life in the wild. As this is a burgeoning field of research, we suspect that novel signals, unique detection and response apparatuses and additional, as yet undescribed, quorum-sensing behaviors await discovery.

Quorum Sensing in Gram-Negative Bacteria: The LuxI/LuxR Paradigm

Initial investigations of quorum sensing centered on *Vibrio fischeri*, a bioluminescent marine bacterium that exists as a symbiont inhabiting specialized light organs of several animal hosts, including the squid *Euprymna scolopes* and the fish *Monocentris japonicus* (for review see Visick and McFall-Ngai, 2000). In the eukaryote-*V. fischeri* association, the animal host provides a nutrient-rich environment for the bacteria, and the bacteria provide light (bioluminescence) to the host. The hosts use the light produced by the bacteria for different purposes including attraction of mates and escape from predators.

In the case of the *E. scolopes*-*V. fischeri* symbiosis, *V. fischeri* exists in pure culture and grows to extremely high cell densities (approximately 10^{11} cells/ml) in the squid light organ (Ruby and McFall-Ngai, 1992). As *V. fischeri* grows, it produces an autoinducer hormone that accumulates in the light organ. Presumably the build-up of autoinducer communicates to the bacteria that they exist "inside" a light organ as opposed to "outside" in the ocean, an environment where the autoinducer would diffuse away and therefore never accumulate to any significant concentration. In the squid light organ, when a critical autoinducer concentration is achieved, a signaling cascade is initiated that results in induction of the expression of the genes required for light production. These genes, *luxCDABE* (*lux*) encode the structural components of the luciferase enzyme complex (Engebrecht and Silverman, 1984). Therefore, *V. fischeri* only produces light at high cell density and only in the light organ of the host.

The squid *E. scolopes*-*V. fischeri* association is fascinating. *Euprymna scolopes* is a nocturnal animal that lives in shallow coastal waters, and it uses the light made from *V. fischeri* for counter-illumination at night. The light organ inhabited by *V. fischeri* resides on the underside of the squid. At night, the squid senses the ambient starlight or moonlight penetrating the water and shining onto its back. By opening and closing a shutter beneath the specialized light organ, the squid is able to modulate the amount of light emanating from the symbiotic *V. fischeri* culture. The squid appropriately opens and closes this shutter to make the amount of light shining down from the light organ exactly match the amount of light shining onto its back from the stars and moon. Therefore, using the light from *V. fischeri*, *E. scolopes* manages to avoid casting a shadow beneath itself and thereby avoids predation (Ruby and McFall-Ngai, 1992).

In summary, *V. fischeri* exists at high cell density only in the light organ of the squid, and this is the only niche where autoinducer concentration is above the required threshold for *lux* expression. Therefore, under this condition, the bacteria make light. Conversely, when the bacteria are shed from the light organ into the seawater (which occurs at sunrise and is regulated by the circadian rhythm of the squid), both the bacterial cell density and the autoinducer diminish to below the required level for signaling, and the bacteria make no light (Lee and Ruby, 1994). Quorum sensing thus enables *V. fischeri* to determine when it exists in a symbiotic association with a eukaryotic host versus when it exists free-living in the ocean. This sensory transduction system thereby grants *V. fischeri* the benefits of life as a symbiont.

The *Vibrio fischeri* LuxI/LuxR System

In *V. fischeri*, quorum sensing is regulated by two proteins called LuxI and LuxR (Engebrecht and Silverman, 1987). The LuxI protein is the autoinducer synthase, and it is responsible for production of the autoinducer signal molecule. The autoinducer is an acylated-homoserine lactone (AHL, described in LuxI-Directed Autoinducer Biosynthesis), and it freely diffuses through the cell membrane (Kaplan and Greenberg, 1985). The second protein, LuxR, is a regulatory protein that binds both the autoinducer and DNA (Stevens and Greenberg, 1999; Salmond et al., 1995).

Engebrecht and Silverman discovered and cloned both the regulatory components (*luxI* and *luxR*) and the luciferase structural genes (*luxCDABE*) from *V. fischeri* (Engebrecht and Silverman, 1984; Engebrecht and Silverman, 1987). They also determined how this first quorum-sensing circuit functioned. Their work demonstrated that the bioluminescence structural and regulatory genes were arranged in two divergently transcribed units, *luxR* and *luxICDABE* (Fig. 1). In dilute culture, the *luxICDABE* operon has weak constitutive expression, and *V. fischeri* produces almost no light. Conversely, significant transcription of *luxR* occurs at low cell density. As the cell density increases, autoinducer accumulates due to the low level expression of the *luxI* gene in the *luxICDABE* operon. Therefore, when a critical concentration of the autoinducer molecule is reached, LuxR binds it

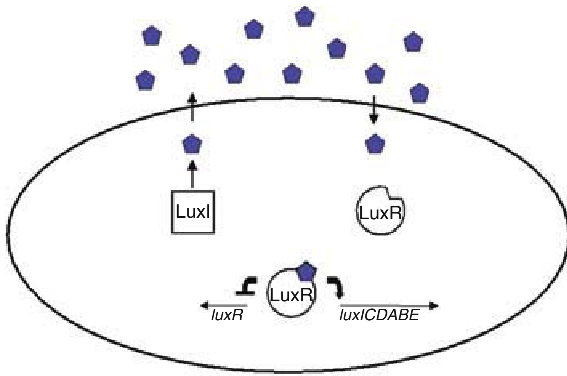

Fig. 1. The LuxI/R Quorum Sensing Paradigm-*V. fischeri*. This figure depicts the prototypical *V. fischeri* quorum sensing system. LuxI is the protein responsible for autoinducer production, and LuxR is the protein necessary for detecting and responding to autoinducer. Following LuxI-directed synthesis, autoinducer molecules (blue pentagons) accumulate thereby allowing interaction with LuxR. The LuxR-autoinducer complex is a transcriptional activator of the *luxICDABE* operon. Activation of the *luxICDABE* operon establishes a positive feedback loop, increasing the level of autoinducer production (*via luxI*) and the amount of light the bacterium emits (*via luxCDABE*). Conversely, the LuxR-autoinducer complex inhibits the transcription of *luxR*, which provides a compensatory mechanism for the regulation of light production. The oval represents a bacterium; the square and circle demarcate the proteins LuxI and LuxR respectively.

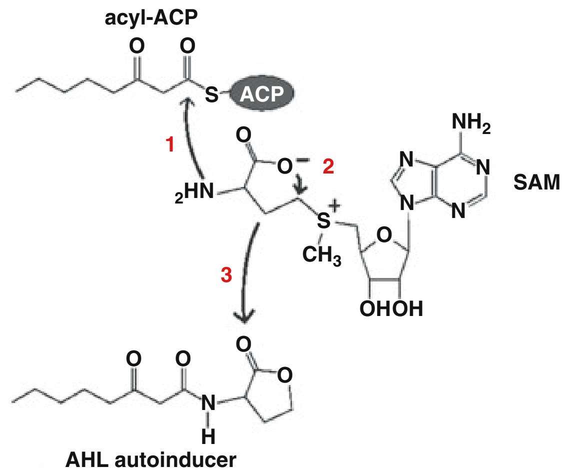

Fig. 2. The Biosynthetic Pathway for Acylated Homoserine Lactone (AHL) Autoinducers. *S*-adenosyl methionine (SAM) and acyl-acyl carrier proteins (acyl-ACP) are the substrates in autoinducer synthesis by LuxI-like enzymes. LuxI promotes the formation of an amide bond between SAM and the acyl side chain from acyl-ACP (1). This intermediate subsequently undergoes lactonization (2) and releases methylthioadenosine (MTA) as a side product. The result is the formation of an acylated-homoserine lactone (3). The autoinducer structure shown in the model is N-(3-oxooctanoyl)-homoserine lactone, the autoinducer of *Agrobacterium tumefaciens*. Adapted and reprinted with permission of S.C. Winans.

and together they activate expression of the *luxICDABE* operon. This action results in a positive feedback circuit. Specifically, an exponential increase in autoinducer production occurs (from the increase in *luxI* transcription), and because the luciferase structural genes *luxCDABE* reside downstream of *luxI*, an exponential increase in light production occurs. Furthermore, the LuxR-autoinducer complex, while acting positively on *luxICDABE* transcription, acts negatively to control *luxR* expression. Negative regulation of *luxR* transcription by the LuxR-autoinducer complex is a compensatory mechanism for modulating *luxICDABE* expression. Together, these two autoregulatory loops tightly control light production in response to autoinducer concentration and therefore in response to increasing cell population density (Engebrecht et al., 1983).

Homologues of the *V. fischeri* LuxI and LuxR proteins have now been identified in over 25 species of Gram-negative bacteria (Fuqua et al., 1996; De Kievit and Iglewski, 2000). In each documented case, the LuxI enzymes control the synthesis of an acylated-homoserine lactone autoinducer. The LuxR proteins bind a specific partner autoinducer and together the cognate pair activates the transcription of some target gene(s) in response to increasing cell population density. Although the genetic arrangement of the regulatory genes and target genes differ, in every case the mechanism of regulation is conserved. A variety of functions are controlled by LuxI-R quorum-sensing systems, as described in The *Pseudomonas aeruginosa* LasI/LasR-RhlI/RhlR Systems, The *Agrobacterium tumefaciens* TraI/TraR System, and The *Erwinia carotovora* ExpI/ExpR System.

LuxI-Directed Autoinducer Biosynthesis: Autoinducer Homoserine Lactone

The autoinducer synthesized by LuxI in *V. fischeri* is *N*-(3-oxohexanoyl)-homoserine lactone (AHL; Eberhard et al., 1981). *S*-Adenosylmethionine (SAM) and acyl-acyl carrier protein (acyl-ACP), an intermediate in fatty acid biosynthesis, are the substrates for AHL synthesis (Hanzelka and Greenberg, 1996; Val and Cronan, 1998). The LuxI enzyme promotes the formation of an amide bond joining the acyl side chain from the acyl-ACP to SAM (More et al., 1996). Lactonization of the ligated intermediate with the concomitant release of methylthioadenosine (MTA) results in AHL. The complete biosynthetic pathway for AHL autoinducers is shown in Fig. 2.

The biochemical mechanism of using SAM and a fatty acid-acyl ACP as substrates for AHL

autoinducer synthesis has been demonstrated for several autoinducers produced by LuxI homologues, indicating that this biosynthetic pathway is likely conserved among the entire family of LuxI autoinducer synthases (Parsek et al., 1999). Although not proven, because the AHL class of autoinducers are very similar, it is assumed that most AHL autoinducers are freely permeable to the Gram-negative cell membrane, similar to what has been demonstrated for the *V. fischeri* AHL (Kaplan and Greenberg, 1985). However, in the case of the *Pseudomonas aeruginosa* autoinducer *N*-(3-oxododecanoyl)-HSL, there is evidence suggesting that the MexAB-OprM multidrug efflux pump is involved in export of that particular AHL signal (Evans et al., 1998). Structurally, AHLs differ only in the acylated side chains, suggesting that the LuxI interaction with a particular acyl-ACP provides the specificity in AHL autoinducer biosynthesis (Fuqua and Eberhard, 1999). However, because AHL autoinducers act by binding to a particular LuxR protein, the LuxR homologues also contribute to the specificity inherent in quorum-sensing systems by binding to their cognate autoinducer at a higher affinity than to other autoinducers.

Specificity appears crucial to the simple signal-response quorum-sensing systems of the LuxI-R type. Whereas the LuxI's produce a highly similar family of signaling molecules, the autoinducers are typically not crossreactive (Gray et al., 1994). The selectivity of the LuxRs for their partner autoinducers presumably makes the LuxI bacterial language quite species-specific. Table 1 lists the bacterial species known to possess LuxI proteins, the structures of the autoinducers and the regulated functions.

The *Pseudomonas aeruginosa* LasI/LasR-RhlI/RhlR Systems

Quorum sensing in the opportunistic pathogen *P. aeruginosa* is controlled by a more complex LuxI/LuxR quorum-sensing circuit than that described for *Vibrio fischeri* (De Kievit and Iglewski, 2000). Specifically, two pairs of LuxI/LuxR homologues have been identified in *P. aeruginosa*, LasI/LasR and RhlI/RhlR. LasI and RhlI are autoinducer synthases that produce the AHL signals *N*-(3-oxododecanoyl)-homoserine lactone and *N*-(butyryl)-homoserine lactone, respectively (Winson et al., 1995; Pearson et al., 1995). These two quorum-sensing systems function in tandem to control virulence in *P. aeruginosa* (Pesci and Iglewski, 1997).

The LasI/LasR system was the first quorum-sensing system identified in *P. aeruginosa*, and as in the *V. fischeri* system, the transcriptional activator LasR was shown to bind to its cognate AHL autoinducer whose synthesis was dependent on LasI (Passador et al., 1993). The LasR-autoinducer complex is responsible for activation of several target virulence genes, the products of which are secreted and are involved in host tissue destruction during the establishment of infection (Jones et al., 1993). These virulence targets include *lasB*, encoding elastase; *lasA*, encoding a protease; *toxA*, encoding exotoxin A; and *aprA*, encoding an alkaline phosphatase. In addition to these virulence factors, and similar to the prototypical *V. fischeri* LuxI/LuxR system, the *P. aeruginosa* LasI/LasR system also activates *lasI* to establish an autoregulatory circuit.

However, in contrast to the *V. fischeri* system, the LasI/LasR system also activates a second quorum-sensing system, composed of RhlI/RhlR, by promoting the expression of the transcriptional activator *rhlR* (Ochsner and Reiser, 1995). Again, like LasI/LasR, in the RhlI/RhlR system, the RhlI synthesized AHL autoinducer binds to the transcriptional activator RhlR to regulate specific genes that display density-dependent expression. The genes regulated by RhlR bound to its cognate autoinducer include *lasB* and *aprA*, which are also under the control of the LasI-R system; *rpoS*, encoding the stationary phase σ factor required for stress response; *rhlAB*, encoding rhamnosyltransferase that is involved in the production of the biosurfactant/hemolysin rhamnolipid; pyocyanin, a phenazine antibiotic; and *rhlI*, encoding the autoinducer synthase (Pesci and Iglewski, 1999a).

In addition to the activation of *rhlR* by the LasI/LasR system, the LasI-dependent AHL autoinducer also acts to inhibit the RhlI autoinducer from binding RhlR, when the concentration of the LasI-dependent autoinducer is significantly higher than the RhlI-dependent autoinducer. It is hypothesized that this dual regulation by the LasI-R quorum-sensing system ensures that the RhlI-R quorum-sensing system will only be activated once the LasI-R controlled regulon has been established. Presumably, this hierarchy allows P. aeruginosa to precisely and specifically time expression of the different density-dependent target genes (Fig. 3).

A third autoinducer has recently been identified in *P. aeruginosa*. This autoinducer is especially interesting because it is not an AHL, but rather, 2-heptyl-3-hydroxy-4-quinolone (Pesci et al., 1999b). This third signal is referred to as the *Pseudomonas* quinolone signal (PQS). Similar to the Las and Rhl quorum-sensing systems, the PQS regulates the expression of *lasB*, the gene encoding the virulence factor elastase. Recent evidence suggests that the PQS could be the link between the Las and Rhl quorum-sensing hierarchy, because PQS production requires LasR, and also because PQS significantly stimulates rhlI expression (McKnight et al., 2000). It is

Table 1. Summary of the LuxI/LuxR-like quorum sensing systems that have been described.

Organism	LuxI/LuxR homologue(s)	Autoinducer identity[a]	Target genes and functions	References
Vibrio fischeri	LuxI/LuxR	*N*-(3-Oxohexanoyl)-HSL	*luxICDABE* (Bioluminescence)	Engebrecht et al., 1983 Eberhard et al., 1981
Aeromonas hydrophila	AhyI/AhyR	*N*-Butanoyl-HSL	Serine protease and metalloprotease production	Swift et al., 1997
Aeromonas salmonicida	AsaI/AsaR	*N*-Butanoyl-HSL	*aspA* (Exoprotease)	Swift et al., 1999
Agrobacterium tumefaciens	TraI/TraR	*N*-(3-Oxooctanoyl)-HSL	*tra, trb* (Tiplasmid conjugal transfer)	Piper et al., 1993 Zhang et al., 1993
Burkholderia cepacia	CepI/CepR	*N*-Octanoyl-HSL	Protease and siderophore production	Lewenza et al., 1999
Chromobacterium violaceum	CviI/CviR	*N*-Hexanoyl-HSL	Violacein pigment, hydrogen cyanide, antibiotics, exoproteases and chitinolytic enzymes	McClean et al., 1997 Chernin et al., 1998
Enterobacter agglomerans	EagI/EagR	*N*-(3-Oxohexanoyl)-HSL	Unknown	Swift et al., 1993
Erwinia carotovora	(1) ExpI/ExpR (2) CarI/CarR	*N*-(3-Oxohexanoyl)-HSL	(1) Exoenzyme synthesis (2) Carbapenem antibiotic synthesis	(1) Pirhonen et al., 1993 Jones et al., 1993 (2) Bainton et al., 1992
Erwinia chrysanthemi	ExpI/ExpR	*N*-(3-Oxohexanoyl)-HSL	*PecS* (Regulator of pectinase synthesis)	Nasser et al., 1998 Reverchon et al., 1998
Erwinia stewartii	EsaI/EsaR	*N*-(3-Oxohexanoyl)-HSL	Capsular polysaccharide biosynthesis, virulence	Beck von Bodman and Farrand, 1995
Escherichia coli	?/SdiA	?	*ftsQAZ* (Cell division), chromosome replication	Sitnikov et al., 1996 Garcia-Lara et al., 1996 Withers and Nordstrom, 1998
Pseudomonas aereofaciens	PhzI/PhzR	*N*-hexanoyl-HSL	*phz* (Phenazine antibiotic biosynthesis)	Pierson et al., 1994 Wood et al., 1997

Pseudomonas aeruginosa	(1) LasI/LasR	(1) *N*-(3-Oxododecanoyl)-HSL	(1) *lasA*, *lasB*, *aprA*, *toxA* (Exoprotease virulence factors), biofilm formation	(1) De Kievit and Iglewski, 2000 Pearson et al., 1994 Davies et al., 1998
	(2) RhlI/Rh1R	(2) *N*-Butyryl-HSL	(2) *lasB*, *rhlAB* (Rhamnolipid), *rpoS* (stationary phase)	(2) Pearson et al., 1995 Latifi et al., 1996 De Kievit and Iglewski, 2000
Ralstonia solanacearum	SolI/SolR	*N*-Hexanoyl-HSL, *N*-Octanoyl-HSL	Unknown	Flavier et al., 1997
Rhizobium ethi	RaiI/RaiR	Multiple, unconfirmed	Restriction of nodule number	Rosemeyer et al., 1998
Rhizobium leguminosarum	(1) RhiI/RhiR	(1) *N*-hexanoyl-HSL	(1) *rhiABC* (Rhizosphere genes) and stationary phase	(1) Cubo et al., 1992 Gray et al.,1996 Rodelas et al., 1999
	(2) CinI/CinR	(2) *N*-(3-Hydroxy-7-*cis*-tetradecenoyl)-HSL	(2) Quorum sensing regulatory cascade	(2) Lithgow et al., 2000
Rhodobacter sphaeroides	CerI/CerR	7,8-*cis*-*N*-(Tetradecanoyl)-HSL	Prevents bacterial aggregation	Puskas et al., 1997
Salmonella typhimurium	?/SdiA	?	*rck* (Resistance to competence killing), ORF on *Salmonella* virulence plasmid	Ahmer et al., 1998
Serratia liquefaciens	SwrI/?	*N*-Butanoyl-HSL	Swarmer cell differentiation, exoprotease	Eberl et al., 1996 Givskov et al., 1997
Vibrio anguillarum	VanI/VanR	*N*-(3-Oxodecanoyl)-HSL	Unknown	Milton et al., 1997
Yersinia enterocolittca	YenI/YenR	*N*-Hexanoyl-HSL, *N*-(3-Oxohexanoyl)-HSL	Unknown	Throup et al., 1995
Yersinia pseudotuberculosis	(1) YpsI/YpsR (2) YtbI/YtbR	(1) *N*-(3-Oxohexanoyl)-HSL (2) *N*-Octanoyl-HSL	Hierarchical quorum sensing cascade regulating bacterial aggregation and motility	Atkinson et al., 1999

[a]Generally, only the primary autoinducer are listed, but it should be noted that in a few species, multiple autoinducers have been demonstrated to be synthesized by the one LuxI-type protein.
Adapted from De Kievit and Iglewski, 2000.

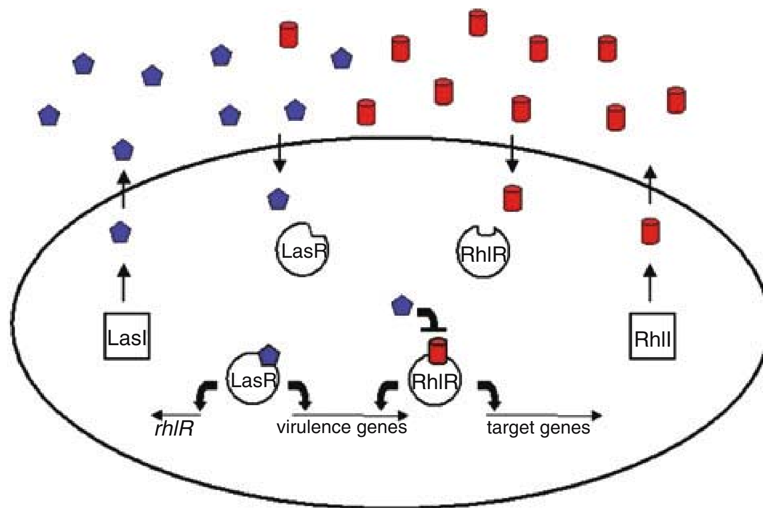

Fig. 3. Hierarchical Quorum Sensing in *P. aeruginosa*. Similar to many Gram-negative bacteria, *P. aeruginosa* uses a LuxI/R type quorum sensing system. *P. aeruginosa* has two LuxI/LuxR homologue pairs (LasI/LasR and RhlI/RhlR) that function in tandem to regulate gene expression in response to cell number. As in the *V. fischeri* model, the AHL autoinducers of *P. aeruginosa* are produced by the synthases LasI and RhlI. As the cell population density increases, and autoinducer concentration increases, the AHL autoinducers bind their corresponding transcriptional activators (LasR and RhlR). The transcriptional activator-autoinducer complexes then positively regulate target gene expression. The target genes for the LasR-autoinducer complex include secreted virulence factors and the *rhlR* gene encoding the activator RhlR of the second quorum sensing system. Upon activation by the LasI/R quorum sensing system, the RhlI/R system induces the transcription of a subset of the LasI/R regulated virulence genes, as well as RhlI/R-specific target genes. In addition to activating the RhlI/R system, the LasI/R system also negatively regulates the binding of the RhlI-dependent autoinducer to RhlR through competitive inhibition by the LasI-dependent autoinducer. The quinolone signal (PQS) also links the regulation of the Las and Rhl systems (see text). The oval represents a bacterium. The LasI-dependent autoinducer is represented by a blue hexagon, while the RhlI-dependent autoinducer is depicted as a red cylinder. The squares represent the autoinducer synthases LasI and RhlI; the circles represent the transcriptional activators LasR and RhlR.

interesting that this autoinducer is a member of the quinolone family because halogenated quinolones are commonly used as potent antibiotics against both Gram-negative and Gram-positive bacteria. It is possible that *P. aeruginosa* uses this compound both for intercellular communication and as an antimicrobial agent.

The *Agrobacterium tumefaciens* TraI/TraR System

The plant pathogen *A. tumefaciens* causes crown gall tumors in part by the transfer of its tumor-inducing (Ti) plasmid to the plant host nuclei. Though quorum sensing is not directly involved in the transfer of the Ti plasmid to the plant host, it does control the inter-bacterial specific transfer of the Ti plasmid by conjugation (Zhang et al., 1993; Piper et al., 1993). Conjugation in *A. tumefaciens* requires two signals: one from the host plant (opine) and an AHL autoinducer signal produced by the autoinducer synthase TraI. Opines produced at the tumor site in plants are a nutritive source for the infecting bacteria, but opines also indirectly activate expression of the quorum-sensing regulator *traR* via an opine-specific regulator. Therefore, in *A. tumefaciens*, bacterial conjugation is jointly controlled by both plant and bacterial signals.

The quorum-sensing system of *A. tumefaciens* is comprised of TraI/TraR, both of which are encoded on the transmissible Ti plasmid. The autoinducer synthase, TraI, produces *N*-(3-oxooctanoyl)-homoserine lactone. As in *Vibrio fischeri*, *traI* is expressed at a low basal level, so only low amounts of autoinducer are produced. After activation of the expression of *traR* by plant opines, TraR binds the autoinducer and together the complex activates the transcription of traI to establish the characteristic autoinduction loop. In addition to activating *traI*, the TraR-autoinducer complex activates the *tra* operon required for mobilization of the Ti plasmid, the *trb* operon that encodes the mating pore, and an additional regulator encoded by *traM* (Winans et al., 1999). The TraM protein, although activated by TraI/TraR, acts as a negative regulator of quorum sensing by inhibiting TraR-autoinducer-specific target activation by binding directly to TraR and inhibiting the DNA-binding and target gene activation functions of TraR (Luo et al., 2000). This additional layer of regulation in the

A. tumefaciens system that apparently does not exist in the *V. fischeri* or the *P. aeruginosa* system indicates that the different quorum-sensing bacteria have evolved specific regulatory controls to precisely adapt their specific density-dependent regulons to particular niches.

The *Erwinia carotovora* ExpI/ExpR System

Erwinia carotovora is a plant pathogen that causes soft rot in its host. Virulence in *E. carotovora* depends on several factors, many of which are exoenzymes that act to degrade plant tissue, enabling the bacterium to successfully establish an infection (Jones et al., 1993). The LuxI/LuxR homologues ExpI/ExpR are hypothesized to be involved in the regulation of many of the secreted enzymes (Pirhonen et al., 1993). An *expI* mutant displays pleiotropic defects in exoenzyme production, but a distinct role for ExpR (and therefore quorum sensing) in regulation of exoenzyme production has not been demonstrated.

The role of a second quorum-sensing system in *E. carotovora*, CarI/CarR, is less ambiguous. The CarI/CarR system positively regulates the biosynthesis of carbapenem antibiotics (Bainton et al., 1992; Williams et al., 1992). Carbapenem production is density dependent, and furthermore, occurs simultaneous with exoenzyme production. It is theorized that, during infection, *E. carotovora* not only destroys the plant tissue for nutrients, but it also kills competing/invading bacteria of other species with antibiotics. In addition, CarI/CarR activate the production of exoenzymes, and this activity is suggested to be coupled to the activity of ExpR through the *rex* (regulation of exoenzymes) gene product (Pierson et al., 1999). Interestingly, both ExpI and CarI produce the same AHL signaling molecule, *N*-(3-oxohexanoyl)-homoserine lactone. It is noteworthy that ExpI and CarI were identified in separate isolates of *E. carotovora*, and whereas the proteins show only 70% identity, they may prove to be functionally identical in the independent isolates. Although the understanding of ExpI and CarI is limited, it is apparent that the functions of the two transcriptional activators, ExpR and CarR, are somehow integrated through the use of the same AHL signal molecule.

Gram-Positive Quorum Sensing: Peptide Signals and Two-Component Signal Transduction

Like Gram-negative bacteria, quorum sensing also occurs in Gram-positive bacterial species. Although the fundamental purpose of quorum sensing in Gram-negative and Gram-positive bacteria is identical, i.e., the density-dependent expression of target genes via the secretion and detection of an autoinducer signaling molecule, the signaling molecules, mechanism of their synthesis and the secretion and detection apparatus used by Gram-positive bacteria are not similar to those of Gram-negative bacteria (see Quorum Sensing in Gram-Negative Bacteria: The LuxI/LuxR Paradigm).

Gram-positive quorum-sensing bacteria use a secreted peptide as the autoinducer. Typically, the peptide signal molecule is secreted by a dedicated ATP-binding cassette (ABC) transporter (Kleerebezem et al., 1997). Also in contrast to the simple detection/response mechanism of the LuxR-like transcriptional regulators, autoinducer detection and response are mediated by two-component adaptive response circuits in Gram-positive bacteria (Kleerebezem et al., 1997). Two-component systems consist of a family of homologous proteins that exist in a wide variety of both Gram-negative and Gram-positive bacteria. These systems enable bacteria to adapt to alterations in a wide variety of environmental conditions. Two-component systems relay sensory information by phosphorylation/dephosphorylation cascades. The two components are a membrane-bound sensor kinase protein, that initiates information transfer by autophosphorylation, and a response regulator protein, which following phosphotransfer from a cognate sensor kinase, typically controls transcription of downstream target genes. (For a detailed review of two-component systems see Stock et al., 1989 and Parkinson, 1995.) Gram-positive quorum-sensing bacteria use two-component systems to detect and respond to the accumulation of a threshold concentration of a peptide autoinducer. A general scheme for Gram-positive quorum sensing is shown in Fig. 4, and several Gram-positive quorum-sensing regulatory systems and the targets they control are described in The *Streptococcus pneumoniae* Competence System, The *Bacillus subtilis* Competence System, and The *Staphylococcus aureus* Agr System.

The *Streptococcus pneumoniae* Competence System

Genetic transformation of bacteria was first described in *S. pneumoniae* (see Havarstein and Morrison, 1999 and references therein). Transformation by foreign DNA requires the bacterium to possess the ability to take up exogenous DNA. This ability is known as "competence" for transformation. *Streptococcus pneumoniae* is a naturally competent bacterium, and it uses peptide quorum sensing to regulate development of

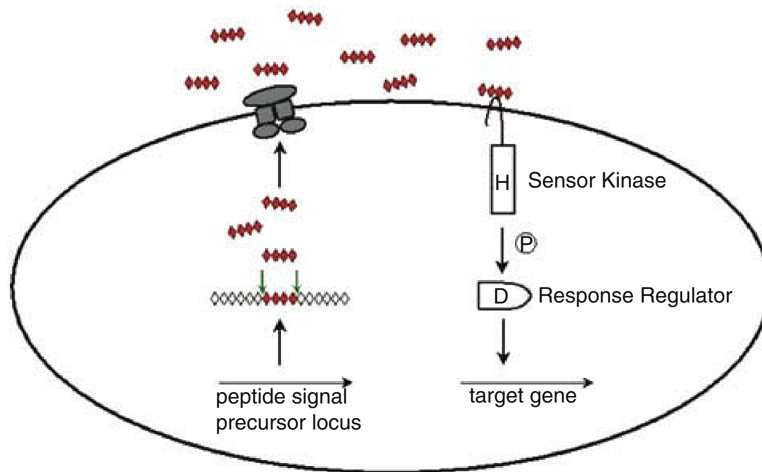

Fig. 4. A General Model for Gram-Positive Quorum Sensing. In Gram-positive bacteria, the autoinducer is a peptide signal that is processed from a larger precursor peptide. The peptide signal precursor locus is translated into a precursor protein, which is subsequently cleaved to produce the peptide signal. In most cases, the peptide signal is transported out of the cell *via* an ATP-binding cassette (ABC) transporter. As the bacterial population grows, the peptide signal accumulates extracellularly, where the signal can then be detected by a two-component system. Following interaction with the peptide signal, the sensor kinase protein of the two-component system autophosphorylates on a conserved histidine residue (H). This autophosphorylation event initiates a phospho-relay cascade that results in phosphorylation of the cognate response regulator protein on a conserved aspartic acid residue (D). The phosphorylated response regulator activates the transcription of the target gene(s). The oval represents a bacterium; diamonds are units of the precursor protein with the red diamonds representing the signal peptide. The green arrows indicate the processing of the precursor protein into the peptide signal. The gray proteins represent an ABC transporter. The P in the circle represents the phosphorylation cascade. Note that the length of the precursor and processed peptides do not imply a specific number of amino acids.

the competent state. Unlike most other naturally competent organisms, *S. pneumoniae* can assimilate DNA regardless of sequence and, thus, regardless of the species of origin of the DNA. By using quorum sensing to regulate competence genes, these promiscuous organisms are presumed to enhance their likelihood of acquiring DNA from a variety of sources that could contain a vast assortment of genes specifying beneficial functions that have not evolved within their own species.

The peptide signal for density-dependent competence development in *S. pneumoniae* is called the "competence-stimulating peptide" (CSP), which contains 17 amino acids and is produced by the cleavage of a 41-residue peptide precursor called "ComC" (Tortosa and Dubnau, 1999). The CSP is secreted by the ABC-transporter encoded by *comAB*. Similar to AHL-autoinducers, the concentration of CSP molecule increases in the extracellular environment as the bacterial population grows. Accumulated CSP is detected by the two-component sensor kinase ComD, which initiates a phosphorylation cascade that results in phosphorylation of the cognate response regulator protein ComE (Kleerebezem et al., 1997). Phosphorylation of ComE activates it, and phospho-ComE, in turn, activates transcription of the gene *comX*. The protein encoded by *comX*, is an alternative σ factor that is required for the downstream expression of genes necessary for development of the competent state (Havarstein and Morrison, 1999). Competence in *S. pneumoniae* occurs only during exponential growth, and competence is transient. The benefit *S. pneumoniae* derives from a temporary competent state is unclear, but it is likely that additional regulatory mechanisms exist to eliminate the transient density-dependent expression of competence.

The *Bacillus subtilis* Competence System

Bacillus subtilis is a commensal soil organism that, like *Streptococcus pneumoniae*, uses quorum sensing to control genes required for the acquisition of extracellular DNA (Lazazzera and Grossman, 1998). The development of competence in *B. subtilis* occurs in about 10% of the bacterial population at the transition between logarithmic growth and stationary phase, when cell lysis and the concomitant release of DNA likely occurs. Therefore, competence for uptake of exogenous DNA in a small fraction of the population could allow this subpopulation to use these fragments of DNA as a repository for repair of mutated and broken chromosomes (Lazazzera et al., 1999). Unlike *S. pneumoniae*, which is postulated to use competence in early log phase to acquire heterologous DNA, *B.*

subtilis is proposed to use quorum sensing and competence at higher cell density to inherit its own species' DNA.

Density-dependent control of competence in *B. subtilis* is mediated by two peptide signals called "ComX" and "CSF" (competence and sporulation factor). These two autoinducer signals are secreted as the cell population density increases. The ComX peptide is translated as a 55-amino-acid precursor protein, but is subsequently post-translationally modified on a tryptophan residue, and the precursor protein is cleaved (Lazazzera and Grossman, 1998). The final exported ComX signal molecule is a modified decapeptide. A protein called "ComQ" is required for production of the ComX peptide. The ComQ protein is hypothesized to be involved in the processing, modification and/or secretion of the ComX peptide, however, the exact function of ComQ has not been established (Lazazzera et al., 1999). The concentration of external ComX increases as the culture grows, and detection of the peptide is via the two-component system ComP/ComA. The ComP protein is the sensor kinase and ComA is the response regulator. Phosphorylated ComA is responsible for the activation of *comS*. The function of the ComS protein is to protect another protein called "ComK" from proteolytic degradation (Tortosa and Dubnau, 1999). The ComK protein is the transcriptional activator of competence genes. Finally, this complicated quorum-sensing circuit allows *B. subtilis* to become competent for transformation by exogenous DNA only at high cell density.

As mentioned, two peptide signals (ComX and CSF) are involved in quorum sensing and competence development in *B. subtilis*. The CSF pentapeptide is produced by the processing of the C-terminus of a peptide precursor called "PhrC" (Lazazzera and Grossman, 1998). Although the CSF peptide signal is produced in a density-dependent manner, its mechanism of action is different than that of other peptide quorum-sensing autoinducers. Specifically, secreted CSF is internalized via an oligopeptide-permease, and intracellular CSF acts to modulate the levels of phosphorylated ComA by inhibiting the activity of a phosphatase called "RapC" (Lazazzera et al., 1999). As described, ComA is a response regulator protein, and the inhibition of a specific ComA phosphatase results in an increase in the level of phospho-ComA in the cell. Phospho-ComA activates expression of a set of genes required for competence development. Thus, whereas CSF may not itself be a typical quorum-sensing signaling molecule, it is clearly involved in the modulation of competence gene expression in a density-dependent manner.

The *Staphylococcus aureus* Agr System

Staphylococcus aureus is an invasive pathogen that can cause disease in almost any tissue or organ in the human body, primarily in compromised individuals. Staphylococcal infections such as pneumonia, endocarditis, septicemia, toxic shock syndrome and food poisoning require several virulence factors, most of which are secreted enzymes or toxins. Expression of many of the virulence factors of *S. aureus* is dependent on cell density and peptide quorum sensing.

Density-dependent virulence in *S. aureus* is regulated by an RNA molecule called "RNAIII" (Kleerebezem et al., 1997). Levels of RNAIII are controlled by three loci, one of which encodes a peptide quorum-sensing system. The *agrBDCA* operon encodes the quorum-sensing components, and this operon is divergently transcribed from the *hld* locus which encodes the RNAIII transcript. In the *S. aureus* quorum-sensing circuit, the 46-residue signal peptide precursor is encoded by *agrD* (Morfeldt et al., 1996). The AgrD precursor protein is subsequently cleaved to an octapeptide, and this processing step requires the product of the *agrB* gene (Novick, 1999). The processed autoinducing peptide (AIP) is unique in that it contains a thiolactone ring (Mayville et al., 1999). The products of the *agrC* and *agrA* genes, AgrC and AgrA respectively, comprise the two-component sensor kinase and response regulator signaling pair. Following the build-up and detection of AIP, the AgrC/AgrA two-component phosphorylation cascade culminates in phosphorylation of AgrA. Phospho-AgrA is responsible for increasing RNAIII levels in the cell, although the exact mechanism of activation has not been determined. The RNAIII, through another unknown mechanism, subsequently functions to activate the expression of a variety of exported virulence factors in *S. aureus*.

Quorum Sensing in *Vibrio harveyi*: Integration of AHL and Two-Component Signaling

Vibrio harveyi is a marine bacterium that uses quorum sensing, among other environmental cues, to modulate bioluminescence (Bassler, 1999a). However, unlike *Vibrio fischeri*, *V. harveyi* is not known to exist in symbiotic relationships, and the benefit it receives from producing light remains a mystery. Although *V. harveyi* is a Gram-negative bacterium, its quorum-sensing circuit possesses features reminiscent of both Gram-negative (see Quorum Sensing in Gram-Negative Bacteria: The LuxI/LuxR Para-

digm) and Gram-positive bacteria (see Gram-Positive Quorum Sensing: Peptide Signals and Two-Component Signal Transduction; Bassler, 1999b). For this reason, we have chosen to discuss the *V. harveyi* quorum-sensing circuit separately from the other Gram-negative systems.

Analogous to other Gram-negative bacteria, *V. harveyi* produces and responds to an AHL autoinducer (Bassler et al., 1993; Cao and Meighen, 1989). In contrast to Gram-negative bacteria, but similar to Gram-positive bacteria, detection of and response to autoinducer is carried out by a two-component circuit (Bassler et al., 1993; Bassler et al., 1994a; Bassler et al., 1994b). Additionally, *V. harveyi* possesses a novel autoinducer signaling molecule, called "AI-2" (Bassler et al., 1993; Surette and Bassler, 1998). The AI-2 molecule and the gene required for its production have recently been demonstrated to occur in a wide variety of Gram-negative and Gram-positive bacteria (Surette and Bassler, 1998; Surette et al., 1999). The AI-2 molecule could be the common link that connects the evolution of the two major classes of quorum-sensing circuits.

Multiple Systems Regulate Quorum Sensing in *Vibrio harveyi*

Vibrio harveyi was the first bacterium in which the use of multiple autoinducers was described (Bassler et al., 1994a). Specifically, two parallel quorum-sensing systems converge to regulate *luxCDABE*, the luciferase structural operon in *V. harveyi*. System 1 is comprised of autoinducer-1 (AI-1) and Sensor 1 (LuxN); System 2 consists of autoinducer-2 (AI-2) and Sensor 2 (LuxPQ) (Bassler et al., 1993; Bassler et al., 1994a). While the two sensors detect independent autoinducer signals, the parallel signaling systems converge at a downstream integrator protein called "LuxU" (Freeman and Bassler, 1999b). Integration of the two autoinducer cues allows light production in *V. harveyi* to be modulated by multiple inputs (Fig. 5).

Both LuxN and LuxQ are members of a family of two-component proteins called "hybrid kinases." Members of the hybrid sensor kinase family contain multiple signaling modules. In the case of LuxN and LuxQ, each possesses both a sensor kinase domain and an attached response-regulator domain. In *V. harveyi*, at low cell density, when little autoinducer is present, the hybrid sensor kinases LuxN and LuxQ initiate a phospho-relay cascade that results in phosphorylation of the signal integrator protein LuxU, and finally the phosphoryl group is transferred to a response regulator protein called "LuxO" (Freeman et al., 2000). When LuxO is phosphorylated, the luciferase operon (*luxCDABE*) is not transcribed, and the bacteria do not make light (Bassler et al., 1994b; Freeman and Bassler,

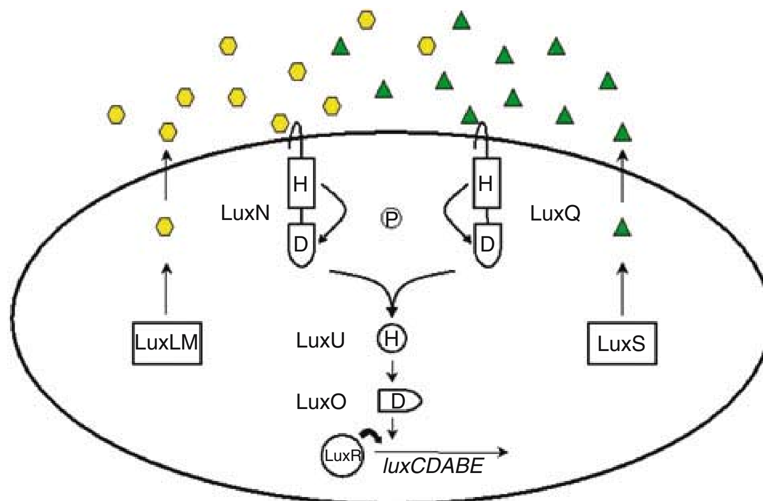

Fig. 5. Quorum Sensing in *V. harveyi*. This model demonstrates how *V. harveyi* uses both an AHL (AI-1, yellow hexagons) and a novel autoinducer signal (AI-2, green triangles) as autoinducers to regulate quorum sensing. *V. harveyi* has two parallel two-component signaling circuits. LuxN is the hybrid sensor kinase for AI-1 (synthesized by LuxLM), and LuxQ senses and responds to AI-2 (synthesized by LuxS). At low cell density and low autoinducer concentration, LuxN and LuxQ autophosphorylate and initiate phosphoryl flow through LuxU to the response regulator, LuxO. When LuxO is phosphorylated, *luxCDABE* is not transcribed and the bacteria make no light. Conversely, at high cell density and high autoinducer concentration, the sensor kinases switch from being kinases to phosphatases, which results in the draining of phosphate out of the system. When LuxO is dephosphorylated, *luxCDABE* is transcribed and the bacteria make light. Additionally, the transcriptional activator LuxR (not homologous to the *V. fischeri* LuxR) is required for the transcription of *luxCDABE*. The oval represents a bacterium, H: Histidine, D: Aspartic Acid. The P in the circle represents the phosphorylation cascade.

1999a). Conversely, at high cell density, when autoinducer is abundant, the sensors switch from being kinases to being phosphatases. Phosphatase activity leads to the dephosphorylation of LuxO and subsequent transcription of *luxCDABE*. As in *V. fischeri*, transcription of *luxCDABE* in *V. harveyi* results in luciferase production and light emission. Additionally, a transcriptional activator, LuxR, is absolutely required for the transcription of the *V. harveyi luxCDABE* operon (Martin et al., 1989; Showalter et al., 1990). However, the *V. harveyi* LuxR protein shares no homology to the *V. fischeri* family of LuxR transcriptional activators (The *Vibrio fischeri* LuxI/LuxR System).

Apparently *V. harveyi* does not possess LuxI/LuxR homologues. This finding is surprising because the *V. harveyi* AI-1 is an AHL, *N*-(3-hydroxybutanoyl)-homoserine lactone (Cao and Meighen, 1989). Synthesis of AI-1 however, is not dependent on a *luxI* gene. Rather synthesis of the *V. harveyi* AHL autoinducer is dependent on the *luxLM* locus (Bassler et al., 1993). The genes luxL and *luxM* share no homology with the *luxI* gene family. The second *V. harveyi* autoinducer, AI-2, is not an AHL, as its structure has recently been determined (Schauder and B.L. Bassler, manuscript in preparation). Synthesis of AI-2 is dependent on the gene *luxS* (Surette et al., 1999). Again, the *luxS* gene is not similar to the *luxI* gene.

Although no quorum-sensing components similar to *V. fischeri* LuxI and LuxR have been identified in *V. harveyi*, the opposite is not the case. A LuxM homologue (called "AinS") and homologues of the two-component proteins LuxU and LuxO have recently been identified in *V. fischeri* (Kuo et al., 1994; Gilson et al., 1995; Miyamoto et al., 2000). The AinS protein directs the synthesis of an AHL autoinducer, and LuxU and LuxO play a role in density-dependent regulation of *lux* expression in *V. fischeri*. It is now becoming apparent that multiple signaling circuits could be involved in quorum sensing in *V. fischeri* similar to what is known about quorum-sensing regulation in *V. harveyi*.

Recently many Gram-negative and Gram-positive bacteria have been shown to produce a *V. harveyi* AI-2-like activity, whereas only one closely related species, *Vibrio parahaemolyticus*, has been identified to produce a *V. harveyi* AI-1-like activity (Bassler et al., 1997). This finding led to the hypothesis that, in *V. harveyi*, AI-1 and System 1 are involved in intraspecies quorum sensing, while AI-2 and System 2 could be used by *V. harveyi* for interspecies cell-cell communication. The convergence of these two quorum-sensing systems allows *V. harveyi* to regulate light production in response to its own high cell density and also in response to the presence of other species of bacteria. This observation is noteworthy because in its natural habitat, *V. harveyi* is expected to exist in mixed populations containing many species of bacteria. Multiple autoinducer languages could grant *V. harveyi* a selective advantage in the wild. If *V. harveyi* can detect its own species as well as the presence of other species of bacteria, this ability could allow *V. harveyi* to determine when it is likely to be in intense competition for scarce nutrients. In addition, *V. harveyi* could specifically and appropriately modulate gene expression in response to the presence of other species of bacteria. Distinct roles for the two *V. harveyi* quorum-sensing systems are further supported by the recent discovery that *V. harveyi* regulates many different genes in addition to *lux* by these two autoinducers. In addition, AI-1- and AI-2-specific targets have now been identified in *V. harveyi* indicating that not all of the quorum-sensing information is channeled to LuxU and LuxO to control gene expression (B.L. Bassler, unpublished data).

LuxS and AI-2: The Language of Inter-Species Communication

As mentioned, a gene called *luxS* is required for the production of AI-2 in *V. harveyi* (Surette et al., 1999). DNA database analysis revealed that highly conserved homologues of this novel gene are present in over 30 species of both Gram-negative and Gram-positive bacteria, including, but not limited to, *Escherichia coli*, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi*, *Hemophilus influenzae*, *Helicobacter pylori*, *B. subtilis*, *Borrelia burgdorferi*, *Neisseria meningitidis*, *Yersinia pestis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Enterococcus faecalis*, *S. pneumoniae*, *Streptococcus pyogenes*, *S. aureus*, *Clostridium perfringens*, *Clostridium difficile* and *Klebsiella pneumoniae*. Most of these species have now been shown to produce an AI-2 activity, and mutation of *luxS* in a number of these species, including *V. harveyi*, *S. typhimurium* and *E. coli*, eliminates AI-2 production. These results suggest that *luxS* is responsible for AI-2 production in all of these bacteria. Because both Gram-negative and Gram-positive bacterial species are represented, and because preliminary evidence indicates that many of these bacteria are producing an identical signaling molecule, AI-2 is considered a universal bacterial language that bacteria could use for interspecies communication (Bassler, 1999b). Therefore, in contrast to AHL and peptide quorum-sensing systems, which represent species-specific bacterial languages, AI-2 and LuxS could be the foundation of a species nonspecific bacterial language.

The identities of the genes regulated by the AI-2 signal are being actively sought in a number of bacteria. Initial evidence in several bacterial species indicates that AI-2 is involved in regulation of pathogenicity. For example, AI-2-specific activation of the locus of enterocyte effacement (LEE) pathogenicity island, encoding a type III secretion system in *E. coli* O157 has been reported (Sperandio et al., 1999). Secretion of virulence factors in *Vibrio vulnificus* has been shown to be controlled by AI-2, and furthermore, the LD50 of a *V. vulnificus luxS* mutant is greatly increased (Kim et al., 2000). Presently there are only a few reports of AI-2 regulated target gene expression, but this is most likely because the *luxS* gene and its widespread nature have only recently been discovered. It will be interesting to determine how bacteria that live in diverse habitats have adapted the use of the AI-2 quorum-sensing language to enhance survival in their particular niches.

Quorum Sensing in *Myxococcus xanthus*: A Unique Sensory System

Myxococcus xanthus is a Gram-negative soil bacterium that displays complex social behaviors. *Myxococcus xanthus* moves by gliding. Specifically, it glides over and colonizes solid surfaces such as decaying plant material that it subsequently uses for nutrients. The *M. xanthus* bacteria hunt for food in swarms, a behavior that allows the individual cells to take advantage of secreted hydrolytic enzymes produced by neighboring cells (Dworkin, 1973; Dworkin and Kaiser, 1985). At high cell density under nutrient limiting conditions, *M. xanthus* forms complex structures called fruiting bodies. Bacterial cells inside the fruiting body undergo a developmental process that leads to spore formation. Spore formation is partially controlled by a quorum-sensing circuit. However, in contrast to other Gram-negative quorum-sensing bacteria, in *M. xanthus*, quorum sensing is not dependent on an AHL autoinducer. Therefore, because *M. xanthus* has a unique quorum-sensing system, as in the *Vibrio harveyi* case (Quorum Sensing in *Vibrio harveyi*: Integration of AHL and Two-Component Signaling), this system is discussed separately.

A secreted signal called "A-signal" is required for quorum sensing in *M. xanthus* (Hagen et al., 1978; LaRossa et al., 1983). The A-signal is a mixture of amino acids that are produced as a consequence of the enzymatic action of extracellular proteases (Kupsa et al., 1992a; 1992b; Plamann et al., 1992). Similar to other quorum-sensing bacteria, the extracellular concentration of the signal increases as the cell population density increases. Three genes are necessary for production of A-signal. These genes are called *asgA*, *asgB* and *asgC*. The AsgA protein is a two-component sensor kinase, AsgB is a DNA-binding transcriptional regulator, and AsgC encodes the housekeeping σ factor for *M. xanthus* (Davis et al., 1995; Plamann et al., 1994; Plamann et al., 1995). These proteins, as well as others that remain to be identified, function in a signaling circuit that activates the expression of genes encoding the secreted proteases required for A-signal generation.

Detection of A-signal is via a two-component sensor kinase called "SasS." The SasS protein transfers phosphate to a response regulator called "SasR" (Kaplan et al., 1991; Yang and Kaplan, 1997). Phosphorylated-SasR, in conjunction with the alternative sigma factor σ^{54}, activates downstream target genes. These genes are hypothesized to encode structural and possibly regulatory proteins that are required for the spore differentiation process. A negative regulatory protein, called "SasN," must also be inactivated for *M. xanthus* to respond to A-signal. The function of SasN is not known, and SasN is not homologous to any other identified protein (Gorski et al., 2000; Xu et al., 1998). This system is remarkable because it is so different from every other described quorum-sensing system. Apparently, some facet(s) of the complicated social lifestyle of *M. xanthus* warrants the use of a distinct quorum-sensing mechanism.

Eukaryotic Interference with Quorum Sensing

Outlined in this chapter (Quorum Sensing in Gram-Negative Bacteria: The LuxI/LuxR Paradigm, Gram-Positive Quorum Sensing: Peptide Signals and Two-Component Signal Transduction, Quorum Sensing in *Vibrio harveyi*: Integration of AHL and Two-Component Signaling, Quorum Sensing in *Myxococcus xanthus*: A Unique Quorum Sensing System) are just a few examples of the many known quorum-sensing systems. Identification of new quorum-sensing systems continues at a rapid pace, in part owing to the use of PCR amplification of quorum-sensing genes based on homology to known quorum-sensing regulators, and also owing to the development of easy-to-use bioassays that facilitate the cloning and identification of new quorum-sensing genes (Swift et al., 1993). However, in general, defining and understanding the regulons controlled by new quorum-sensing regulatory proteins typically lags behind the identification of the autoinducer/sensor pair.

Although we know that over 25 LuxI/LuxR systems exist, and at least as many species of bacteria produce AI-2 and possess a LuxS homologue, in most cases we do not yet know what targets these autoinducer systems control.

It is clear, however, that several quorum-sensing systems have been demonstrated to modulate the expression of virulence factors, and interest in designing and implementing novel antimicrobial strategies that target quorum sensing in pathogenic bacteria is high (Zhu et al., 1998). In addition to synthetic strategies for drug design based on autoinducers, it seemed likely that eukaryotes that are susceptible to infection by quorum-sensing bacteria could have already evolved natural therapies to thwart bacterial invasion by inhibiting quorum sensing. One such example of the evolution of a naturally occurring antibacterial agent that specifically counteracts invasion of a eukaryotic host by quorum-sensing bacteria is documented (Givskov et al., 1996). The seaweed *Delisea pulchra* produces halogenated-furanones, molecules that are structurally related to AHL autoinducers. The furanone of *D. pulchra* has the ability to inhibit a social motility phenotype called "swarming" in *Serratia liquefaciens* and other bacterial species. Swarming motility allows bacteria to move over and colonize a surface. Swarming in *S. liquefaciens* is controlled by an AHL quorum-sensing system (Eberl et al., 1996). The halogenated-furanone produced by the eukaryotic host specifically binds with high affinity to the *Vibrio fischeri* LuxR protein, and presumably to the S. liquefaciens LuxR homologue to inhibit motility (Manefield et al., 1999). Although *D. pulchra* and *S. liquefaciens* do not encounter each other in nature, the ability of *D. pulchra* to inhibit swarming is likely a general host defense mechanism that prevents colonization of the plant surface by bacteria (Givskov et al., 1997). While the details and in vivo significance of eukaryotic interference with bacterial quorum sensing are yet to be defined, it remains an intriguing eukaryotic defense mechanism that may have long-range implications in antimicrobial therapy. Likewise, competing bacterial populations might also inhibit each other's quorum sensing by developing autoinducer antagonists. To date, there is only one documented example of such a process between populations of coexisting bacteria (Dong et al., 2000). It has recently been shown that AiiA, an enzyme produced by *B. subtilis* inactivates the *E. carotovora* AHL quorum-sensing signal, thereby attenuating the virulence of *E. carotovora*. The AiiA enzyme is similar to members of a zinc-binding metallohydrolase family of proteins. Much emphasis is now being placed on identifying such antagonistic eukaryotic-bacterial and bacterial-bacterial interactions and the molecules that control these interactions.

Conclusions

Quorum-sensing systems have been widely adapted for a variety of uses by bacteria. However, in every case, quorum sensing confers on bacteria the ability to communicate, and further to alter gene expression in response to the presence of other bacteria. This ability allows a population of small organisms to behave as a multicellular unit, and to gain power and reap benefits that would otherwise be exclusive to eukaryotes. The study of quorum sensing is in its infancy. We need to learn more about how quorum sensing is used by bacteria to communicate both within and between species, how it is used by bacteria to act synergistically and to overcome competitors. We need to understand the variety of signals produced and how information contained within those signals is transduced and integrated to control an elaborate series of responses. Further, the knowledge we gain from studies of quorum sensing can be used as the basis for the design of novel antibacterial therapies. This is especially important at a time when new antibacterial pharmaceuticals are required to combat the ever-increasing problem of multidrug resistance in bacteria.

Finally, the phenomenon of quorum sensing and how bacteria talk to each other is a fascinating one, and its study could reveal fundamental principles about cell-cell communication and information flow. Additionally, if antibiotics can be designed that specifically counteract quorum sensing, these fundamental quorum-sensing studies could prove to have enormous practical application.

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Prokaryotes (2006) 2:354–420
DOI: 10.1007/0-387-30742-7_13

CHAPTER 1.13

Acetogenic Prokaryotes

HAROLD L. DRAKE, KIRSTEN KÜSEL AND CAROLA MATTHIES

Introduction to Acetogenic Bacteria and the Process of Acetogenesis

This chapter presents an overview of the history, taxonomy, phylogenetics, biochemistry, physiology, ecology, and applied aspects of acetogens. Acetogenic prokaryotes have only been found in the domain Bacteria. These prokaryotes utilize a reductive one-carbon pathway for the synthesis of acetyl-CoA, a metabolic precursor of both acetate and biomass. This pathway fixes CO_2 and is termed "the acetyl-CoA pathway." This pathway is often referred to as "the Wood-Ljungdahl pathway" in recognition of the two individuals, Harland G. Wood and Lars G. Ljungdahl, who were responsible for elucidating most of its enzymological features from the model acetogen *Moorella thermoacetica* (Fig. 1; see the section on Historical Perspectives in this Chapter). Acetogenesis (i.e., the process by which acetogens synthesize acetate) is often regarded as a fermentation process; however, as outlined in the subsection on CO_2 as Terminal Electron Acceptor and the Concept of Fermentation, acetogenesis is very dissimilar to classic fermentations. Purinolytic bacteria that synthesize acetate via the glycine pathway will not be considered in this chapter. However, certain features of this CO_2-fixing, glycine reductase-dependent pathway are similar to those of the acetyl-CoA pathway, and the reader is directed to the review of Andreesen (1994) for a detailed assessment of this pathway and organisms that use it.

Acetogens Defined

Usage of the term "acetogen" has not been consistent in the literature, and this inconsistent usage has caused a small amount of confusion regarding which organisms utilize the acetyl-CoA pathway for the synthesis of acetate. The following definition for the term acetogen has been previously proposed (Drake, 1994) and is applied in this chapter:

> Acetogen: an anaerobe that can use the acetyl-CoA pathway as a 1) mechanism for the reductive synthesis of acetyl-CoA from CO2, 2) terminal electron-accepting, energy-conserving process, and 3) mechanism for the fixation (assimilation) of CO2 in the synthesis of cell carbon.

Per this definition, the formation of acetate as an end product is unimportant, i.e., the fate of acetyl-CoA is less important than the process by which it is formed. For example, *Eubacterium limosum*, "*Butyribacterium methylotrophicum*" and *Caloramator pfennigii* (formerly *Clostridium pfennigii*), organisms that qualify as acetogens per the above definition, form butyrate from the acetyl-CoA that is formed via the acetyl-CoA pathway (Lynd and Zeikus, 1983; Zeikus, 1983; Krumholz and Bryant, 1985; Zeikus et al., 1985; Loubiere et al., 1992). Likewise, the acetogen *Acetobacterium woodii* forms ethanol from acetyl-CoA under certain conditions (Buschhorn et al., 1989).

The term "acetogenic" is an adjective that could be used to describe any organism that makes acetate or acetic acid. However, the metabolic processes by which acetate can be formed during either the aerobic or anaerobic growth of diverse microorganisms might not be equivalent. The mechanism by which acetate is formed via the oxidation of ethanol by *Acetobacter aceti* is fundamentally different from that used by certain obligate anaerobes that synthesize acetate from CO_2 via the reductive acetyl-CoA pathway. Thus, it is important that a differential nomenclature be applied to distinguish between acetate-forming bacteria because failure to do so results in unnecessary confusion in the literature. For example, *Thermobacteroides proteolyticus* and the syntroph PA-1 have been referred to as acetogens because they form acetate from glucose (Ollivier et al., 1985b; Brulla and Bryant, 1989). However, these organisms use protons, not CO_2, as terminal electron acceptors and form H_2, not acetate, as their main reduced end product; in short, they do not appear to use the acetyl-CoA pathway for the synthesis of acetate. Likewise, the butyrate-degrading syntroph *Syntrophomonas wolfei* has been described as an

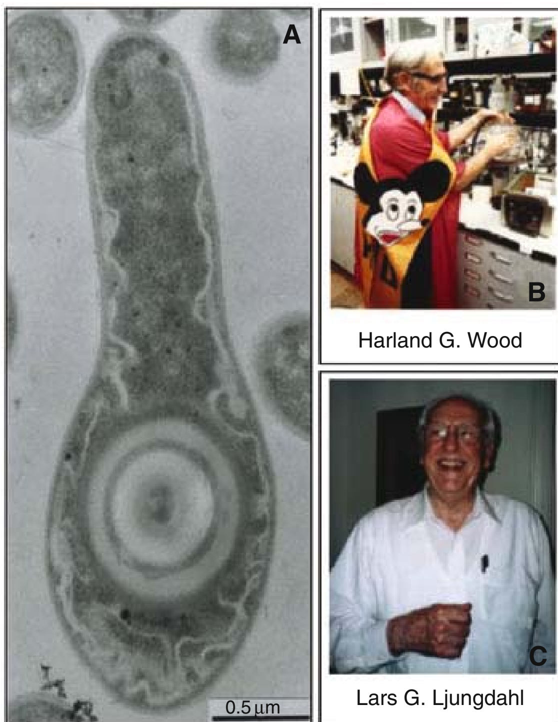


Fig. 1. A. Electron micrograph of a sporulated cell of *Clostridium thermoaceticum*, which was reclassified as *Moorella thermoacetica* (Collins et al., 1994). From Drake (1994), used with permission from Kluwer Academic. B, C. The two biochemists who were primarily responsible for resolving the enzymological features of the acetyl-CoA "Wood-Ljungdahl" pathway in *M. thermoacetica*. From Drake and Daniel (2004), used with permission from Elsevier. The dates of the photos for B and C are September 1977 (taken after Harland Wood's 70th birthday celebration/ symposium at Case Western Reserve University, during which Wood was "roasted" and given the honorary degree of Doctor of Mouse Science [the image on the hood symbolizes the shape of transcarboxylase as seen by electron microscopy]) and May 2000 (taken during the symposium honoring Harry D. Peck, Jr., at The University of Georgia), respectively.

acetogen (Stams and Doug, 1995). However, this organism 1) converts butyrate to acetate and H_2 (which can subsequently be used to reduce CO_2 to formate) by β-oxidation via the crotonyl-CoA pathway (Wofford et al., 1986), 2) does not reduce CO_2 to acetate, and 3) is not known to utilized the acetyl-CoA pathway.

Usage of the Terms "Acetogenesis," "Homoacetogen," and "Homoacetogenesis"

The term "acetogenesis" could be used to describe the process by which any organism forms acetate. For example, the term acetogenesis has been used to describe the 1) oxygen-dependent process by which *Enterococcus* RfL6 oxidizes lactate to acetate (Tholen et al., 1997) and 2) the production of acetate during proteolysis by *Treponema denticola* (Mikx, 1997). No evidence suggests that these organisms utilize the acetyl-CoA pathway for acetate synthesis. Since such usage makes it difficult to understand what process is being referred to, it has been suggested that usage of the term acetogenesis be restricted to processes by which two molecules of CO_2 are used to form one molecule of acetate (Wood and Ljungdahl, 1991b). Unfortunately such usage fails to adequately distinguish between the three known metabolic processes by which acetate is formed from CO_2: 1) the acetyl-CoA pathway, 2) the glycine synthase-dependent pathway, and 3) the reductive citric acid cycle (Fuchs, 1986; Fuchs, 1989; Thauer, 1988; Wood and Ljungdahl, 1991b).

The term "homoacetogen" is often used to distinguish between organisms that use the acetyl-CoA pathway and those that do not (Schink and Bomar, 1992). This term implies that acetate is the sole product formed by a particular organism. However, organisms that use the acetyl-CoA pathway usually do not form acetate as their sole end product. Their capacity to form any particular end product, including acetate, is dependent upon cultivation conditions. Butyrate (Lynd and Zeikus, 1983; Krumholz and Bryant, 1985; Worden et al., 1989; Grethlein et al., 1991), ethanol (Buschhorn et al., 1989), lactate (Lorowitz and Bryant, 1984; Drake, 1993; Misoph and Drake, 1996a), succinate (Dorn et al., 1978; Lorowitz and Bryant, 1984; Matthies et al., 1993; Misoph and Drake, 1996a), reduced aromatic acrylates (Tschech and Pfennig, 1984; Parekh et al., 1992; Misoph et al., 1996b), reduced aromatic aldehydes (Lux et al., 1990), CO (Diekert et al., 1986), H_2 (Martin et al., 1983; Lorowitz and Bryant, 1984; Savage et al., 1987), CH_4 (Savage et al., 1987; Buschhorn et al., 1989), sulfide (Heijthuijsen and Hansen, 1989; Beaty and Ljungdahl, 1991), dimethylsulfide (Beaty and Ljungdahl, 1991), nitrite (Seifritz et al., 1993; Fröstl et al., 1996), and ammonium (Seifritz et al., 1993; Seifritz et al., 2003; Fröstl et al., 1996) are examples of reduced end products of so-called "homoacetogens." Indeed, the production of such products can constitute the sole energy-conserving, growth-supportive process of the cell (see the subsection on Use of Diverse Terminal Electron Acceptors in this Chapter). Thus, the conditions under which an acetogen forms acetate should be qualified, rather than merely referring to the organism as a homoacetogen. For example, *Ruminococcus productus* (formerly *Peptostreptococcus productus*) is homoacetogenic on pyruvate but forms acetate, lactate, succinate, and formate when cultivated on fructose; this acetogen can also form large amounts of ethanol during glycerol-dependent growth

(Misoph and Drake, 1996a). Likewise, *Moorella thermoacetica* is homoacetogenic when cultivated on H_2/CO_2, but does not form acetate when cultivated on H_2/CO_2 in the presence of nitrate; under this condition, the dissimilation of nitrate is used preferentially to acetogenesis for the conservation of energy (Fröstl et al., 1996). Lastly, the term "homoacetate production" has been used to describe the process by which a genetically modified strain of *Escherichia coli* anaerobically produces 2 moles of acetate per mole glucose fermented (Causey et al., 2003), yet this process is not homoacetogenic (i.e., does not yield 3 moles of acetate per mole glucose) and the acetyl-CoA pathway is not involved.

Independent of these problems of usage, the production of acetate as the sole end product from certain sugars, H_2/CO_2, or CO/CO_2 strongly suggests that the organism in question utilizes the acetyl-CoA pathway per the definition for the term acetogen (see the subsection on Acetogens Defined in this Chapter).

Global Impact and Evolutionary Perspectives

Acetogens were initially viewed as obscure, poorly defined microorganisms. For nearly five decades following the discovery of acetogens in the 1930s, the major interest in them was restricted to resolving the biochemical features of the acetyl-CoA pathway (see the section on Historical Perspectives in this Chapter). The microbiology of acetogens drew little interest until the 1980s when it started to become apparent that acetogens were a widely distributed, phylogenetically diverse group of microorganisms. Added interest in the acetyl-CoA pathway occurred when it was discovered that methanogens and sulfate-reducing bacteria used metabolic pathways that contained acetyl-CoA synthase, one of the key enzymes in the acetyl-CoA pathway (Fuchs, 1986; Fuchs, 1989; Schauder et al., 1986; Thauer et al., 1989; see the subsection on Occurrence of the Acetyl-CoA Pathway in Nonacetogenic Microorganisms in this Chapter). Major bacterial groups employing this pathway in either the direction of acetate/biomass synthesis or acetate degradation include acetogens, methanogens, and sulfate-reducing bacteria.

It is not possible to determine how much carbon is processed globally via acetogens and pathways that make use of acetyl-CoA synthase. However, several facts are noteworthy:

1) The Calvin cycle, the reductive tricarboxylic acid cycle, the hydroxypropionate cycle, and the acetyl-CoA Wood-Ljungdahl pathway facilitate the complete autotrophic fixation of CO_2. Of these pathways, the one-carbon acetyl-CoA pathway is biochemically the most simple. For example, the acetyl-CoA pathway requires less ATP to fix a molecule CO_2 than does the Calvin cycle. Furthermore, the acetyl-CoA pathway is a linear process that does not depend on preformed, complex molecules to which CO_2 is fixed in a cyclic process (e.g., the Calvin cycle, the reductive tricarboxylic acid cycle, the hydroxypropionate cycle are dependent upon ribulose biphosphate, oxalacetate, and acetyl-CoA, respectively, for the fixation of CO_2) (see section on The Acetyl-CoA Pathway and Bioenergetics in this Chapter). Methanogens utilize an acetyl-CoA synthase-dependent pathway that is biochemically very similar to the acetyl-CoA pathway utilized by acetogenic bacteria (see the subsection on Occurrence of the Acetyl-CoA Pathway in Nonacetogneic Microorganisms in this Chapter), and methanogens (or ancestors of methanogens) may have been the first autotrophs (Schopf et al., 1983; Brock, 1989). Thus, and since life originated under anoxic conditions, the acetyl-CoA pathway or a pathway closely related to it may have been the first process used for the autotrophic fixation of CO_2 (Fuchs, 1986; Wood and Ljungdahl, 1991b; Lindahl and Chang, 2001).

2) Approximately half of the human population contain low numbers of methanogens in their gastrointestinal systems and produce relatively little CH_4; the colon of these individuals, as well as those who more actively emit CH_4, are heavily colonized by acetogens (Wolin and Miller, 1983). Indeed, the gastrointestinal systems of mammals, whether they harbor methanogens or not, are heavily colonized with acetogens (Prins and Lankhorst, 1977; Breznak and Kane, 1990; Mackie and Bryant, 1994; Wolin and Miller, 1994; Leedle et al., 1995).

3) Acetogens inhabit the human colon. In this habitat, acetogens produce 10^{10} kg of acetate per year from H_2-CO_2, and acetogenesis is one of the dominant processes in the overall metabolism of carbohydrate in the human colon (Lajoie et al., 1988; Wolin and Miller, 1994; Doréet al., 1995; Bernalier et al., 1996a; Bernalier et al., 1996b; Miller and Wolin, 1996; Wolin et al., 1999).

4) Totally, 10^{12} kg of acetate are produced each year via the reduction of CO_2 by acetogens in the hindgut of termites, a number that is fivefold greater than the annual amount of methane formed globally via the biogenic reduction of CO_2 (Breznak and Kane, 1990). One-third of the energy requirements of the termite is provided by the acetate that is synthesized by the reduction of CO_2 by gut acetogens (Breznak, 1994).

5) Totally, 10^{13} kg of acetate are formed and further metabolized annually in terrestrial habitats such as soils and sediments, and a minimum of 10% of this acetate is likely formed by the reduction of CO_2 via the acetyl-CoA pathway (Wood and Ljungdahl, 1991b).

6) Up to 25% of the total organic carbon of soil can be turned over through acetate under low temperature, anoxic conditions (equivalent to nearly 40 g acetate per kg dry wt. of soil; Küsel and Drake, 1994). The capacity to form acetate in soils is concomitant with acetogenic activities and the occurrence of H_2-utilizing acetogens (Küsel and Drake, 1995; Wagner et al., 1996; Küsel et al., 1999c). Acetate is a dominant organic compound in soil solution (Tani et al., 1993), and concentrations can be in the mM range following a rainfall event (Küsel and Drake, 1999a). Assuming a weight of 10^{17} kg for the first meter of the global terrestrial surface (based on a surface area of 10^{14} m^2 [Whitman et al., 1998] and using a weight conversion of 10^3 kg per m^3) and an acetate concentration of 0.1 mmol per kg of this material, it can be estimated that 10^{12} kg of acetate is present in the first meter of the terrestrial surface at any one moment (i.e., per "snapshot"). Even if only a small percentage of this acetate were formed by acetogens, given the turnover dynamics of acetate, the annual magnitude of the acetogen-derived acetate in the terrestrial biosphere would be enormous. The number of prokaryotes in the terrestrial subsurface might exceed that of the terrestrial surface by a factor of ten (Whitman et al., 1998). It can be projected that acetate and acetogens are also involved in the cycling of carbon in this poorly explored compartment of the terrestrial ecosphere (see the subsection on Diverse Habitats in this Chapter).

7) The acetate formed by acetogenesis is an essential trophic link during the turnover of carbon in diverse anoxic habitats (McInerney and Bryant, 1981).

Such observations not only illustrate that nature's ability to form acetate is enormous, they also demonstrate that the acetyl-CoA Wood/Ljungdahl pathway is fundamental to the carbon cycle of earth.

Historical Perspectives

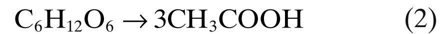
Discovery of Acetogenic Bacteria and Acetogenesis

Acetogenesis was first reported in 1932, when unknown organisms in sewage were shown to catalyze the H_2-dependent reduction of CO_2 to acetate (Fischer et al., 1932). Shortly thereafter, the Dutch microbiologist Wieringa reported the isolation of the first acetogen (Wieringa, 1936; Wieringa, 1939–40; Wieringa, 1941). The organism, *Clostridium aceticum*, was a sporeforming, mesophilic rod, and grew at the expense of the following reaction:

$$4H_2 + 2CO_2 \rightarrow CH_2COOH + 2H_2O \quad (1)$$

Such a reaction had not been observed earlier. With the exception of a small study on the nutritional requirements of *C. aceticum* (Karlsson et al., 1948), no further work was published with this acetogen until it was reisolated in 1980–1981 (Adamse, 1980; Braun et al., 1981; Gottschalk and Braun, 1981; Fig. 2).

Clostridium thermoaceticum was discovered a few years after the isolation of *C. aceticum* (Fontaine et al., 1942) and was the only acetogen available for laboratory study for several decades (Fig. 1). This bacterium was reclassified as *Moorella thermoacetica* (Collins et al., 1994) and will be referred to by this name hereafter. *Moorella thermoacetica* was isolated as an obligate heterotroph and was observed to convert glucose to acetate; the stoichiometry of this process approximated the following reaction:



$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (2)$$

In the early 1940s, no known metabolic process could explain this reaction, and it was proposed that the CO_2 produced via oxidation was subsequently utilized in the synthesis of acetate:

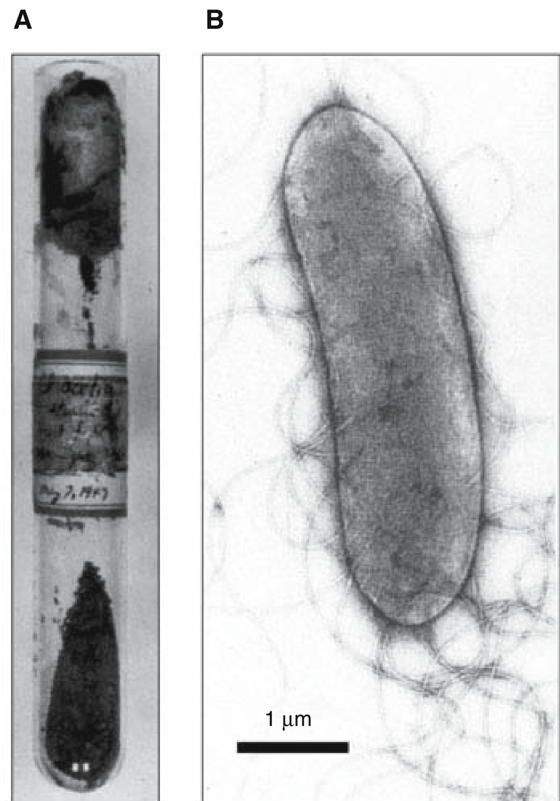
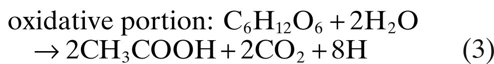


Fig. 2. A. Tube containing dried soil and spores of the first acetogen to be isolated, *Clostridium aceticum*. The tube was obtained from H. A. Barker; the date on the tube is May 7, 1947. B. Electron micrograph of a peritrichously flagellated cell of *C. aceticum*. From Braun et al. (1981), used with permission from Springer. The photograph (panel A) was kindly provided by G. Gottschalk.

> Since, in this fermentation, 2.5 moles of a two-carbon compound (acetic acid) are obtained from 1 mole of glucose, it seems probable that either there is some primary cleavage of glucose other than the classical 3-3 split, or that a one-carbon compound is being reabsorbed. Of these two possibilities, the recent work on carbon dioxide uptake makes the latter seem more likely (Fontaine et al., 1942).

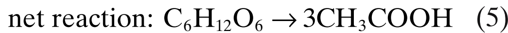
The latter statement was in reference to the discovery of CO_2 fixation in heterotrophs (Wood and Werkman, 1936; Wood and Werkman, 1938; Wood et al., 1941a; Wood et al., 1941b). Subsequent proposals for the acetogenic conversion of glucose or pyruvate to acetate made it possible to see that both the autotrophic and heterotrophic acetogenic processes likely involved the reductive synthesis of acetate from CO_2 (Barker, 1944).

Conversion of glucose to acetate:



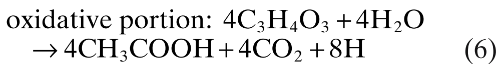
$$\text{oxidative portion: } C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H \quad (3)$$

$$\text{reductive portion: } 8H + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (4)$$

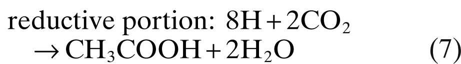


$$\text{net reaction: } C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (5)$$

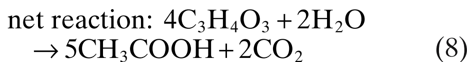
Conversion of pyruvate to acetate:



$$\text{oxidative portion: } 4C_3H_4O_3 + 4H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H \quad (6)$$



$$\text{reductive portion: } 8H + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (7)$$



$$\text{net reaction: } 4C_3H_4O_3 + 2H_2O \rightarrow 5CH_3COOH + 2CO_2 \quad (8)$$

The overlap between reactions 1, 4, and 7 indicated that a unique reductive process was likely responsible for acetate synthesis from CO_2.

Resolution of the Acetyl-CoA "Wood/Ljungdahl" Pathway

Barker and Kamen (1945) demonstrated in the first published biological experiments with ^{14}C (Kamen, 1963) that *M. thermoacetica* incorporated $^{14}CO_2$ equally into both carbon atoms of acetate. This landmark experiment with ^{14}C demonstrated that the capacity of *M. thermoacetica* to synthesize acetate from glucose was, in fact, similar to the capacity of *C. aceticum* to synthesize acetate from H_2/CO_2:

> *It may be concluded that the acetic acid fermentation of glucose by C. thermoaceticum involves a partial oxidation of the substrate to two moles each of acetic acid and carbon dioxide followed by a reduction and condensation of the carbon dioxide to a third mole of acetic acid* (Barker and Kamen, 1945).

In 1952, Wood repeated the ^{14}C-experiments of Barker and Kamen with $^{13}CO_2$ and confirmed that *M. thermoacetica* synthesized acetate from two molecules of CO_2 (Wood, 1952a). In this work, mass spectrometry conclusively demonstrated that CO_2 was uniformly fixed into both the carboxyl and methyl carbons of the third molecule of acetate from glucose. Utilizing [3,4-^{14}C]-glucose, it was also shown that carbons 3 and 4 of glucose were converted to CO_2 (Wood, 1952b). These early studies by Barker, Kamen and Wood demonstrated that 1) glucose was subject to a classic 3-3 split between carbons 3 and 4, and 2) CO_2 was fixed via an unknown CO_2-fixing process into acetate (Fig. 3).

It took decades of continued research before the enzymology of this CO_2-fixing process was fully resolved (see the section on The Acetyl-CoA Pathway and Bioenergetics in this Chapter). It is an irony of the history of acetogenesis that the model organism (i.e., *M. thermoacetica*)

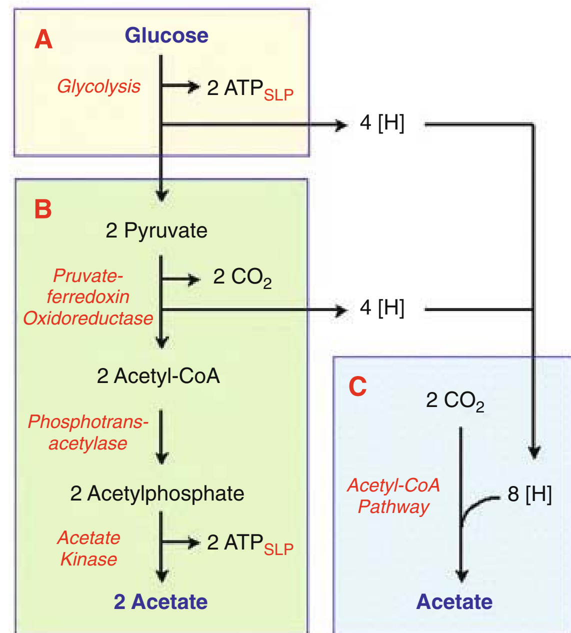


Fig. 3. Homoacetogenic conversion of glucose to acetate. Glucose is first converted to two molecules of pyruvate via glycolysis (Box A); glycolysis yields ATP by substrate-level phosphorylation (SLP). Pyruvate is then oxidized and decarboxylated, yielding acetyl-CoA, CO_2, and reducing equivalents (Box B). The two acetyl-CoA molecules that are produced from pyruvate are converted to two molecules of acetate; this process yields additional ATP by SLP. The 8 reducing equivalents that are produced via glycolysis and pyruvate-ferredoxin oxidoreductase are utilized in the acetyl-CoA pathway to reduce two molecules of CO_2 to an additional molecule of acetate (Box C). The CO_2 that is reduced in the acetyl-CoA pathway is likely derived primarily from supplemental CO_2 rather than the CO_2 derived via the decarboxylation of pyruvate. Modified from Drake (1994).

used to resolve the biochemistry of this autotrophic process was thought to be an obligate heterotroph during these decades of research. Indeed, the chemolithoautotrophic nature of *M. thermoacetica* (Daniel et al., 1990) was resolved nearly five decades after its isolation and well after the enzymological details of the acetyl-CoA pathway were firmly established. The milestones of the numerous studies that resolved both the enzymology of the acetyl-CoA pathway and the chemolithoautotrophic abilities of the model acetogen used in these studies can be found in numerous review articles (Ljungdahl and Wood, 1969; Wood, 1972; Wood, 1976; Wood, 1982; Wood, 1985; Wood, 1989; Wood, 1991a; Ljungdahl, 1986; Wood and Ljungdahl, 1991b; Drake, 1992; Drake, 1994; Ragsdale, 1991; Ragsdale, 1994; Ragsdale, 1997) and are outlined in Table 1. For additional insights into the early career years of Harland G. Wood, see the recent excellent historical treatments by Singleton (Singleton, 1997a; Singleton, 1997b; Singleton, 2000).

Isolates to Date and Microbiological Methods

The number of known acetogens has increased significantly in the last two decades, and approximately 100 different species have been isolated to date from extremely diverse habitats. Acetogens can be found in almost all anoxic environments, including some extreme habitats, as indicated by the isolation of strain SS1 (Liu and Suflita, 1993) and "*Acetobacterium psammolithicum*" from deep subsurface sediment and sandstone, respectively (Krumholz et al., 1999). Although most isolates to date are mesophilic, thermophilic and psychrotolerant species have also been isolated. The occurrence and ecological roles of acetogens in various habitats is discussed in the section on acetogen ecology (see the section on Ecology of Acetogens in this Chapter).

Bacteria considered to be acetogens as defined above (see the subsection on Acetogens Defined in this Chapter) are listed in Table 2.

Table 1. Milestones that led to resolving the acetyl-CoA pathway and chemolithoautotrophic abilities of *Moorella thermoacetica*.

Year	Event*
1932	H_2-dependent conversion of CO_2 to acetate in sewage sludge (Fischer et al., 1932)
1936	Isolation of the first acetogen, *Clotridium aceticum*; total synthesis of acetate from H_2-CO_2 (Note: culture was lost, Wieringa, 1936, 1939–40)
1942	Discovery of the second acetogen, *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*); conversion of one glucose to three acetate molecules (Fontaine et al., 1942)
1944	Acetogenic conversion of pyruvate to acetate (Barker, 1944)
1945–52	Synthesis of acetate from $^{14}CO_2$ (Barker and Kamen, 1945) or $^{13}CO_2$ (Wood, 1952a, b)
1955	Formate as a methyl-group precursor (Lentz and Wood, 1955)
1964	Methylcobalamin as methyl-group precursor (Poston et al., 1964)
1965	Autotrophic synthesis of cell-carbon precursors from CO_2 (Ljungdahl and Wood, 1965)
1966–69	Proposal of one-carbon pathway for the tetrahydrofolate/corrinoid-mediated synthesis of acetate from CO_2 (Ljungdahl and Wood, 1966, 1969)
1973–86	Resolution of the tetrahydrofolate pathway (reviewed in Ljungdahl [1986])
1978–80	Discovery of CO dehydrogenase as a nickel-containing enzyme (Diekert and Thauer, 1978; Drake et al., 1980)
1981	Resolution of enzymes required for synthesis of acetyl-CoA from pyruvate and methyltetrahydrofolate (Drake et al., 1981a)
1981–82	Demonstration that CO replaces the carboxyl-group of pyruvate and undergoes an exchange reaction with acetyl-CoA (Drake et al., 1981b; Hu et al., 1982)
1982	Discovery of hydrogenase (Drake, 1982)
1983	Purification of CO dehydrogenase (Diekert and Ritter, 1983; Ragsdale et al., 1983)
1983	Use of H_2 and CO under organotrophic conditions (Kerby and Zeikus, 1983)
1984	Resolution of nutritional requirements (Lundie and Drake, 1984)
1984	Enzyme system for H_2-dependent synthesis of acetyl-CoA (Pezacka and Wood, 1984b)
1984–86	CO dehydrogenase is acetyl-CoA synthase (Pezacka and Wood, 1984a, b; Regsdale and Wood, 1985) and CO is the carbonyl precursor in the acetyl-CoA pathway under growth conditions (Diekert et al., 1984; Martin et al., 1985)
1985–91	Catalytic mechanism of acetyl-CoA synthase (reviewed in Ragsdale [1991])
1986–90	H_2- and CO-dependent electron transport system coupled to the synthesis of ATP (Ivey and Ljungdahl, 1986; Hugenholtz and Ljungdahl, 1989, 1990; Das et al., 1989)
1990	Chemolithoautotrophic growth on H_2-CO_2 and CO-CO_2 (Daniel et al., 1990)
1991	Integrated model for catabolic, anabolic, and bioenergetic features of the acetyl-CoA "Wood-Ljungdahl" pathway (Wood and Ljungdahl, 1991)

*Events prior to the isolation of *Moorella thermoacetica*.
Modified from Drake 1994.

Table 2. Acetogenic bacteria isolated to date.[a]

Acetogen	Source of isolate	Gram type[b]	Cell morphology	Growth temperature[c]	G+C (mol%)	Deposited as	References
Acetoanaerobium ruminis	Rumen fluid, steer	+	Rod	Mesophilic	34	ATCC 43876[I]	Greening and Leedle, 1989
Acetoanaerobium noterae	Sediment	–	Rod	Mesophilic	37	ATCC 35199[I]	Sleat et al., 1985
"Acetoanaerobium romashkovii"	Oil field	+	Rod	Mesophilic	40	n.d.	Davydova-Charakhch'yan et al., 1992
Acetobacterium baloï	Wastewater sediment	+	Rod	Psychrotrophic	42	DSM 8239[I]	Kotsyurbenko et al., 1995
Acetobacterium carbinolicum	Freshwater sediment	+	Rod	Mesophilic	38	DSM 2925[I]	Eichler and Schink, 1984
"Acetobacterium dehalogenans"	Sewage digester sludge	+	Coccus	Mesophilic	48	DSM 11527	Traunecker et al., 1991
Acetobacterium fimetarium	Digested cattle manure	+	Rod	Psychrotrophic	46	DSM 8238[I]	Kotsyurbenko et al., 1995
Acetobacterium malicum	Freshwater sediment	+	Rod	Mesophilic	44	DSM 4132[I]	Tanaka and Pfennig, 1988
Acetobacterium paludosum	Fen sediment	+	Rod	Psychrotrophic	42	DSM 8237[I]	Kotsyurbenko et al., 1995
"Acetobacterium psammolithicum"	Subsurface sandstone	–	Rod	Mesophilic	n.r.	SMCC/W 751[I]	Krumholz et al., 1999
Acetobacterium tundrae	Tundra soil	+	Rod	Psychrotrophic	39	DSM 9173[I]	Simankova et al., 2000
Acetobacterium wieringae	Sewage digester	+	Rod	Mesophilic	43	DSM 1911[I]	Braun and Gottschalk, 1982
Acetobacterium woodii	Marine sediment	+	Rod	Mesophilic	39	ATCC 29683[I]	Balch et al., 1977
Acetobacterium sp. AmMan1	Freshwater sediment	+	Rod	Mesophilic	36	n.d.	Dörner and Schink, 1991
Acetobacterium sp. B10	Wastewater pond	+	Rod	Mesophilic	n.r.	n.d.	Sembining and Winter, 1989, 1990
Acetobacterium sp. HA1	Sewage sludge	+	Rod	Mesophilic	n.r.	n.d.	Schramm and Schink, 1991
Acetobacterium sp. HP4	Lake sediment	+	Rod	Psychrotrophic	n.r.	n.d.	Conrad et al., 1989
Acetobacterium sp. KoB58	Sewage sludge	+	Rod	Mesophilic	44	n.d.	Wagener and Schink, 1988
Acetobacterium sp. LuPhet1	Sewage sludge	+	Rod	Mesophilic	44	DSM 9077	Frings and Schink, 1994
Acetobacterium sp. LuTria3	Sewage sludge	+	Rod	Mesophilic	35	DSM 8909	Frings et al., 1994
Acetobacterium sp. MrTac1	Marine sediment	+	Rod	Mesophilic	n.r.	n.d.	Emde and Schink, 1987
Acetobacterium sp. OyTac1	Freshwater sediment	+	Rod	Mesophilic	n.r.	n.d.	Emde and Schink, 1987
Acetobacterium sp. RMMac1	Marine sediment	–	Rod	Mesophilic	48	n.d.	Schuppert and Schink, 1990
Acetobacterium sp. 69	Sea sediment	+	Rod	Mesophilic	48	n.d.	Inoue et al., 1992
Acetobacterium sp.	Tundra wetland soil	+	Rod	Psychrotrophic	39	n.d.	Kotsyurbenko et al., 1996
Acetohalobium arabaticum	Saline lagoon	–	Rod	Mesophilic	34	DSM 5501[I]	Zhilina and Zavarzin, 1990
Acetonema longum	Wood-eating termite, gut	–	Rod	Mesophilic	52	DSM 6540[I]	Kane and Breznak, 1991
"Bryantella formatexigens"	Human feces	+	Rod	Mesophilic	50	DSM 14469[I]	Wolin et al., 2003
"Butyribacterium methylotrophicum"	Sewage digester	+	Rod	Mesophilic	49	ATCC 33266[I]	Zeikus et al., 1980
Caloramator fervidus (?)	Hot spring	–	Rod	Thermophilic	39	ATCC 43204[I]	Patel et al., 1987

Clostridium aceticum	Soil	–	Rod	Mesophilic	33	DSM 1496[T]	Wieringa, 1936
							Braun et al., 1981[d]
"Clostridium autoethanogenum" (?)	Rabbit feces	+	Rod	Mesophilic	26	DSM 10061	Abrini et al., 1994
Clostridium coccoides	Mouse feces, human feces	+	Coccoid rod	n.r.	46	DSM 935[T]	Kaneuchi et al., 1976
							Kamlage et al., 1997
Clostridium difficile AA1	Rumen, newborn lamb	+	Rod	Mesophilic	31	DSM 12056	Rieu-Lesme et al., 1998
Clostridium formicaceticum	Sewage	–	Rod	Mesophilic	34	DSM 92[T]	Andreesen et al., 1970[e]
Clostridium glycolicum 22	Sewage	+	Rod	Mesophilic	n.r.	ATCC 29797 (22)	Ohwaki and Hungale, 1977
Clostridium glycolicum RD-1	Seagrass roots	+	Rod	Mesophilic	32	DSM 13865 (RD-1)	Küsel et al., 2001
Clostridium ljungdahlii	Chicken waste	+	Rod	Mesophilic	22	ATCC 49587[T]	Barik et al., 1988
							Tarmer et al., 1993
Clostridium magnum	Freshwater sediment	–	Rod	Mesophilic	29	DSM 2767[T]	Schink, 1984
Clostridium mayombei	Soil-feeding termite, gut	+	Rod	Mesophilic	26	DSM 6539[T]	Kane et al., 1991
Clostridium methoxybenzovorans	Olive oil mill wastewater	+	Rod	Mesophilic	44	DSM 12182[T]	Mechichi et al., 1999
Clostridium scatologenes	Soil, coal mine pond sediment	+	Rod	Mesophilic	30	DSM 757[T]	Weinberg and Ginsbourg, 1927
						DSM 12750 (SL1)	Küsel et al., 2000
Clostridium ultunense	Swine manure digester	+	Rod	Mesophilic	32	DSM 10521[T]	Schnurer et al., 1996
Clostridium sp. CV-AA1	Sewage sludge	–	Rod	Mesophilic	42	n.d.	Adamse and Velzeboer, 1982
Clostridium sp. M5a3	Human feces	+	Rod	n.r.	n.r.	n.d.	Bernalier et al., 1996a
							Leclerc et al., 1997a, b
Clostridium sp. F5a15	Human feces	+	Rod	n.r.	n.r.	n.d.	Bernalier et al., 1996a
							Leclerc et al., 1997a, b
Clostridium sp. Ag4f2	Human feces	+	Rod	n.r.	n.r.	n.d.	Bernalier et al., 1996a
Clostridium sp. TLN2	Human feces	+	Coccobacillus	n.r.	n.r.	n.d.	Bernalier et al., 1996a
Eubacterium aggregans	Olive oil mill wastewater	+	Rod	Mesophilic	55	DSM 12183[T]	Mechichi et al., 1998
Eubacterium limosum	Rumen fluid, sheep	+	Rod	Mesophilic	48	ATCC 8486[T]	Sharak-Genthner et al., 1981[r]
Holophaga foetida	Freshwater ditch mud	–	Rod	Mesophilic	62	DSM 6591[T]	Bak et al., 1992
							Liesack et al., 1994
Moorella glycerini	Hot spring sediment	+	Rod	Thermophilic	54	DSM 11254[T]	Slobodkin et al., 1997
"Moorella mulderi"	Bioreactor	+	Rod	Thermophilic	53	DSM 14980[T]	Balk et al., 2003
Moorella thermoacetica	Horse manure	+/–	Rod	Thermophilic	54	ATCC 35608[T]	Fontaine et al., 1942
Moorella thermoautotrophica	Hot spring	+/–	Rod	Thermophilic	54	ATCC 33924[T]	Wiegel et al., 1981
Moorella sp. F21[g]	Soil	+	Rod	Thermophilic	n.r.	n.d.	Karita et al., 2003

(*Continued*)

Table 2. *Continued*

Acetogen	Source of isolate	Gram type[b]	Cell morphology	Growth temperature[c]	G+C (mol%)	Deposited as	References
Natroniella acengena	Soda lake deposits	–	Rod	Mesophilic	32	DSM 9952[I]	Zhilina et al., 1996
Natronincola histidinovorans	Soda lake deposits	+	rod	Mesophilic	32	DSM 11416[I]	Zhilina et al., 1998
Oxobacter pfennigii	Rumen fluid, steer	+	Rod	Mesophilic	38	DSM 3222[I]	Krumholz and Bryant, 1985
Ruminococcus hydrogenotrophicus	Human feces	+	Coccobacillus	Mesophilic	45	DSM 10507[I]	Bernalier et al., 1996b
Ruminococcus productus U1	Sewage digester	+	Coccus	Mesophilic	45	ATCC 35244	Lorowilz and Bryant, 1984
Ruminococcus productus Marburg	Sewage digester	+	Coccus	Mesophilic	46	ATCC 43917	Geerligs et al., 1987
Ruminococcus schinkii	Rumen, 3-day-old lamb	+	Coccoid rod	Mesophilic	46	DSM 10518[I]	Rieu-Lesme et al., 1996b
Ruminococcus sp. TLF1	Human feces	+	Coccobacillus	n.r.	n.r.	n.d.	Bernalier et al., 1996a
Sporomusa acidovorans	Distillation waste water	–	Rod	Mesophilic	42	DSM 3132[I]	Ollivier et al., 1985a
Sporomusa aerivorans	Soil-eating termite, gut	–	Rod	Mesophilic	n.r.	DSM 13326[I]	Boga et al., 2003
Sporomusa malonica	Freshwater sediment	–	Rod	Mesophilic	44	DSM 5090[I]	Dehning et al., 1989
Sporomusa ovata	Silage	–	Rod	Mesophilic	42	DSM 2662[I]	Möller et al., 1984
Sporomusa paucivorans	Lake sediment	–	Rod	Mesophilic	47	DSM 3697[I]	Hermann et al., 1987
Sporomusa silvacetica	Beech forest soil	+	Rod	Mesophilic	43	DSM 10669[I]	Kubner et al., 1997
Sporomusa sphaeroides	River mud	–	Rod	Mesophilic	47	DSM 2875[I]	Möller et al., 1984
Sporomusa termitida	Wood-eating termite, gut	–	Rod	Mesophilic	49	DSM 4440[I]	Breznak et al., 1988
Sporomusa sp. DR6[h]	Rice field soil	+	Rod	n.r.	n.r.	n.d.	Rosencrantz et al., 1999
Sporomusa sp. DR1/8	Rice field soil	+	Rod	n.r.	n.r.	n.d.	Rosencrantz et al., 1999
Syntrophococcus sucromutans	Rumen fluid, steer	–	Coccus	Mesophilic	52	DSM 3224[I]	Krumholz and Bryant, 1986
Thermoacetogenium phaeum	Pulp waste water reactor	+	Rod	Thermophilic	54	DSM 12270[I]	Hattori et al., 2000
Thermoanaerobacter kivui	Lake sediment	–	Rod	Thermophilic	38	ATCC 33488[I]	Leigh et al., 1981
"*Treponemaprimitia*" sp. ZAS-2	Termite, hindgut	n.r.	Spirochete	Mesophilic	51	DSM 1247	Graber et al., 2004
Unclassified							
AG (?)	Granular reactor sludge	+	Rod	Thermophilic	n.r.	n.d.	Davidova and Stams, 1996
AOR	Thermophilic digester	+	Rod	Thermophilic	47	n.d.	Lee and Zinder, 1988
CS1 Van	Human feces	+	Rod	Mesophilic	n.r.	n.d.	Wolin and Miller, 1993
CS3Glu	Human feces	+	Coccoid rod	Mesophilic	n.r.	n.d.	Wolin and Miller, 1993
CS7H	Human feces	+	Rod	Mesophilic	n.r.	n.d.	Wolin and Miller, 1993
D	Rumen fluid, deer	–	Rod	Mesophilic	53	n.d.	Rieu-Lesme et al., 1995
DMG58	River mud	–	Rod	Mesophilic	43	DSM 3301	Möller et al., 1984
EE121	Granular reactor sludge	+	Rod	n.r.	n.r.	n.d.	Plugge et al., 1990

HA	Horse feces	–	Coccobacillus	n.r.	n.r.	n.d.	Miller and Wolin, 1995
I52	Human feces	–	Coccoid rod	Mesophilic	n.r.	n.d.	Wolin and Miller, 1994
S5a2[h]	Human feces	+	Coccus	n.r.	n.r.	n.d.	Bernalier et al., 1996a Leclerc et al., 1997a, b
Ser8	Rumen, newborn lamb	n.r.	n.r.	n.r.	n.r.	n.d.	Chaucheyras et al., 1995
SS1	406-m deep sediment	+	Oval rod	Mesophilic	n.r.	n.d.	Liu and Suflita, 1993
TH-001	Sewage sludge	–	Rod	Mesophilic	n.r.	n.d.	Frazer and Young, 1985
VK64[i]	Human feces	+	Coccus	n.r.	n.r.	n.d.	Bernalier et al., 1996a
X-8	Vegetable wastewater	–	Rod	Mesophilic	n.r.	n.d.	Samain et al., 1982
ZJ[j]	Tundra soil	+	Rod	Psychrophilic	n.r.	n.d.	Kotsyurbenko et al., 1992 Nozhevnikova et al., 1994
417/2	Oil field	–	Rod	Mesophilic	43	n.d.	Davydova-Charakhchyan et al., 1992
417/5	Oil field	–	Rod	Mesophilic	43	n.d.	Davydova-Charakhchyan et al., 1992
"New acetogenic bacterium"	Rumen, 15-hour-old lamb	+	Coccoid rod	Mesophilic	46	DSM 12568	Rieu-Lesme et al., 1996a

Symbols and Abbreviations: +, positive; –, negative; +/–, variable; n.d., not deposited; n.r., not reported; [T], type strain; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; SMCC, and Subsurface Microbial Culture Collection.

[a]Bacteria listed appear to use the acetyl-CoA pathway for the synthesis of acetate and growth (modified from Drake [1992, 1994]). If the acetogenic nature of an organism is uncertain, a question mark occurs after the name of the organism (see text). Organisms not having validated names are enclosed in quotation marks. Unless otherwise indicated, type strains (marked with a "T" adjacent to the deposition number) are available from American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia, U.S.A., and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) Mascheroder Weg 1b, Braunschweig, Germany.

[b]Gram type is based on electron microscopic analyses of the cell wall structure if reported. Otherwise, Gram type is based on the Gram-stain reaction. (Note: results of the Gram-stain reaction are not always in agreement with the electron microscopic analysis of the cell wall.)

[c]General temperature preference: psychrophilic (5–10°C), psychrotolerant (16–18°C), mesophilic (31–34°C), and thermophilic (58–62°C).

[d]See also Adamse (1980).

[e]See also E1 Ghazzawi (1967).

[f]See also Moore and Cato (1965).

[g]Isolate F21 is phylogenetically closely related to *M. thermoacetica* ET-5a, which was also isolated from soil (Gößner et al., 1999; Karita et al., 2003).

[h]*Sporomusa* sp. DR6 appears to be identical to *Sporomusa* sp. DR15 and *Sporomusa* sp. DR16, which were isolated in the same study (Rosencrantz et al., 1999).

[i]Strain may be a species of *Streptococcus* (Bernalier et al., 1996a).

[j]Strain may be a species of *Acetobacterium* (Nozhevnikova et al., 1994).

However, relatively few of these bacteria have been examined in detail, and a good understanding of the metabolic capabilities of most of the isolates is lacking. In compiling the list, the apparent acetogenic capability (see the subsection on Usage of the Terms "Acetogenesis," Homoacetogen" and "Homoacetogenesis" in this Chapter) of each organism has been taken into account. In this regard, three main metabolic features of these organisms (Drake, 1994) are 1) the use of chemolithoautotrophic substrates (H_2-CO_2 or CO-CO_2) as sole sources of carbon and energy under anoxic conditions, 2) the capacity to convert certain sugars stoichiometrically to acetate, and 3) the ability to *O*-demethylate methoxylated aromatic compounds and metabolize the *O*-methyl group via the acetyl-CoA pathway. Many acetogens display all three of these metabolic capabilities.

Most acetogenic isolates are rod-shaped, but coccoid forms have also been observed (Table 2). Staining properties vary, sometimes within a genus, and both Gram-negative and Gram-positive species have been reported (Table 2). Some acetogens have flagella and are motile. Some form spores that remain viable for long periods; the thermophilic spore-formers are fairly resistant to high temperatures. Indeed, spores of *M. thermoacetica* have a decimal reduction time (i.e., the time required to decrease the population of viable spores by 90%) of 111 min at 121°C (Byrer et al., 2000). Cells of the acetogen *Clostridium glycolicum* RD-1 are tethered by connecting filaments, a morphological structure recently described for *Clostridium akagii* and *Clostridium uliginosum* (Kuhner et al., 2000; Matthies et al., 2001). Thus, the ultrastructural features of acetogens are highly variable.

Description of Species

Acetogens have been assigned to 21 different genera and differ in their morphological, cytological and physiological properties (Table 2). The genera *Clostridium* and *Acetobacterium* harbor the most acetogenic species isolated to date. The first acetogen was classified as a clostridial species, *C. aceticum* (Wieringa, 1936). The second acetogenic genus *Acetobacterium* was established when the first Gram-positive, nonsporeforming acetogen (*Acetobacterium woodii*; Balch et al., 1977; Fig. 4) was isolated and could not be grouped with the acetogenic clostridia. Conspicuously, all the heretofore isolated psychrotolerant acetogens and many N_2-fixing acetogens belong to the genus *Acetobacterium* (Schink and Bomar, 1992; Table 2). About half of the genera that harbor acetogens only contain one acetogenic species (e.g., *Holophaga foetida*, *Acetohalobium arabaticum*, *Oxobacter pfennigii*, *Acetonema longum*; Table 2). Recently, acetogenesis has been observed in spirochetes ("*Treponema primitia*") isolated from termite guts (Leadbetter et al., 1999; Graber and Breznak, 2004a; Graber et al., 2004b; Fig. 5).

A brief overview of acetogenic species having validated names is given in the following paragraphs. The names of those organisms not validated are in quotation marks. Earlier compilations include Breznak (1992), Diekert (1992), Hippe et al. (1992), Schink and Bomar (1992), Mackie and Bryant (1994), and Schink (1994). Although relatively few of the acetogens listed below have been evaluated for their ability to tolerate O_2, it should be anticipated that many acetogens possess the ability to both tolerate and consume small amounts of oxygen (Küsel et al., 2001; Karnholz et al., 2002; Boga and Brune, 2003).

ACETITOMACULUM RUMINIS. This species was isolated from steer rumen fluid (Greening and Leedle, 1989). Cells are Gram-positive, nonsporeforming, motile, slightly curved rods. Growth-supportive substrates include H_2-CO_2, CO, formate, cellobiose, glucose, ferulate and syringate. With all substrates, acetate is the sole reduced end product (Greening and Leedle, 1989).

ACETOANAEROBIUM NOTERAE. This species was isolated from sediment samples of the Notera oil exploration site in Israel (Sleat et al., 1985). Cells are Gram-positive, nonsporeforming, motile, straight rods. *Acetoanaerobium noterae* grows with H_2-CO_2, glucose, and maltose, and produces acetate as the sole product. Propionate, butyrate, isobutyrate, and isovalerate are also formed when yeast extract serves as the growth-supportive substrate (Sleat et al., 1985).

"ACETOANAEROBIUM ROMASHKOVII." This organism was isolated from the Romashkin oil field in Tatarstan (Davydova-Charakhch'yan et al., 1992). Cells are Gram-positive, nonsporeforming, motile rods with rounded ends. Growth-supportive substrates include H_2-CO_2, formate, methanol, pyruvate, lactate, ethylene glycol, sugars, and amino acids. Acetate is the sole product from carbohydrates and H_2-CO_2; propionate is also formed during growth on sucrose. "*Acetoanaerobium romashkovii*" produces and excretes polysaccharides during growth on H_2-CO_2 or methanol (Davydova-Charakhch'yan et al., 1992).

ACETOBACTERIUM BAKII, ACETOBACTERIUM FIMETARIUM, AND ACETOBACTERIUM PALUDOSUM. These species were isolated from cold habitats (<6°C) or from

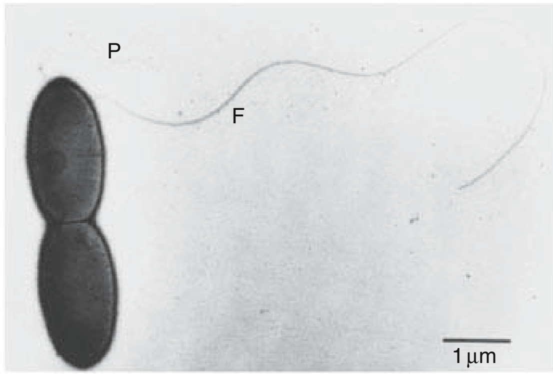


Fig. 4. Electron micrograph of cells of *Acetobacterium woodii* (ATCC 29683[T], DSM 1030[T]) with a single subterminal flagellum (F) and pili-like structures (P). From Balch et al. (1977), used with permission from International Union of Microbiological Societies. The micrograph was kindly provided by R. S. Wolfe.

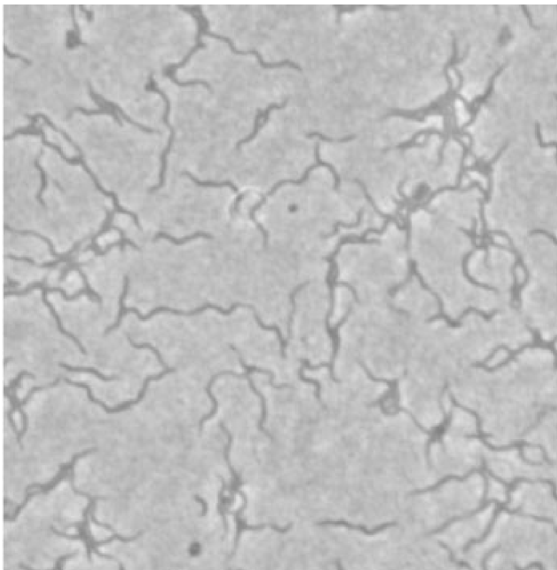


Fig. 5. Phase contrast micrograph of spirochete *Treponema* sp., strain ZAS-2. The length of cells varies from 3 to 7 µm. The micrograph was kindly provided by J. Breznak.

samples that were kept for more than one year at 6°C (Kotsyurbenko et al., 1995). Cells of all three species are Gram-positive, oval-shaped, and motile rods. Although these species were isolated at 6°C, their optimum growth temperatures are 20°C (*A. bakii* and *A. paludosum*) and 30°C (*A. fimetarium*). However, their ability to grow at a temperature as low as 1°C qualifies them as psychrotolerant bacteria. *Acetobacterium bakii*, *A. fimetarium* and *A. paludosum* grow with H_2-CO_2, CO, formate and certain sugars, and stoichiometrically convert these substrates to acetate as the sole product (Kotsyurbenko et al., 1995). Although these three species are very similar, DNA-DNA hybridization supported the designation of three different species.

ACETOBACTERIUM CARBOLINICUM. This species was isolated from freshwater sediments (Eichler and Schink, 1984). Cells are Gram-positive, nonsporeforming, rods with slightly pointed ends; some strains are motile. The type strain grows with ethanol, propanol, butanol, 1,2-propanediol, and 2,3-butanediol and oxidizes these substrates incompletely to the corresponding fatty acids and uses the reducing equivalents to reduce CO_2 to acetate via the acetyl-CoA pathway (Eichler and Schink, 1984). Other growth-supportive substrates include H_2-CO_2, formate, pyruvate, lactate, methanol, hexoses, ethylene glycol, and methoxylated aromatic acids. Strain *A. carbinolicum* KoMac1 (DSM 5193) was isolated with the methylalkylether methoxyacetate and can also grow on glycol ethers. The ether bond of these compounds is cleaved and acetate is formed as the sole product (Schuppert and Schink, 1990). Strain KoMac1 also utilizes the *O*-methyl group of methoxylated aromatics and betaine (Schuppert and Schink, 1990).

"ACETOBACTERIUM DEHALOGENANS." This organism (also termed "strain MC") was isolated from sewage sludge (Traunecker et al., 1991). Cells are Gram-positive, nonsporeforming, nonmotile, elongated cocci. "*Acetobacterium dehalogenans*" is the only known acetogen able to utilize and grow with methyl chloride. Methyl chloride is dehalogenated via a methyl chloride dehalogenase (Meßmer et al., 1996) and is further metabolized to acetate via the acetyl CoA pathway. Other substrates supporting growth of the organism include H_2-CO_2, CO, glucose, fumarate, methanol, and methoxylated aromatic compounds.

ACETOBACTERIUM MALICUM. This species was enriched and isolated with 2-methoxyethanol as growth substrate from a freshwater sediment (Tanaka and Pfennig, 1988). Cells are Gram-positive, nonsporeforming, motile rods with slightly pointed ends. Similar to a few other species of *Acetobacterium*, *A. malicum* can grow at the expense of the ether compounds 2-methoxymethanol and 2-ethoxyethanol, which are metabolized to acetate and the corresponding alcohols. Other growth-supportive substrates include malate, H_2-CO_2, formate, pyruvate, fructose, betaine, and the *O*-methyl groups of methoxylated aromatic compounds (Tanaka and

Pfennig, 1988). Acetate is the sole product of these substrates.

"ACETOBACTERIUM PSAMMOLITHICUM." This species was isolated from subsurface sandstone and represents the second acetogen isolated from a subsurface ecosystem (Krumholz et al., 1999). (The first acetogenic isolate from a subsurface habitat was the unclassified strain SS1; Liu and Suflita, 1993). Cells are Gram-negative, nonsporeforming, nonmotile rods. Growth is very slow in mineral medium. In medium supplemented with yeast extract, growth is very good on H_2-CO_2, methanol, formate, glucose, syringate, alcohols, and organic acids (Krumholz et al., 1999). Acetate is the product of H_2-CO_2-dependent growth; products of other growth-supportive substrates are not reported.

ACETOBACTERIUM TUNDRAE. This organism is psychrotolerant and was isolated from a tundra wetland soil (Simankova et al., 2000). Cells are Gram-positive, nonsporeforming, motile rods. Growth-supportive substrates include H_2-CO_2, CO, formate, methanol, and sugars; acetate is the sole reduced end product. As with the psychrotrophic acetogens *A. bakii* and *A. paludosum*, *A. tundrae* has a minimum growth temperature of 1°C and an optimal growth temperature of 20°C.

ACETOBACTERIUM WIERINGAE. This species was isolated from a sewage digester (Braun and Gottschalk, 1982). Cells are Gram-positive, nonsporeforming, motile rods. H_2-CO_2, fructose, and lactate are growth-supportive substrates; acetate is the sole reduced end product (Braun and Gottschalk, 1982). *Acetobacterium wieringae* tolerates 300 mM acetate (Menzel and Gottschalk, 1985).

ACETOBACTERIUM WOODII. The type strain of *A. woodii* was enriched and isolated from black sediment of a marine estuary with H_2-CO_2 as substrate (Balch et al., 1977). Cells are Gram-positive, nonsporeforming, motile rods with slightly pointed ends (Fig. 4). Growth-supportive substrates include H_2-CO_2, CO, formate, methanol, 2,3-butandiol, ethylene glycol, acetoin, glycerol, sugars, betaine, and several methoxylated aromatic acids (Balch et al., 1977; Bache and Pfennig, 1981; Eichler and Schink, 1984; Sharak Genthner and Bryant, 1987; Schink and Bomar, 1992). Cultures demethylate the osmolytes dimethylsulfoniopropionate and glycine-betaine to methylthiopropionate and dimethylglycine, respectively; however, only the demethylation of glycine-betaine supported growth of the organism (Jansen and Hansen, 2001). *Acetobacterium woodii* growths mixotrophically on (i.e., can simultaneously utilize) H_2-CO_2 and organic compounds (e.g., fructose; Braun and Gottschalk, 1981) and can use aromatic acrylates as energy-conserving, growth-supportive terminal electron acceptors (Bache and Pfennig, 1981; Tschech and Pfennig, 1984). Growth, motility, and acetate formation from H_2-CO_2 are strictly dependent on sodium ions (Heise et al., 1989; Müller and Bowien, 1995; Aufurth et al., 1998). Several Na^+-dependent reactions in the metabolism of *A. woodii* have been identified, and associated enzymes have been purified and characterized (Heise et al., 1989; Heise et al., 1991; Heise et al., 1992; Heise et al., 1993; Müller and Gottschalk, 1994; Reidlinger and Müller, 1994a; Reidlinger et al., 1994b; Müller et al., 2001). Cells reductively dechlorinate carbon tetrachloride (Egli et al., 1988; Stromeyer et al., 1992); dechlorination is enhanced by the addition of hydroxocobalamin (Hashsham and Freedman, 1999). Cells also tolerate and consume small amounts of oxygen (Karnholz et al., 2002).

ACETOHALOBIUM ARABATICUM. This organism was isolated from a cyanobacterial mat in a saline lagoon and was the first obligately halophilic acetogen to be described (Zhilina and Zavarzin, 1990). Sodium chloride (10–25%) is necessary for growth. Cells are motile, straight rods often aggregated in palisades. H_2-CO_2, CO, trimethylamine, formate, betaine, lactate, pyruvate, and histidine are growth-supportive substrates. Acetate is the main product during growth on trimethylamine and betaine and is accompanied by minor amounts of methylamines (Zhilina and Zavarzin, 1990; Zavarzin et al., 1994). Cell extracts have CO dehydrogenase and hydrogenase activities, which are stimulated by increased salt concentrations.

ACETONEMA LONGUM. This organism was isolated from the gut contents of the wood-feeding termite *Pterotermes occidentis* (Kane and Breznak, 1991a). Cells are sporeforming, motile rods of unusually large size; cells can be up to 60 µm in length. Growth-supportive substrates include H_2-CO_2, pyruvate, fumarate, glucose, mannitol, and ribose; poor growth occurs on citrate, propanol, ethylene glycol, and 3,4,5-trimethoxybenzoate. Homoacetogenesis only occurs with H_2-CO_2. Butyrate and acetate are the main products from carbohydrates and pyruvate; fumarate is metabolized to propionate and acetate, and rhamnose yields 1,2-propanediol as the major product (Kane and Breznak, 1991a).

"BRYANELLA FORMATEXIGENS." This species was isolated from human feces (Wolin et al., 2003). Cells are Gram-positive, nonmotile short rods (approx. 1.2 × 0.7 µm). Single cells, and pairs and short chains of cells, are apparent. Upon isolation, the type strain (I-52; Wolin and Miller, 1994) fermented vegetable cellulose and carboxymethylcellulose but lost this ability after storage under frozen conditions. No growth occurs on H_2-CO_2 or formate, and formate is

required for optimal homoacetogenic conversion of glucose. The lack of supplemental formate yields succinate, lactate and acetate as products from glucose. These characteristics indicate that the formate dehydrogenase is negliable. Growth is supported by stachyose, sucrose, lactose, maltose, galactose, mannose, and xylose. Cells are catalase and oxidase negative, and nitrate is not reduced.

"BUTYRIBACTERIUM METHYLOTROPHICUM." This organism was isolated from a sewage digestor (Zeikus et al., 1980). Cells are Gram-positive, sporeforming, nonmotile rods. Growth is supported by H_2-CO_2, formate, methanol, glucose, fructose, sucrose, pyruvate, lactate and glycerol (Zeikus et al., 1980; Kerby and Zeikus, 1987). Homoacetogenic utilization of substrates only occurs with H_2-CO_2 and formate. With other substrates, butyrate and H_2 are also produced (Zeikus et al., 1980; Lynd and Zeikus, 1983). After prolonged incubation in medium with CO in the gas phase, the type strain grew on and utilized CO; acetate was the sole product from CO (Lynd et al., 1982). There is substantial evidence that "*B. methylotrophicum*" and *Eubacterium limosum* are the same species: 1) the metabolic properties of the two organisms are nearly identical, and 2) the 16S rRNA gene sequences of the two organisms are very similar (99.4% sequence similarity; Moore and Cato, 1965; Sharak Genthner et al., 1981; Tanner et al., 1981; Sharak Genther and Bryant, 1982; Tanner and Woese, 1994; Jansen and Hansen, 2001).

CALORAMATOR FERVIDUS. This species was isolated from a hot spring in New Zealand and was first described as *Clostridium fervidus* (Patel et al., 1987). Cells are Gram-negative, sporeforming, motile rods. Carbohydrates support growth, and acetate is the major end product. However, growth on one-carbon compounds (e.g., formate) or other typical acetogenic substrates (e.g., H_2-CO_2) has not been reported, and substrate/product stoichiometries of carbohydrate utilization are not available. Thus, the true acetogenic nature of this organism has not been estabilished. Until otherwise proven, one should assume that the organism might not be an acetogen.

CLOSTRIDIUM ACETICUM. This species was the first acetogen to be isolated (Fig. 2). It was isolated from soil and described by Wieringa (Wieringa, 1936; Wieringa, 1939–40). After early studies with the organism (Karlsson et al., 1948), it was lost for about 30 years. However, *C. aceticum* was reisolated from soil using Wieringa's enrichment procedure, and almost at the same time spores of the original Wieringa strain in sterile dried soil were found in Barker's laboratory and revived (Adamse, 1980; Braun et al., 1981). Cells are Gram-negative, sporeforming, motile rods (Wieringa, 1939–40; Braun et al., 1981). Growth-supportive substrates include H_2-CO_2, CO, fructose, glutamate fumarate, pyruvate, aldehyde groups of aromatic compounds, and methoxylated aromatic compounds (Wieringa, 1939–40; Braun et al., 1981; Lux and Drake, 1992; Matthies et al., 1993; Gößer et al., 1994). As with *C. formicoaceticum* (see below), fumarate is dismutated by *C. aceticum* to acetate and succinate, and is metabolized independent of the acetyl-CoA pathway; fumarate also serves as an alternative electron acceptor and is reduced to succinate (Matthies et al., 1993). N_2 is fixed (Cato et al., 1986).

"CLOSTRIDIUM AUTOETHANOGENUM." This organism was isolated from rabbit feces (Abrini et al., 1994). Cells are Gram-positive, sporeforming, motile rods. The range of substrates includes H_2-CO_2, CO, pyruvate, hexoses, pentoses, and glutamate, and is similar to the range of substrates used by *Clostridium ljungdahlii* (Abrini et al., 1994; Tanner et al., 1993). CO is converted to acetate and ethanol (Abrini et al., 1994). Ethanol production from CO was also reported for *C. ljungdahlii*; however, this metabolic potential is not necessarily stable (Barik et al., 1988; Tanner et al., 1993). The 16S rRNA gene sequences of "*C. autoethanogenum*" and *C. ljungdahlii* are essentially identical (Stackebrandt et al., 1999).

CLOSTRIDIUM COCCOIDES. Two acetogenic strains of *C. coccoides* (strains 1410 and 3110) were isolated from the human intestinal tract (Kamlage et al., 1997). The type strain of *C. coccoides* isolated from mouse feces was not initially described as an acetogen; however, it has recently been shown to contain all the enzymes of the acetyl CoA pathway when grown on H_2-CO_2-formate (Kaneuchi et al., 1976; Kamlage et al., 1997). Cells of *C. coccoides* strain 1410 (which is probably identical to strain 3110) are Gram-variable, coccoid rods. *Clostridium coccoides* strain 1410 grows on a variety of hexoses, pentoses, sugar alcohols, H_2-CO_2-formate, and H_2-CO_2-vanillate. Products from growth have not been reported. However, resting cells convert formate, H_2-CO_2, and *O*-methyl groups of vanillate to acetate at stoichiometries indicative of acetogenesis; the aromatic ring of vanillate remains intact (Kamlage et al., 1997). Resting cells of *C. coccoides* strain 1410 convert glucose to acetate, succinate, and D-lactate.

CLOSTRIDIUM DIFFICILE. Five acetogenic strains of *C. difficile* were isolated from the rumen of newborn lambs; strain AA1 is considered as a representative strain (Rieu-Lesme et al., 1998). No acetogenic potentials have been documented for the type strain of *C. difficile*. Cells of *C. difficile* strain AA1 are Gram-positive, sporeforming, giant filamentous rods. Growth of

strain AA1 is supported by H_2-CO_2, fructose, glucose, cellobiose, maltose, mannose, and syringate. Acetate is the sole product from H_2-CO_2 and the substrate/product stoichiometry is indicative of acetogenesis; however, glucose and fructose are metabolized to almost equal amounts of acetate and butyrate, and small amounts of ethanol and isovalerate (Rieu-Lesme et al., 1998).

CLOSTRIDIUM FORMICOACETICUM. The first strain of *C. formicoaceticum* was probably isolated from pond sediment by El Ghazzawi (1967). Although the organism was called *Clostridium aceticum* in the title of the German publication, El Ghazzawi stated that his isolate differed from *C. aceticum* and tentatively named his organism "*Clostridium formicoaceticum*" because it produced both formate and acetate (El Ghazzawi, 1967). The type strain of *C. formicoaceticum* was isolated from sewage sludge (Andreesen et al., 1970). Cells are Gram-negative, sporeforming, motile, straight or slightly curved rods. The range of substrates is very similar to that of *C. aceticum* (see above) but also includes glycerol, gluconate, glucuronate, and glycerate (Andreesen et al., 1970). *Clostridium formicoaceticum* can be differentiated from *C. aceticum* by its inability to grow with H_2-CO_2 and its ability to grow with methanol and lactate (Andreesen et al., 1970; Lux and Drake, 1992). As with *C. aceticum*, the utilization of fumarate by *C. formicoaceticum* does not involve the acetyl-CoA pathway; fumarate is dismutated to acetate and succinate (Dorn et al., 1978). Fumarate can also serve as an alternative electron acceptor (Matthies et al., 1993), and N_2 is fixed (Bogdahn et al., 1983). Reductant derived from the oxidation of the aldehyde groups of certain aromatic compounds (e.g., 4-hydroxybenzaldehyde) is growth supportive (Gößer et al., 1994), preferentially used in the acetyl-CoA pathway, and inhibits the use of fructose (Frank et al., 1998).

CLOSTRIDIUM GLYCOLICUM. Two acetogenic strains of *C. glycolicum* have been isolated. Strain 22 was isolated from sewage sludge, grows on H_2-CO_2, and produces mainly acetate; cells are Gram-positive rods that form oval, subterminal spores (Ohwaki and Hungate, 1977). Strain 22 has been deposited at the American Type Culture Collection (ATCC) and has been identified as a strain of *Clostridium glycolicum*; however, the 16S rRNA gene sequence is not available (per information from the ATCC Bacteriology Program). Strain RD-1 was isolated from sea grass roots and was identified as an acetogenic strain of *C. glycolicum* by analysis of the 16S rRNA gene sequence (Küsel et al., 2001). Cells of strain RD-1 are Gram-positive, sporeforming, motile rods that can be linked by connecting filaments. Growth-supportive substrates of strain RD-1 include H_2-CO_2, formate, pyruvate, lactate, ethylene glycol, and certain sugars. Except for growth on sugars and ethylene glycol, acetate is the sole reduced end product. Strain RD-1 is aerotolerant and grows at O_2 concentrations of up to 6% in the headspace of static liquid cultures and up to 4% in the headspace of shaken liquid cultures; ethanol, lactate and H_2 are the reduced end products under oxic conditions (Küsel et al., 2001; see the subsection on Tolerance to Oxic Conditions and Metabolism of O_2 in this Chapter). No acetogenic potentials have been found for the type strain of *C. glycolicum* (Gaston and Stadtman, 1963; Küsel et al., 2001).

CLOSTRIDIUM LJUNGDAHLII. This organism was isolated from chicken manure/waste (Barik et al., 1988; Tanner et al., 1993). Cells are Gram-positive, sporeforming, motile rods (Fig. 6). The organism grows autotrophically on H_2-CO_2 and CO; heterotrophic growth occurs on formate, ethanol, pyruvate, fumarate, and sugars (including fructose and xylose; Tanner et al., 1993). The sole product from H_2-CO_2 and fructose is acetate; however, from synthesis gas (a mixture of H_2, CO, and CO_2), acetate and ethanol are produced (Tanner et al., 1993; Phillips et al., 1994). Nitrate is reduced to ammonium; however, unlike the dissimilation of nitrate by *M. thermoacetica* (see the sections on Use of Diverse Terminal Electron Acceptors and Regulation of the Acetyl-CoA Pathway and Other Metabolic Abilities in this Chapter), the reduction of nitrate does not have a regulatory effect on acetogenesis and likewise does not enhance the growth of the organism (Seifritz et al., 1993; Fröstl et al., 1996; Laopaiboon and Tanner, 1999).

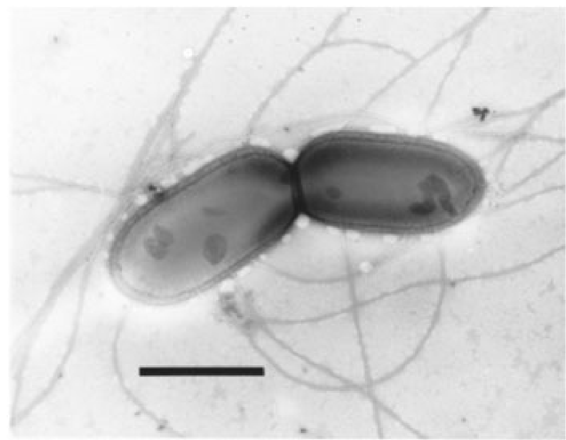


Fig. 6. Electron micrograph of cells from a young culture (16 h, fructose-grown) of *Clostridium ljungdahlii* (ATCC 55383^T) with peritrichously inserted flagella. Bar equals 1 µm. The micrograph was kindly provided by R.S. Tanner.

CLOSTRIDIUM MAGNUM. This species was isolated from pasteurized freshwater sediment (Schink, 1984). Cells are Gram-positive, sporeforming, motile, large straight rods. H_2-CO_2, formate, methanol, 2,3-butandiol, acetoin, malate, citrate, and a few sugars are substrates, and acetate is the sole reduced end product. N_2 is fixed (Bomar et al., 1991) and small amounts of O_2 are tolerated and consumed (Karnholz et al., 2002).

CLOSTRIDIUM MaOMBEI. This organism was isolated from the gut of a soil-feeding termite (Kane et al., 1991b). Cells are Gram-positive, sporeforming, motile, straight rods. Growth occurs on H_2-CO_2, sugars, sugar alcohols, organic acids, and amino acids. The main reduced end product is acetate; however, succinate is metabolized to CO_2 and propionate (Kane et al., 1991b).

CLOSTRIDIUM METHOXYBENZOVORANS. This species was isolated from an olive mill wastewater digester (Mechichi et al., 1999). Cells of *C. methoxybenzovorans* are Gram-positive, sporeforming, nonmotile rods. Growth occurs on H_2-CO_2, methanol, lactate, sugars, methoxylated aromatic compounds, betaine, dimethylglycine, dimethylsulfide, casaminoacids, and peptone. H_2-CO_2 is metabolized to acetate and formate. Metabolism of betaine, dimethylglycine, and dimethylsulfide yields acetate, and sugars are metabolized to acetate, formate, ethanol, H_2, and CO_2. *O*-methyl groups, methanol, and lactate are metabolized to acetate and butyrate (Mechichi et al., 1999). Since no substrate/product stoichiometries have been reported for the organism, the acetogenic utilization of most substrates is uncertain.

CLOSTRIDIUM SCATOLOGENES. An acetogenic strain of *C. scatologenes* (SL1) was isolated from sediment of an acidic coal mine pond (Küsel et al., 2000). The type strain of *C. scatologenes* was isolated from soil and was not originally described as an acetogen (Holdeman et al., 1977). However, both strain SL1 and the type strain utilize H_2 and CO with the concomitant production of acetate, and cell extracts of both organisms have CO dehydrogenase, hydrogenase, and formate dehydrogenase activities (Küsel et al., 2000). Cells are Gram-positive, sporeforming, motile, long rods and produce skatole, a dung odor component (Holdeman et al., 1977; Küsel et al., 2000). Substrates include fructose, arabinose, ethanol, formate, vanillate, H_2-CO_2, and CO. The major reduced end product is acetate. However, in addition to acetate, butyrate and traces of H_2 are also produced from sugars (Küsel et al., 2000).

CLOSTRIDIUM ULTUNENSE. This species was isolated from an anaerobic acetate-oxidizing triculture that was enriched from a digester fed with swine manure (Schnürer et al., 1994; Schnürer et al., 1996). Cells are Gram-positive, sporeforming rods that change cell size, cell form, and motility during growth. The only known growth-supportive substrates are formate, betaine, glucose, pyruvate, ethylene glycol, and cysteine. The main end products are acetate, formate, and traces of H_2. H_2-CO_2 does not support growth; however, H_2-CO_2 is converted to acetate by resting cells (Schnürer et al., 1996). Acetate is oxidized in coculture with a methanogen, and the oxidation of acetate appears to occur via a reversal of the acetyl-CoA pathway (Schnürer et al., 1997). An acetogen (strain AOR) that also oxidized acetate in coculture with a methanogen was previously isolated (Lee and Zinder, 1988); however, this strain has been lost (S.H. Zinder, personal communication).

EUBACTERIUM AGGREGANS. This organism was isolated from an olive mill wastewater digestor (Mechichi et al., 1998). Cells are Gram-positive, nonsporeforming, nonmotile rods that form aggregates. Substrates include H_2-CO_2, glucose, fructose, sucrose, lactate, formate, methanol, betaine, and numerous methoxylated aromatic compounds. Although *E. aggregans* is described as homoacetogenic, H_2, formate, acetate, and butyrate are produced from sugars (Mechichi et al., 1998). Acetate is the sole reduced end product with formate and methanol. Methoxylated aromatic compounds are *O*-demethylated, and acetate, butyrate, and the corresponding hydroxylated aromatic compounds are formed. Aldehyde groups of methoxylated aromatic compounds are oxidized to carboxylate groups.

EUBACTERIUM LIMOSUM. This species was isolated from sheep rumen and digester sludge (Sharak Genthner et al., 1981). Cells are Gram-positive, nonsporeforming, nonmotile straight rods that become more pleomorphic after prolonged incubation. *Eubacterium limosum* is metabolically very versatile; its substrate range includes sugars, amino acids, methoxylated aromatic compounds, glycine, betaine, lactate, methanol, H_2-CO_2 and CO (Sharak Genthner et al., 1981; Sharak Genthner and Bryant, 1982; Sharak Genthner and Bryant, 1987; Jansen and Hansen, 2001). Both acetate and butyrate are produced from one-carbon compounds (Sharak Genthner et al., 1981; Pacaud et al., 1985; see "*Butyribacterium methylotrophicum*," above). Cultures demethylate the osmolytes dimethylsulfoniopropionate and glycine-betaine to methylthiopropionate and dimethylglycine, respectively; however, only the demethylation of glycine-betaine supports growth of the organism (Jansen and Hansen, 2001).

HOLOPHAGA FOETIDA. This organism (strain TMBS4) was isolated from freshwater

sediment (Bak et al., 1992; Liesack et al., 1994). Cells are Gram-negative, nonsporeforming, nonmotile rods. The substrate range is rather small and mainly consists of pyruvate and aromatic compounds, especially methylated and nonmethylated trihydroxybenzenes. Acetate is the main reduced end product. In contrast to other acetogens, *H. foetida* degrades aromatic rings to acetate (Bak et al., 1992; Kreft and Schink, 1993). Dimethylsulfide and methanediol are produced from methoxylated aromatic compounds when cells are cultured in sulfide-containing media, indicating that sulfide can serve as a methyl acceptor (Bak et al., 1992). CO_2 and CO can also be used as methyl acceptors with the subsequent formation of acetate. CO dehydrogenase activity is present in cells grown on methoxylated aromatic compounds (Kreft and Schink, 1993). *Holophaga foetida* occupies a fairly isolated position in the phylogenetic tree of the Bacteria (Liesack et al., 1994; Ludwig et al., 1997; see the section on Taxonomy and Phylogeny).

MOORELLA GLYCERINI. This species is a thermophilic acetogen and was isolated from the sediment of a hot spring at Yellowstone National Park (Slobodkin et al., 1997). The cells are Gram-positive, sporeforming, motile, straight rods. Growth is supported by glycerol, sugars, lactate, glycerate, pyruvate, and yeast extract; however, H_2-CO_2 is not growth supportive. Acetate is the only product from glycerol and glucose. Fumarate is reduced to succinate, and the reduction of thiosulfate yields elemental sulfur. Nitrate is not dissimilated. Optimum growth occurs at 58°C.

"MOORELLA MULDERI." This organism is a thermophilic acetogen and was isolated from a high-temperature bioreactor (Balk et al., 2003). The cells are Gram-positive, sporeforming rods. Growth is supported by H_2-CO_2, formate, methanol, hexoses, cellobiose, lactate and pyruvate. The reduction of thiosulfate yields sulfide. Nitrate is not dissimilated.

MOORELLA THERMOACETICA. This organism is a thermophilic acetogen that was isolated from horse manure and was first described as *Clostridium thermoaceticum* (Fontaine et al., 1942). On the basis of phylogenetic analysis of the 16S rRNA gene sequence, *C. thermoaceticum* was reclassified as *M. thermoacetica* (Collins et al., 1994). Although the organism was originally isolated from horse manure, the organism is a common inhabitant of soils (Gößner and Drake, 1997; Gößner et al., 1998; Gößner et al., 1999; Karita et al., 2003). Cells are Gram-variable, sporeforming, variably motile, straight rods (Fig. 1). The optimum temperature of growth is 55–60°C (Fontaine et al., 1942), and the vitamin nicotinic acid is required for growth (Lundie and Drake, 1984). *Moorella thermoacetica* was the first bacterium that was shown to produce 3 moles of acetate from 1 mole of hexose (Fontaine et al., 1942), and is one of the most metabolically robust acetogens characterized to date. *Moorella thermoacetica* was originally isolated as an obligate heterotroph (Fontaine et al., 1942), but nearly five decades later, it was shown to be capable of autotrophic growth (Daniel et al., 1990). This bacterium displays very diverse physiological capabilities (Drake and Daniel, 2004). Growth-supportive substrates include CO, H_2-CO_2, formate, methanol, hexoses, pentoses, methoxylated benzoic acids, and several two-carbon compounds (e.g., oxalate, glycolate, and glyoxylate; Fontaine et al., 1942; Daniel et al., 1990; Daniel and Drake, 1993; Daniel et al., 2004; Drake et al., 1997; Seifritz et al., 1999; Kim et al., 2002). Carboxyl groups of aromatic compounds can serve as CO_2 equivalents in the acetyl-CoA pathway (Hsu et al., 1990a; Hsu et al., 1990b). Thiosulfate (Beaty and Ljungdahl, 1990; Beaty and Ljungdahl, 1991), nitrate (Seifritz et al., 1993), and nitrite (Seifritz et al., 2003) serve as alternative electron acceptors. Nitrate is dissimilated to both nitrite and ammonium, and nitrite is dissimilated to ammonium. Ethanol and *n*-propanol are oxidized and are growth-supportive substrates when nitrate is dissimilated; neither ethanol nor *n*-propanol is utilized as an acetogenic substrate (Fröstl et al., 1996). Reductively dechlorinates carbon tetrachloride (Egli et al., 1988). Tolerates and consumes small amounts of oxygen (Karnholz et al., 2002). A recent isolate that is phylogenetcially nearly identical to *M. thermoacetica* is cellulolytic (Karita et al., 2003). *Moorella thermoacetica* is the most studied acetogen, and the enzymology of the acetyl-CoA pathway was resolved with this organism (see the section on Historical Perspectives and Table 1 in this Chapter).

MOORELLA THERMOAUTOTROPHICA. This organism is a thermophilic acetogen that was isolated from a hot spring at Yellowstone National Park and was first described as *Clostridium thermoautotrophicum* (Wiegel et al., 1981). On the basis of phylogenetic analysis of the 16S rRNA gene sequence, *C. thermoautotrophicum* was reclassified as *M. thermoautotrophica* (Collins et al., 1994). Cells are Gram-variable, sporeforming, motile rods (Wiegel et al., 1981). *Moorella thermoautotrophica* was initially described as being metabolically distinct from the closely related *M. thermoacetica* (Collins et al., 1994); this distinction was primarily based on the H_2-dependent acetogenic abilities of the former bacterium (Wiegel et al., 1981). However, later studies demonstrated that *M. thermoacetica* grows chemolithoautotrophically on H_2-CO_2 (Daniel et al., 1990). Both of these species of

Moorella display a similar substrate range. Both species also require the vitamin nicotinic acid for growth (Lundie and Drake, 1984; Savage and Drake, 1986). The substrate range of *M. thermoautotrophica* includes H_2-CO_2, CO, formate, methanol, glucose, fructose, glycerate, glycolate, and methoxylated aromatic compounds (Wiegel et al., 1981; Fröstl et al., 1996; Seifritz et al., 1999). Nitrate is utilized as an alternative electron acceptor and is dissimilated to nitrite and ammonium; ethanol and *n*-propanol are growth-supportive substrates only when nitrate is available for dissimilation (Fröstl et al., 1996).

NATRONIELLA ACETIGENA. This organism is a haloalkaliphilic acetogen and was isolated from the soda deposits at Lake Magadi, Kenya (Zhilina et al., 1996). Cells are Gram-negative, sporeforming, motile, large rods. The substrate range is limited and includes lactate, pyruvate, ethanol, glutamate, and propanol. Growth does not occur on H_2-CO_2 or CO-CO_2. Acetate is the sole reduced end product. Propionate is formed during growth on propanol. The optimal pH is 10, and the optimal salinity for growth is 12% NaCl (w/v).

NATRONINCOLA HISTINOVORANS. This species is a moderately haloalkaliphilic acetogen and was isolated from soda deposits at Lake Magadi, Kenya (Zhilina et al., 1998). Cells are Gram-positive, motile rods; sporeforming and nonsporeforming strains have been isolated. *Natronincola histidinovorans* is specialized in using amino acids (histidine, glutamate, and casaminoacids) as sources of energy. Neither H_2-CO_2 nor CO-CO_2 support growth. Optimal growth occurs at pH 9 and a salinity of 9% NaCl. Acetate and ammonium are the main end products.

OXOBACTER PFENNIGII. This organism was isolated from the rumen fluid of a steer and was first described as *Clostridium pfennigii* (Krumholz and Bryant, 1985). On the basis of phylogenetic analysis of the 16S rRNA gene sequence, *C. pfennigii* was reclassified as *O. pfennigii* (Collins et al., 1994). Cells are Gram-positive, motile, sporeforming, slightly curved rods. Substrates include CO, pyruvate, vanillate, vanillin, ferulate, syringate, and trimethoxybenzoate. In contrast to most other acetogens, acetate is not produced from methoxybenzenoids (*O*-methyl groups are utilized, and butyrate and the respective hydroxybenzenoids are formed; Krumholz and Bryant 1985). During growth on CO or pyruvate, acetate is formed in addition to butyrate or is the sole product, respectively.

RUMINOCOCCUS HYDROGENOTROPHICUS. This species is a nonsporeforming coccobacillus that was isolated from human feces (Bernalier et al., 1996c). *Ruminococcus hydrogenotrophicus* grows on H_2-CO_2, formate, pyruvate, and several sugars. Acetate is the sole product from H_2-CO_2-dependent growth; however, glucose and fructose are metabolized to acetate, lactate, ethanol, and small amounts of isobutyrate and isovalerate (Bernalier et al., 1996c). Thus, the metabolism of sugars involves several fermentative processes.

RUMINOCOCCUS PRODUCTUS. This organism was originally isolated from various mammalian gastrointestinal tracts and was described as *Peptostreptococcus productus*; the original isolates were not described as acetogens (Moore and Holdeman, 1974; Varel et al., 1974; Holdeman-Moore et al., 1986). On the basis of phylogenetic analysis of the 16S rRNA gene sequence, *P. productus* was reclassified as *R. productus* (Ezaki et al., 1994). Two acetogenic strains (strain U-1 [ATCC 35244] and strain Marburg [ATCC 43917]) of *R. productus* have been isolated from sewage sludge (Lorowitz and Bryant, 1984; Geerligs et al., 1987). Cells are Gram-positive, nonsporeforming, nonmotile elongated cocci occuring often in pairs or chains (Lorowitz and Bryant, 1984; Holdeman-Moore et al., 1986; Geerligs et al., 1987). Growth-supportive substrates of the acetogenic strains include CO, H_2-CO_2, monomeric and dimeric sugars, and methoxylated aromatic compounds; growth is particularly good on CO (Lorowitz and Bryant, 1984; Geerligs et al., 1987; Parekh et al., 1992). The acrylate side chain of methoxylated and nonmethoxylated phenylacrylates can be used as alternative electron acceptor (Parekh et al., 1992; Misoph et al., 1996b). The major reduced end product is acetate; however, under CO_2-limited conditions or when substrate concentrations are high (e.g., 10 mM fructose), lactate, succinate, and formate are also formed (Misoph and Drake, 1996a).

RUMINOCOCCUS SCHINKII. This organism was isolated from rumen content of 1–3 days-old lambs (Rieu-Lesme et al., 1996b). Cells are Gram-positive, nonsporeforming, nonmotile cocci. Substrates include H_2-CO_2, various sugars, glycerol, syringate, and ferulate. Acetate is the sole reduced end product.

SPOROMUSA ACIDOVORANS. This species was isolated from a distillation wastewater fermentor (Ollivier et al., 1985a). Cells are Gram-negative, sporeforming, motile, curved rods. Growth-supportive substrates mainly include organic acids, H_2-CO_2, methanol, glycerol, and a few sugars; acetate is the sole reduced end product with all substrates.

SPOROMUSA AERIVORANS. This organism was isolated from a soil-feeding termite (Boga et al., 2003). Cells are Gram-negative, sporeforming, motile, curved rods. Growth-supportive substrates include H_2-CO_2, formate,

methanol, ethanol, lactate, pyruvate, mannitol, citrate, and various methoxylated aromatic compounds; hexoses are not utilized. Cells tolerate and consume small amounts of oxygen and are catalase positive (Boga and Brune, 2003).

SPOROMUSA MALONICA. This species was isolated from freshwater sediment (Dehning et al., 1989). Cells are Gram-negative, sporeforming, motile, curved rods. The organism exhibits a very versatile metabolism and utilizes H_2-CO_2 and numerous organic compounds, including formate, pyruvate, alcohols, dicarboxylic acids, fructose and trimethoxycinnamate. Acetate is the reduced end product when typical acetogenic substrates such as H_2-CO_2, formate, methanol, fructose, pyruvate, or the *O*-methyl groups of trimethoxycinnamate are metabolized (Dehning et al., 1989). Alcohols yield acetate and the respective fatty acids, and crotonate and 3-hydroxybutyrate yield acetate and butyrate. As with relatively few anaerobes, *S. malonica* metabolizes simple dicarboxylic acids (e.g., malonate and succinate) by decarboxylation to the respective fatty acids.

SPOROMUSA OVATA. This organism was isolated from sugar beet leaf silage (Möller et al., 1984). Cells are Gram-negative, sporeforming, motile, curved rods. Growth is supported by a variety of substrates including H_2-CO_2, pyruvate, lactate, alcohols, fructose, betaine, dimethylglycine, and sarcosine. Acetate is the sole reduced end product; methylamines are formed from *N*-methyl compounds. Reductively dechlorinates tetrachloroethylene to trichloroethylene (Terzenbach and Blaut, 1994). Cultures demethylate the osmolytes dimethylsulfoniopropionate and glycine-betaine to methylthiopropionate and dimethylglycine, respectively; however, only the demethylation of glycine-betaine supports growth of the organism (Jansen and Hansen, 2001).

SPOROMUSA PAUCIVORANS. This species was isolated from lake sediment (Hermann et al., 1987). Cells are Gram-negative, nonsporeforming, motile, slightly curved rods. H_2-CO_2, formate, methanol, pyruvate, serine, betaine, alcohols, and ethylene glycol support growth. Acetate is the sole reduced end product. Oxidation of alcohols yields the corresponding fatty acids. Sugars are not utilized.

SPOROMUSA SILVACETICA. This organism was isolated from forest soil (Kuhner et al., 1997). Cells are Gram-negative, sporeforming, motile, slightly curved rods (Fig. 7). Growth occurs on H_2-CO_2, formate, methanol, pyruvate, vanillate, ferulate, fructose, betaine, fumarate, 2,3-butanediol, ethanol, lactate and glycerol. With most substrates, acetate is the main reduced end product. Fumarate is dismutated to acetate and succinate. Vanillate and ferulate are *O*-demethylated and reduced, respectively. Cells tolerate and consume small amounts of oxygen (Karnholz et al., 2002).

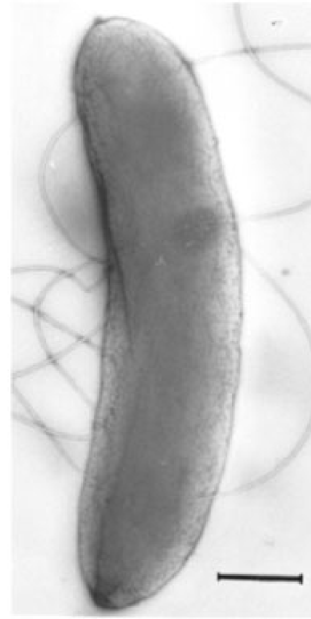


Fig. 7. Electron micrograph of a vegetative cell of *Sporomusa silvacetica* (DSM 10669^T) showing flagella inserting at the concave side of the cell. From Kuhner et al. (1997), used with permission from International Union of Microbiological Societies.

SPOROMUSA SPHAEROIDES. This species was isolated from river mud (Möller et al., 1984). Cells are Gram-negative, sporeforming, motile, curved rods. Growth occurs on H_2-CO_2, pyruvate, lactate, alcohols, glycerol, serine, ethyleneglycol, betaine, and other N-methyl compounds. Acetate is the sole reduced end product; methylamines are formed from N-methyl compounds. Cultures demethylate the osmolytes dimethylsulfoniopropionate and glycine-betaine to methylthiopropionate and dimethylglycine, respectively; however, only the demethylation of glycine-betaine supports growth of the organism (Jansen and Hansen, 2001).

SPOROMUSA TERMITIDA. This organism was isolated from the gut of a wood-feeding termite (Breznak et al., 1988). Cells of *S. termitida* are Gram-negative, sporeforming, motile, straight to slightly curved rods. Substrates include H_2-CO_2, CO, formate, methanol, ethanol, betaine, sarcosine, lactate, pyruvate, oxaloacetate, citrate, malonate, succinate, mannitol and trimethoxybenzoate. Acetate is the main reduced end product. As with *S. malonica*, *S. termitida* decarboxylates succinate to propionate (Breznak et al., 1988; Dehning et al., 1989). *Sporomusa termitida* grows mixotrophically, e.g., by utilizing H_2 and methanol or lactate at the same time (Breznak and Switzer Blum, 1991).

SYNTROPHOCOCCUS SUCROMUTANS. This organism is a Gram-negative, nonsporeforming, nonmotile, coccoid bacterium that was isolated as a dominant methoxybenzenoids-utilizer from the rumen contents of a steer (Krumholz and Bryant, 1986). *Syntrophococcus sucromutans* has a unique metabolism: growth with carbohydrates or pyruvate is only possible in the presence of electron acceptors such as formate, *O*-methyl groups, or a hydrogenotrophic methanogen (Krumholz and Bryant, 1986). Formate and *O*-methyl groups are metabolized via an acetyl CoA pathway that lacks formate dehydrogenase and is therefore incomplete (Doré and Bryant, 1990).

THERMOACETOGENIUM PHAEUM. This species is a thermophilic acetogen that was isolated from an anoxic pulp wastewater reactor (Hattori et al., 2000). Cells are Gram-positive, sporeforming, nonmotile, straight or slightly curved rods. Substrates include H_2-CO_2, formate, methanol, *n*-propanol, methoxylated benzoic acids, glycine and cysteine. Acetate is the sole reduced end product. Acetate is oxidized in the presence of hydrogenotrophic methanogens or an alternative electron acceptor (e.g., sulfate or thiosulfate); concomitantly, methane is produced by the syntrophic methanogen or the alternative electron acceptor is reduced. Its ability to oxidize acetate in syntrophic association with hydrogenotrophic methanogens is similar to that of two other anaerobic acetate oxidizers, strain AOR and *Clostridium ultunense* (Zinder and Koch, 1984; Lee and Zinder, 1988; Schnürer et al., 1996).

THERMOANAEROBACTER KIVUI. This species is a thermophilic acetogen that was isolated from lake sediments of Lake Kivu, Africa, and was first described as *Acetogenium kivui* (Leigh et al., 1981). On the basis of phylogenetic analysis of the 16S rRNA gene sequence, *A. kivui* was reclassified as *T. kivui* (Rainey et al., 1993; Collins et al., 1994). Cells are nonmotile, nonsporeforming rods often occurring in pairs or chains (Leigh et al., 1981; Fig. 8). The cell wall is covered by a hexagonally structured S-layer consisting of an 80-kDa protein (Rasch et al., 1984; Lupas et al., 1994). The temperature optimum is 66°C. Autotrophic growth occurs on H_2-CO_2, and heterotrophic growth occurs on glucose, mannose, fructose, pyruvate and formate; acetate is the main reduced end product (Leigh et al., 1981). Growth does not occur on CO-CO_2 (Daniel et al., 1990). *Thermoanaerobacter kivui* grows robustly on H_2-CO_2, a substrate that yields very slow, poor growth with most acetogens, and displays exceptionally high specific activities of hydrogenase and CO dehydrogenase (i.e., acetyl-CoA synthase) when cultivated chemolithoautotrophically on H_2-CO_2 (Daniel et al., 1990).

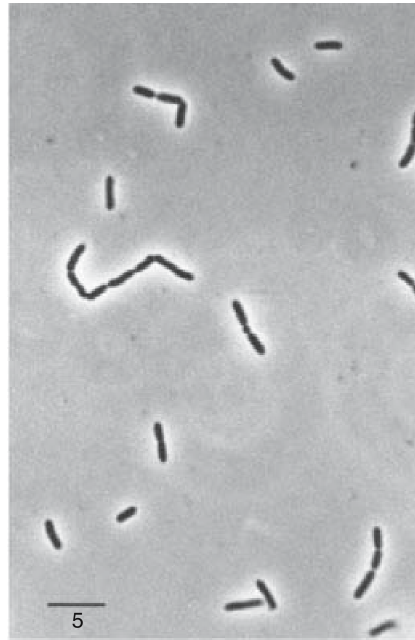


Fig. 8. Phase contrast photomicrograph of cells of *Thermoanaerobacter kivui*. Bar equals 5 μm. From Leigh et al. (1981), used with permission from Springer. The micrograph was kindly provided by R.S. Wolfe.

Cells tolerate and consume small amounts of oxygen (Karnholz et al., 2002).

"TREPONEMA PRIMITIA." This acetogenic spirochete was isolated from the hindgut of termites (Graber et al., 2004b; Graber and Breznak, 2004a). Growth occurs on H_2-CO_2, certain mono- and disaccharides, and methoxybenzoids. Can use H_2-CO_2 and organic compounds simultaneously. Requires folate for growth. Can tolerant low amounts of O_2; cells have NADH peroxidase and NADH oxidase activities.

Cultivation Methods

Even though many acetogens likely have the ability to tolerate small amounts of O_2 (Küsel et al., 2001; Karnholz et al., 2002; Boga and Brune, 2003), acetogens should be considered obligate anaerobes, and care should be taken in the laboratory to protect them from oxic conditions. The Hungate technique (Hungate, 1969) or modifications thereof are recommended for cultivation purposes. Growth media should be anoxic; sodium sulfide, cysteine, dithionite, or dithiothreitol are often used in cultivation media. Titanium (III) reducer has also been used; it may be less toxic than sulfide-based reducers (Zehnder and Wuhrman, 1976; Moench and Zeikus, 1983). Sulfide-based reducers can decrease the number of acetogens obtained from aerated soils (Küsel et al., 1999c). Cadmium

chloride ($CdCl_2$) has also been used as reducing agent for acetogen media (Breznak and Switzer, 1986; Breznak et al., 1988).

Table 3 outlines the contents of a medium that can be used for the cultivation of most known acetogens. In general, acetogens prefer near neutral to slightly alkaline pH. However, many decades ago, Wieringa (1941) reported that enrichment of acetogens under alkaline conditions (pH 8–9) might favor their isolation. It is thus noteworthy that the first haloalkaliphilic acetogens, *N. histidinovorans* and *N. acetigena*, were recently isolated from soda-depositing lakes; these acetogens only grow between pH 8 and 11 (Zhilina et al., 1996; Zhilina et al., 1998). Obviously, designing media for the cultivation of acetogens must take into consideration the in situ conditions of the habit under investigation.

Since CO_2 is used as a terminal electron acceptor by acetogens, many acetogens cannot grow under certain conditions unless CO_2 is readily available (see the section on CO_2 as Terminal Electron Acceptor and the Concept of Fermentation in this Chapter). Thus, acetogenesis is optimized in acetogen media containing a source of CO_2. Although some chemolithoautotrophic acetogens grow without any trace organic nutrients (e.g., *T. kivui*; Leigh et al., 1981), many acetogens require supplemental vitamins. The inclusion of trace metals in acetogen media is important because acetogens are rich in metalloenzymes (Ljungdahl, 1986). Many acetogens have unknown nutritional factors. For example, the protocol used to elucidate the nutritional requirements of *M. thermoacetica* (Lundie and Drake, 1984) and *M. thermoautotrophica* (Savage and Drake, 1986) was not successfully applied to *R. productus* (supplemental vitamins, amino acids, fatty acids, and other nutrients did not substitute for unknown growth factors in yeast extract; thus, the nutritional requirements for *R. productus* remain unresolved; H. L. Drake and coworkers, unpublished data).

The substrate used to enrich and isolate an acetogen can be selective for a certain catabolic type. H_2-CO_2 is quite often selective for acetogens and has been used for the isolation of numerous acetogens (e.g., *T. kivui* [Leigh et al., 1981], *A. woodii* [Balch et al., 1977], and *S. termitida* [Breznak et al., 1988]). CO-CO_2 is also selective (e.g., *R. productus*; Lorowitz and Bryant, 1984; Geerligs et al., 1987). The capacity to utilize methoxylated aromatic compounds is a widespread catabolic potential of acetogens; thus, methoxylated aromatic compounds can also be used to selectively enrich and isolate acetogens (e.g., *A. woodii*; Bache and Pfennig, 1981). Many isolates have been enriched and isolated with compounds not typically utilized by anaerobes. Examples of such substrates include mandelate (*Acetobacterium* strain AmMan1; Dörner and Schink, 1991), trimethylamine (*A.*

Table 3. Contents of a typical acetogen medium.[a]

Salts	mg/liter	Vitamins	mg/liter
NaCl	400	Nicotinic acid	0.25
NH_4Cl	400	Cyanocobalamin	0.25
$MgCl_2 \cdot 6H_2O$	330	*p*-Aminobenzoic acid	0.25
$CaCl_2 \cdot 2H_2O$	50	Calcium d-pantothenate	0.25
		Thiamine·HCl	0.25
Trace elements	**mg/liter**	Riboflavin	0.25
$MnSO_4 \cdot H_2O$	2.5	Lipoic acid	0.30
$FeSO_4 \cdot 7H_2O$	0.5	Folic acid	0.1
$Co(NO_3)_2 \cdot 6H_2O$	0.5	Biotin	0.1
$ZnCl_2$	0.5	Pyridoxal HCl	0.05
$NiCl_2 \cdot 6H_2O$	0.25		
H_2SeO_4	0.25	**Buffer for pH 6.7 media**	**mg/liter**
$CuSO_4 \cdot 5H_2O$	0.05	$NaHCO_3$	7,500
$AlK(SO_4)_2 \cdot 12H_2O$	0.05	KH_2PO_4	500
H_3BO_3	0.05	Gas phase	100% CO_2
$Na_2MoO_4 \cdot 2H_2O$	0.05		
$Na_2WO_4 \cdot 2H_2O$	0.05		
		Buffer for pH 7.8 media	**mg/liter**
Reducers	**mg/liter**	$NaCO_3$	6,000
Cysteine·HCl·H_2O	250	$Na_2CO_3 \cdot 10H_2O$	16,500
Sodium sulfide	250	KH_2PO_4	500
		Gas phase	100% N_2

[a]Media should be prepared using anoxic techniques, and resazurin (1 mg/liter) can be used as a redox indicator. Media can be enriched with yeast extract (0.5–2 g/liter), tryptone (0.5–2 g/liter), clarified rumen fluid (50 ml/liter), or casamino acids (0.5–2 g/liter). Crimp-sealed tubes or bottles are recommended for cultivation. Compiled from Daniel et al. (1990) and Matthies et al. (1993).

arabaticum; Zhilina and Zavarzin, 1990), methoxyacetate (strain RMMac1; Schuppert and Schink, 1990), and methyl chloride ("*Acetobacterium dehalogenans*" [formerly named "strain MC"; Traunecker et al., 1991]). Isolation can be accomplished by various methods, such as the roll-tube method of Hungate (1969), agar shake dilution series (Pfennig, 1978), or streaking/plating on agar or Gelrite® (preferred for thermophiles because of its increased capacity to remain solid at higher temperatures). Methanogens can usually be excluded during enrichment and isolation with 10–50 mM bromoethanesulfonate (an analog of coenzyme M; Gunsalus et al., 1978; Zehnder et al., 1980; Smith and Mah, 1981; Greening and Leedle, 1989; Kane and Breznak, 1991a). Lumazine (2,4-[1H,3H]-pteridinedione) is also an inhibitor of methanogens (*Methanobacterium thermoautotrophicum* does not grow in media containing 0.1 mM Lumazine; Nagaranthal and Nagle, 1992) and might also be useful in such enrichments. Lumazine appears to inhibit methyl-S-CoM reductase (Nagaranthal and Nagle, 1992). Likewise, N-substituted derivatives of *p*-aminobenzoic acid inhibit the enzyme responsible for the synthesis of methanopterin (an intermediate in methanogenesis) and can block the growth of certain methanogens but do not interfere with the growth of acetogens (Dumitru et al., 2003).

Taxonomy and Phylogeny

Acetogens do not form a taxonomic group of closely related organisms. Although utilization of the acetyl-CoA pathway unifies them, they are extremely diverse genetically; the G+C content of their genomes varies from 22 mol% for *C. ljungdahlii* to 62 mol% for *H. foetida* (Table 2). However, all acetogenic isolates to date are members of the domain Bacteria, including the extreme halophilic and haloalkaliphilic isolates *A. arabaticum* and *N. acetigena*, respectively (Zhilina and Zavarzin, 1990; Zhilina et al., 1996). The phylogeny of most acetogens with validated genus and species names and a couple of other acetogens not yet included in the "List of Bacterial Names with Standing in Nomenclature" (http://www.bacterio.cict.fr) has been determined in either comprehensive analyses of bacteria (e.g., within a certain bacterial group, such as the clostridia) or in the first description of a respective species. Analyses of phylogenetic relationships resulted in the reclassification of several acetogenic species (Table 4).

Within the phylogenetic tree of the Bacteria, acetogens are found in more than one phylum and do not form tight clusters like methanogens or sulfate-reducing bacteria. However, most acetogens are affiliated with the phylum of the Gram-positive bacteria with low DNA G+C-content (Fig. 9). One genus that harbors acetogenic species, *Treponema* (Fig. 5), is affiliated with the phylum Spirochetes. *Holophaga*, a genus with only one species isolated to date, *H. foetida*, represents a new line of descent; together with the non-acetogen *Geothrix fermentans*, *H. foetida* was one of the two founding species of the phylum *Holophaga*/*Acidobacterium* (Liesack et al., 1994; Ludwig et al., 1997; Leadbetter et al., 1999; Lilburn et al., 1999). The 16S rRNA genes of the species belonging to the genus *Acetoanaerobium* have not been sequenced; thus, the phylogenetic positions of the acetogens *A. notorae* and "*A. romashkovii*" are unclear. On the basis of morphological and physiological properties, *A. notorae* has been tentatively assigned to the Bacteroidaceae (Sleat et al., 1985).

Acetogens affiliate with 8 (I, V, VI, IX, XI, XII, XIVa, XV) out of the 19 *Clostridium* clusters within the phylum of the Gram-positive bacteria with low DNA G+C-content (Collins et al., 1994; Zhilina et al., 1998; Stackebrandt et al., 1999; Fig. 9). Although some genera (e.g., *Acetobacterium*, *Sporomusa* and *Moorella*) are composed exclusively of acetogenic species, many acetogens in these phylogenetic clusters are very closely related to non-acetogenic bacteria (Fig. 10). Indeed, genera often include acetogenic and non-acetogenic species (e.g., *Clostridium*, *Ruminococcus*, *Eubacterium* and *Thermoanaerobacter*). For example, the closest relative of *C. formicoaceticum* is the non-acetogen *Clostridium felsineum* (99.3% sequence similarity). In

Table 4. Acetogens that have been reclassified.

Described as	Reclassified as	Reference
Acetogenium kivui	*Thermoanaerobacter kivui*	Collins et al., 1994
Clostridium fervidus[a]	*Caloramator fervidus*	Collins et al., 1994
Clostridium thermoaceticum	*Moorella thermoacetica*	Collins et al., 1994
Clostridium thermoautotrophicum	*Moorella thermoautotrophica*	Collins et al., 1994
Clostridium pfennigii	*Oxobacter pfennigii*	Collins et al., 1994
Peptostreptococcus productus	*Ruminococcus productus*	Ezaki et al., 1994

[a]It is not certain that this organism is an acetogen.

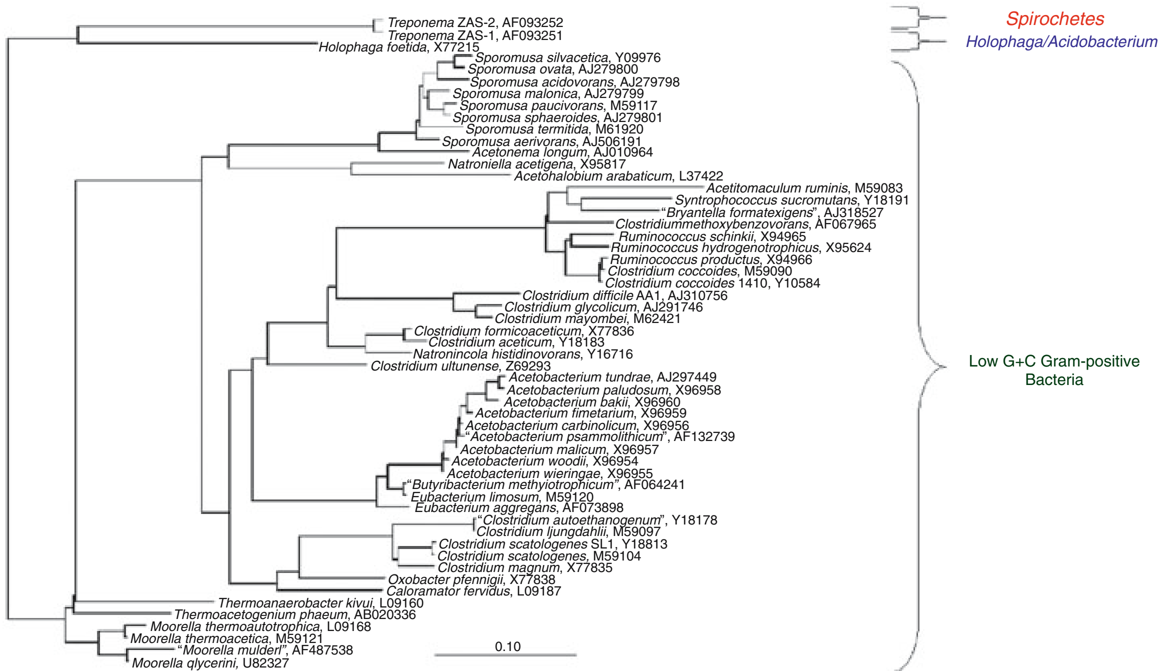


Fig. 9. Parsimony tree containing 20 genera of acetogenic bacteria; tree is based on full length 16S rRNA sequences (ARB-release June 2002). The genus *Acetoanaerobium* (Table 2) is not represented in the tree because a sequence for a species of this genus is not available. Comparison of the tree topology with maximum-likelihood and neighbor-joining based trees suggests that the phylogeny of acetogens remains to be resolved. Numbers are sequence accession numbers. Bar corresponds to 10 nucleotide substitutions per 100 sequence positions. Though unavailable when the tree was constructed, please note that the two *Treponema* strains have been recently named "*T. primitia*" (Graber et al., 2004b).

some cases, clostridial species were not originally described as acetogens but were later discovered to possess acetogenic capabilities. For example, *C. coccoides* strain 1410 and *C. scatologenes* strain SL1 were isolated as acetogens, and subsequent physiological studies revealed that the type strains of these two species also have acetogenic capabilities (Kamlage et al., 1997; Küsel et al., 2000). The opposite is also true, as seen in the case of *C. glycolicum*. Two strains of *C. glycolicum*, strain RD-1 and strain 22, are acetogens, yet the type strain of *C. glycolicum* does not grow acetogenically (Ohwaki and Hungate, 1977; Küsel et al., 2001). Thus, certain acetogens might lose their acetogenic capabilities after prolonged laboratory cultivation. An acetogenic strain of *C. difficile* (AA1) has been isolated from the rumen contents of a newborn lamb; whether the pathogenic type strain of *C. difficile* has acetogenic capabilities is unknown (Hall and O'Toole, 1935; Cato et al., 1986; Rieu-Lesme et al., 1998).

Detection of Acetogens

On agar or Gelrite®acetogenic colonies can be differentiated from non-acetogenic colonies by their ability to form 1) clear zones on media containing calcium carbonate (Balch et al., 1977) or 2) colored zones on media supplemented with a pH indicator (e.g., bromocresol green; Braun et al., 1979; Leedle and Greening, 1988). A colorimetric most-probable-number assay can be utilized to enumerate acetogens that are capable of *O*-demethylating methoxylated lignin derivatives (e.g., vanillate; Harriott and Frazer, 1997). This method based on a yellowing colorimetric reaction between Ti(III) and the vicinal hydroxyl groups of the *O*-demethylated aromatic product (Moench and Zeikus, 1983; Kreft and Schink, 1993; Harriott and Frazer, 1997) has been used to detect acetogens on the roots of the sea grass *Halodule wrightii* (Küsel et al., 1999b).

The occurrence of acetyl-CoA synthase in an isolate can be considered good evidence that the organism is an acetogen. As outlined in the subsection on Enzymology of the Acetyl-CoA Pathway, this enzyme catalyzes two reactions that can be assayed. One of these reactions, i.e., that of CO dehydrogenase, is inexpensive and easily measured by monitoring the reduction of an artificial electron carrier (e.g., methyl viologen) with a spectrophotometer. Thus, CO dehydrogenase activity is often used to assess

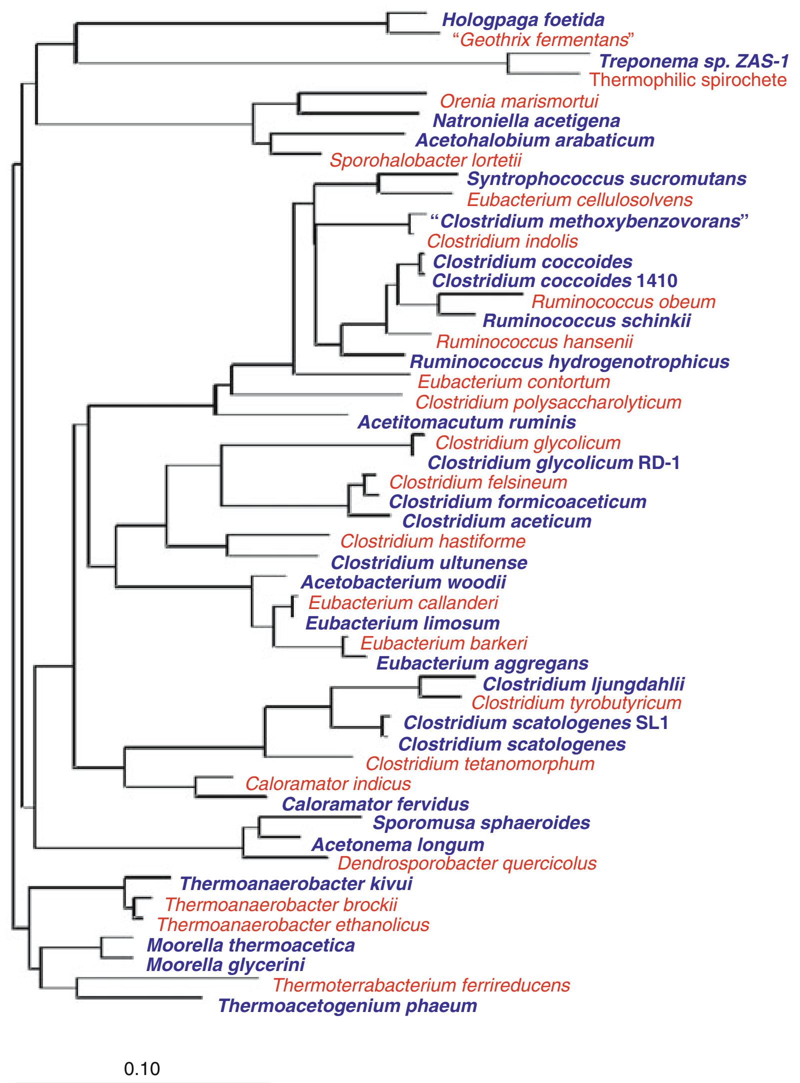


Fig. 10. Parsimony tree of selected acetogenic bacteria (blue, bold font) and their closest nonacetogenic relatives (red, non-bold font) based on full length 16S rRNA sequences (ARB-release December 1998). Bar corresponds to 10 nucleotide substitutions per 100 sequence positions.

acetyl-CoA synthase. However, the occurrence of CO dehydrogenase activity can be misleading and cannot be taken as definitive evidence that an organism utilizes the acetyl-CoA pathway. For example, the CO dehydrogenase activity of *Clostridium pasteurianum* (Fuchs et al., 1974; Thauer et al., 1974; Diekert and Thauer, 1978) implies that this bacterium is an acetogen, but it is not. It is therefore essential that additional evidence (e.g., precise substrate/product stoichiometries or additional enzymological information) be obtained before concluding that an organism is an acetogen (i.e., utilizes the acetyl-CoA pathway). Although the acetyl-CoA synthase assay is more difficult than that for CO dehydrogenase, acetyl-CoA synthase activity can be assayed with the pyruvate/CO-coupled methyltetrahydrofolate assay (Schulman et al., 1973; Drake et al., 1981a; Hu et al., 1982). This activity is much more conclusive relative to the acetogenic nature of an organism. The acetyl-CoA synthase assay is classically based on the use of [$^{14}CH_3$]-methyltetrahydrofolate and the measurement of ^{14}C-labeled acetate (which is derived from the ^{14}C-labeled acetyl-CoA that is formed by acetyl-CoA synthase). However, a method that does not involve the use of ^{14}C can also be used; in this assay, the acetyl-CoA formed by acetyl-CoA synthase is converted to acetate, which is then measured by high performance liquid chromatography (Fröstl et al., 1996). The activities obtained with the CO dehydrogenase and acetyl-CoA synthase activity assays do not always yield parallel results, and the two respective activities can vary differentially (Kellum and Drake, 1986). In general, caution is needed when drawing conclusions based on results obtained with these assays.

The apparent importance of acetogens to the overall flow of carbon, reductant, and energy in

many habitats has generated an interest in assessing acetogenic populations in environmental samples with molecular methods. Unfortunately, based on their 16S rRNA gene sequences, acetogens are not a monophyletic group, and many acetogens are very closely related to non-acetogenic species (see the subsection on Taxonomy and Phylogeny in this Chapter). Thus, the development of 16S rRNA oligonucleotide probes and primers that exclusively target all known acetogens is likely an impossible task. Nonetheless, a 16S rRNA oligonucleotide probe (probe AW) has been developed that targets the acetogenic species that form a phylogenetically tight cluster in the genus *Acetobacterium*; this probe is also specific for the acetogen *E. limosum* (Küsel et al., 1999b). Another 16S rRNA oligonucleotide probe (probe Clost I) is specific for a few acetogens and many non-acetogens in clostridial clusters I and II (Küsel et al., 1999b).

Oligonucleotide probes or primers that target functional genes can also be used to assess microorganisms in environmental samples, and this approach has been used to evaluate several microbial groups, including ammonia-oxidizing bacteria, denitrifying bacteria, and nitrogen-fixing bacteria (Rotthauwe et al., 1997; Braker et al., 2000; Lovell et al., 2000). Formyltetrahydrofolate synthetase (FTHFS) is one of the key enzymes in the acetyl-CoA pathway and catalyzes the ATP-dependent synthesis of formyltetrahydrofolate from formate and tetrahydrofolate. FTHFS is highly conserved in acetogens. A FTHFS-based functional group-specific DNA probe and FTHFS-based primers for polymerase chain reaction (PCR) amplification of partial FTHFS gene sequences have been utilized for evaluating the occurrence of acetogens in environmental samples and complex natural populations (Lovell and Hui, 1991; Lovell, 1994; Leaphart and Lovell, 2001; Leaphart et al., 2003; Salmassi and Leadbetter, 2003).

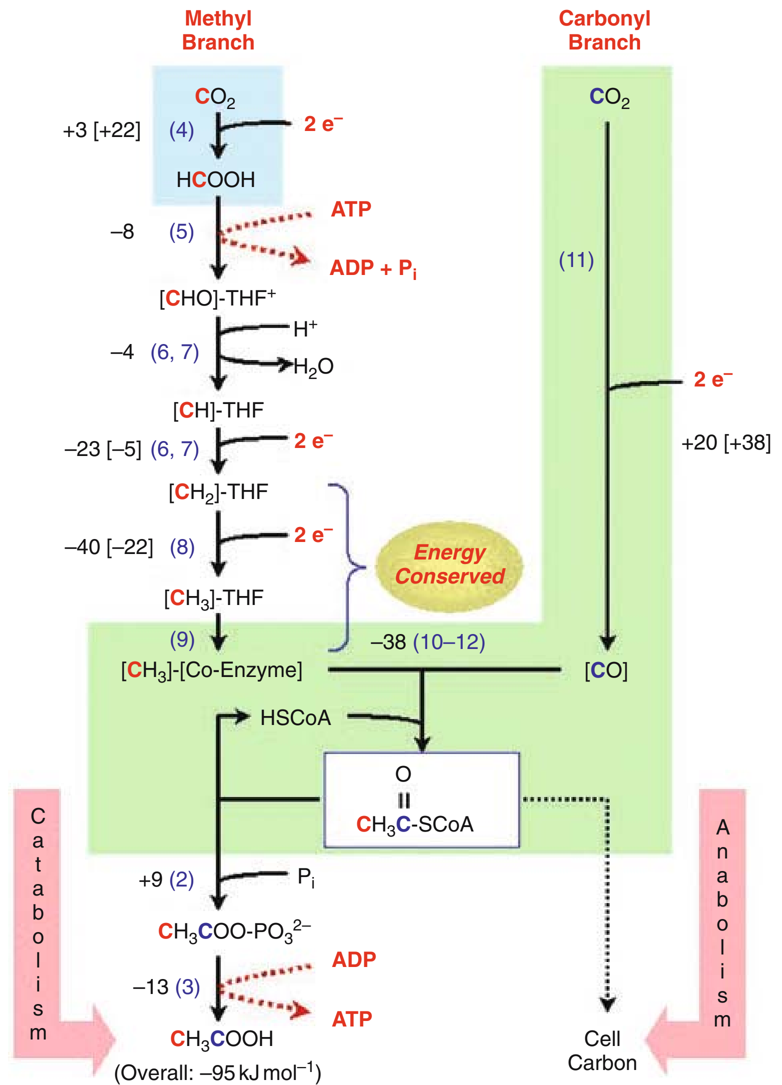
The Acetyl-CoA Pathway and Bioenergetics

The acetyl-CoA pathway is composed of two reductive branches (i.e., the methyl branch and the carbonyl branch), both of which reduce CO_2 and fix CO_2-derived carbon into covalently bond forms (Fig. 11). The acetyl-CoA pathway can be presented in a cyclic form (e.g., Wood and Ljungdahl, 1991b; Ragsdale, 1997); however, it is a linear process that does not depend on multicarbon intermediates to which CO_2 is fixed in a cyclic fashion (e.g., the Calvin cycle and the reductive tricarboxylic acid cycle are cyclic, CO_2-fixing processes that are dependent upon ribulose biphosphate and oxalacetate, respectively, for the initial fixation of CO_2). Although the cofactors and electron carriers of the pathway cycle between different states, the pathway itself is linear relative to carbon flow (Fig. 11).

The acetyl-CoA pathway is a terminal electron-accepting process that also provides a mechanism for the assimilation of CO_2 and other sources of carbon into biomass (Ljungdahl and Wood, 1965; Eden and Fuchs, 1982; Eden and Fuchs, 1983; Ljungdahl, 1986). The main function of the pathway may vary with the growth conditions of the cell. The biochemistry of the acetyl-CoA pathway has been described in numerous reviews (Ljungdahl, 1986; Ragsdale, 1991; Ragsdale, 1994; Ragsdale, 1997; Ragsdale, 2004; Wood, 1991a; Wood and Ljungdahl, 1991b; Diekert and Wohlfarth, 1994a; Diekert and Wohlfarth, 1994b; Drake, 1994; Ragsdale and Kumar, 1996; Drake et al., 1997; Das and Ljungdahl, 2000; Drake and Küsel, 2003); many of these references also provide historical perspectives on the studies that resolved the enzymological features of the pathway. A gateway to some of the more recent studies on the enzymology of the acetyl-CoA pathway can be found in the following references: Maynard and Lindahl (Maynard and Lindahl, 1999; Maynard and Lindahl, 2001), Furdui and Ragsdale (2000), Radfar et al. (2000), Ragsdale (Ragsdale, 2000; Ragsdale, 2003a; Ragsdale, 2003b; Ragsdale, 2004), Müller et al. (2001), Müller et al. (2004), Leaphart et al. (2002), Lindahl (2002), Banerjee and Ragsdale (2003), Das and Ljungdahl (2003), Bramlett et al. (2003), Darnault et al. (2003), Grahame (2003), and Loke and Lindahl (2003).

Acetyl-CoA synthase is a centrally important enzyme of the acetyl-CoA pathway, and many prokaryotes make use of enzymes that are closely related to the acetyl-CoA synthase of acetogens (see the subsections on Enzymology of the Acetyl-CoA Pathway and Occurrence of the Acetyl-CoA Pathway in Nonacetogenic Microorganisms in this Chapter). Numerous theortetical considerations suggest that an acetyl-CoA synthase-dependent pathway may have constituted the first autotrophic process on earth (Fuchs, 1986; Wood and Ljungdahl, 1991b; Lindahl and Chang, 2001). It is uncertain whether the pathway first evolved for the purpose of carbon assimilation (i.e., the reduction and fixation of CO_2) or the oxidation of acetate. Phylogenetic evaluations of acetyl-CoA synthases indicate that microorganisms (e.g., acetogens and methanogens) that have this enzyme, or enzymes that are closely related to it, had a common ancestor (Lindahl and Chang, 2001). This section will focus on some of the biochemical and enzymological features of the acetyl-CoA pathway of acetogens.

Fig. 11. The acetyl-CoA "Wood-Ljungdahl" pathway. The numbers (in black) adjacent to the reactions are standard Gibb's free energies in kJ mol^{-1} (values have been rounded off). For the four reactions in which reducing equivalents are involved, the bracketed and non-bracketed values are Gibb's free energies when the reducing equivalents are derived from either H_2 or reduced NAD/NADP, respectively. For the reactions in which acetyl-CoA is synthesized from CH_3-THF, HSCoA, and [CO], the Gibb's free energy is not known. Different values have been calculated for this reaction (e.g., –22 kJ mol^{-1} in Fuchs [1986], and –49 kJ mol^{-1} in Fuchs [1994]). The value shown in the figure is an estimate that is based on the overall thermodynamic value of the pathway (i.e., –95 kJ mol^{-1} [Fuchs, 1986; Fuchs, 1994]). The parenthetically enclosed numbers (in blue) identify the different enzymes involved (see Table 5 for characteristics of enzymes). The two enzymes responsible for the initial reduction of CO_2 are formate dehydrogenase and acetyl-CoA synthase; reactions in which these two enzymes are involved are shaded in blue and green, respectively. Abbreviations: THF, tetrahydrofolate; HSCoA, coenzyme A; P_i, inorganic phosphate; e^-, reducing equivalent; and Co-Enzyme, corrinoid enzyme.



CO_2 as Terminal Electron Acceptor and the Concept of Fermentation

The main function of the acetyl-CoA pathway during growth on sugars is the recycling of reduced electron carriers (NAD, ferredoxin, etc.; Fig. 12). The 8 reducing equivalents that are collectively generated during glycolysis and the oxidation of pyruvate are used to reduce CO_2 to acetate via the acetyl-CoA pathway. During growth on glucose, the cell has ready access to ATP formed via substrate-level phosphorylation and to biosynthetic precursors (via the breakdown of glucose). Thus, the lithoautotrophic functions of the pathway (i.e., the conservation of energy and the production of acetyl-CoA for anabolism and the assimilation of carbon) are probably of minor importance under such conditions.

CO_2 is the terminal electron acceptor of acetogens when they are grown under acetogenic conditions (see the subsection on Use of Diverse Terminal Electron Acceptors in this Chapter); it is therefore important to include an adequate supply of CO_2 (or carbonates) in the growth medium when acetogens are cultivated in the laboratory (see the subsection on Cultivation Methods in this Chapter). This point may seem obvious, but it is not generally appreciated why supplemental CO_2 is essential to the growth of acetogens, since the stoichiometries for the synthesis of acetate from numerous substrates do not indicate that supplemental CO_2 is required. For example, even though the stoichiometric conversion of sugars (e.g., glucose or fructose) to acetate (reaction 2 above) indicates that supplemental CO_2 is not required for acetogenesis, growth on sugars, as well as the metabolism of

Table 5. Properties of the enzymes in the Acetyl CoA pathways.[a]

Number in the figures[a]	Enzyme	M_r	Subunit composition	Function in pathways[a]	ΔG^{r}_{o} ($kJ\,mol^{-1}$)[b]	Primary/historical reference
1	Pyruvate-Fd oxidoreductase	225,000	α_2	Oxidation/decarboxylation of pyruvate to acetyl-CoA	–19	Drake et al., 1981a
2	Phosphotransacetylase	88,100	α_4	Conversion of acetyl-CoA to acetylphosphate	+9	Drake et al., 1981a
3	Acetate kinase	60,000	n.r.	Conversion of acetyl-CoA and ADP to acetate and ATP	–13	Schaupp and Ljungdahl, 1974
4	Formate dehydrogenase	340,000	$\alpha_2\beta_2$ (96,000 and 76,000)	NADPH-dependent reduction of CO_2 to formate	+3 (+22)	Yamamoto et al., 1983
5	Formyltetrahydrofolate (HCO-THF) synthetase	240,000	α_4	Conversion of formate to HCO-THF	–8	Mayer et al., 1982
6, 7	Methenyltetrahydrofolate (CH-THF) cyclohydrolase and methylenetetrahydrofolate (CH_2-THF) dehydrogenase complex	55,000	α_2	HCO-THF converted to CH-THF, and CH-THF reduced to CH_2-THF	–4 –23 (–5)	O'Brien et al., 1973
8	Methylenetetrahydrofolate (CH_2-THF) reductase	289,000	α_8	Reduction of CH_2-THF to CH_3-THF	–40 (–22)	Park et al., 1991
9	Methyltransferase	58,900	α_2	Transfer of methyl unit from CH_3-THF to coninoid enzyme		Drake et al., 1981a, b
10	Coninoid enzyme	89,000	$\alpha\beta$ (34,000 and 55,000)	methylation of acetyl-CoA synthase		Hu et al., 1984
11	Acetyl-CoA synthase	440,000	$\alpha_3\beta_3$ (78,000 and 71,000) (81,730 and 72,928)[c]	Reduction of CO_2 to the CO level, and synthesis of acetyl-CoA from CH_3-THF, CO, and CoASH	+20 (+38) –22, –49[d]	Ragsdale et al., 1983 Diekert and Ritter, 1983
12	Acetyl-CoA synthase disulfide reductase	225,000	α_4	Reduction of disulfide of CoA binding site of acetyl-CoA synthase		Pezacka, Wood, 1986

Abbreviations: n.r., not reported; THF, tetrahydrofolate; CoA, coenzyme A; and Fd, ferredoxin.

[a]See Figs. 11 and 12. Enzymes indicated have been purified from *M. thermoacetica*.

[b]Gibb's free energies (G^{r}_{o}) have been rounded off and were obtained from Thauer et al. (1977) and Fuchs (1986). Parenthetical values for reactions catalyzed by oxidoreductases are the Gibb's free energies when the reducing equivalents are derived from reduced NAD/NADP rather than H_2.

[c]Based on amino acid composition. Current information indicates that the correct composition is $\alpha_2\beta_2$.

[d]Actual value is unknown. Different values have been calculated for this reaction (e.g., $-22\,kJ\,mol^{-1}$ in Fuchs [1986], and $-49\,kJ\,mol^{-1}$ in Fuchs [1994]).

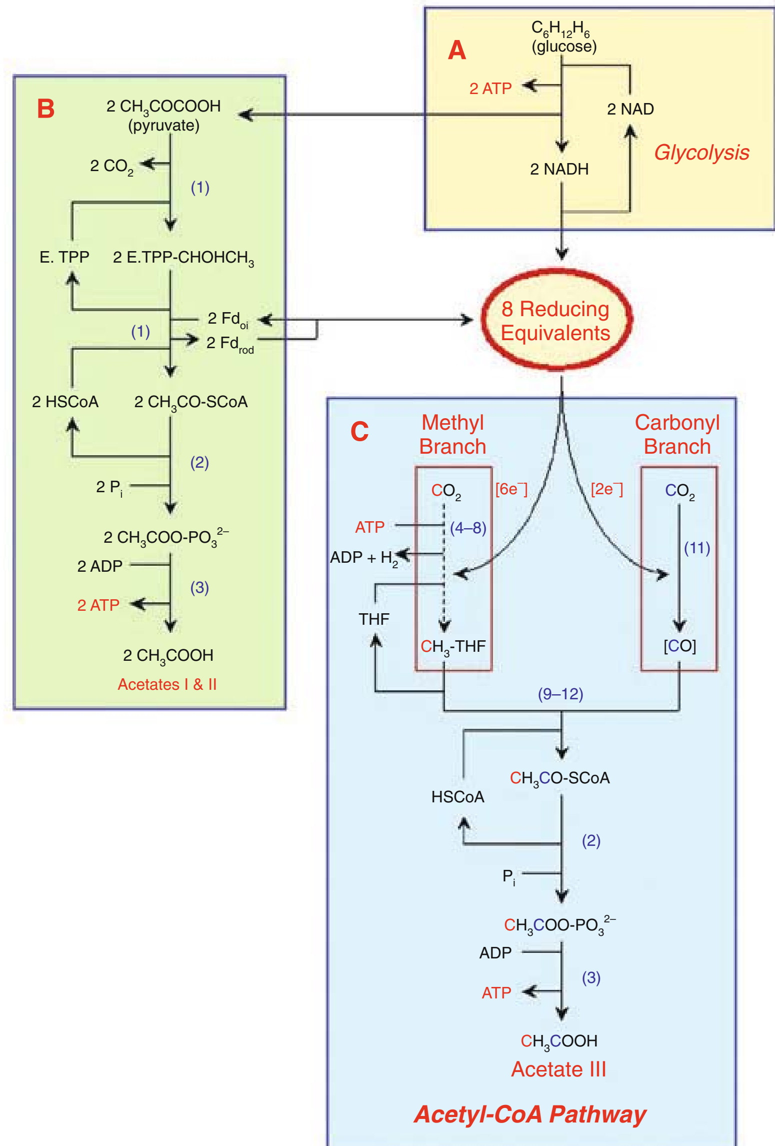


Fig. 12. Transfer of reductant from glycolysis (Box A) and the oxidation of pyruvate (Box B) to the acetyl-CoA pathway (Box C). The overall scheme was elucidated from studies with *Moorella thermoacetica*. The parenthetically enclosed numbers (in blue) identify the different enzymes involved (see Table 5 for characteristics of enzymes). Abbreviations: THF, tetrahydrofolate; E.TPP, enzyme.thiamine pyrophosphate; Fd, ferredoxin; HSCoA, coenzyme A; P_i, inorganic phosphate; and e^-, reducing equivalent.

sugars, may be significantly impaired in the absence of supplemental CO_2 (Andreesen et al., 1970; O'Brien and Ljungdahl, 1972; Braun and Gottschalk, 1981).

The reason why exogenous CO_2 is required for optimal growth on glucose is illustrated in Fig. 13. Supplemental CO_2 is required for the recycling of reduced electron carriers, and this intracellular management of reductant must occur before glucose-derived CO_2 becomes available via the decarboxylation of pyruvate. In support of the scheme illustrated in Fig. 12, early ^{14}C studies demonstrated that carbons 3 and 4 of glucose mostly enter the pool of CO_2 rather than acetate (Wood, 1952b; O'Brien and Ljungdahl, 1972). Thus, approximately one-third of the ^{14}C from [U-^{14}C]glucose is recovered in the exogenous CO_2 pool when *M. thermoacetica* is grown on [U-^{14}C]glucose; the other two-thirds of the ^{14}C is recovered as [^{14}C]acetate (D. R. Martin and H. L. Drake, unpublished data; see also Martin et al., 1985).

Exogenous CO_2 can even be required for CO-dependent acetogenesis, a process that generates excess CO_2 (Savage et al., 1987):

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (9)$$

The acetogenic utilization of highly reduced one-carbon substrates is strictly dependent upon the availability of exogenous CO_2. Thus, acetogenic cultures of *M. thermoacetica* and *C. formicoaceticum* cannot be maintained on methanol in the absence of supplemental CO_2 (Hsu et al., 1990a; Matthies et al., 1993). The importance of CO_2 to acetogens is exemplified by their ability to

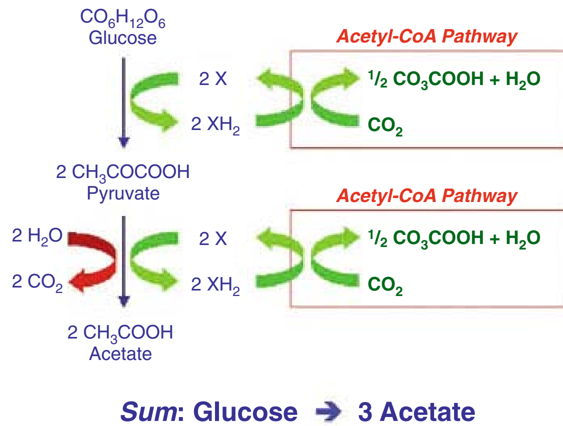


Fig. 13. Importance of CO_2 for the recycling of reduced electron carriers (XH_2) during the acetogenic oxidation of glucose. Under certain growth conditions, exogenous CO_2 is required for growth, even though CO_2 is produced during catabolism (e.g., via the decarboxylation of pyruvate). Modified from Drake (1994).

generate growth-essential CO_2-equivalents from various compounds, including carboxylated lignin derivatives (e.g., vanillate; Hsu et al., 1990a; Hsu et al., 1990b).

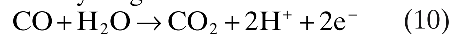
Acetogenesis is often referred to as a fermentation. Since the term "fermentation" implies that an organism uses a partially oxidized carbonaceous intermediate as a terminal electron acceptor (e.g., when pyruvate is reduced to lactate in lactate fermentation), usage of this term for acetogenesis in not fully valid. For example, as outlined above, it appears that exogenous CO_2 rather than that generated from the decarboxylation of pruvate is used as the terminal electron acceptor when glucose is metabolized. Likewise, the CO_2 that is derived from the decarboxylation of aromatic compounds and subsequently used as a terminal electron acceptor in the acetyl-CoA pathway is fundamentally dissimilar to the use of an oxidized intermediate as a terminal electron acceptor in fermentation. Furthermore, referring to acetogenesis as a fermentation does not properly reflect the chemiosmotic manner in which energy is conserved via the acetyl-CoA pathway and plasma membrane ATPases (see the subsection on Conservation of Energy and Bioenergetics in this Chapter).

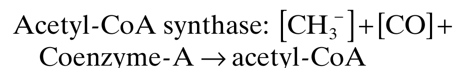
Enzymology of the Acetyl-CoA Pathway

The initial reactions on the methyl and carbonyl branches of the pathway are catalyzed by formate dehydrogenase and acetyl-CoA synthase, respectively. These two enzymes are responsible for reductive reactions that are thermodynamically very unfavorable (i.e., have positive Gibb's free energies under standard conditions; Fig. 11). Formate dehydrogenase from *M. thermoacetica* is rich in metals (2 moles of selenium, 2 moles of tungsten, and approximately 36 moles of iron per mole of enzyme) and was the first enzyme in which tungsten was shown to be a biologically active trace metal (Yamamoto et al., 1983; Ljungdahl, 1986). The tetrahydrofolate pathway facilitates the subsequent reduction of formate to the methyl level (Ljungdahl, 1986). Acetyl-CoA synthase is a nickel-containing enzyme, reduces CO_2 to the carbonyl (CO) level, and facilitates the synthesis of acetyl-CoA (Wood and Ljungdahl, 1991b). The acetyl-CoA that is formed by acetyl-CoA synthase is subsequently converted to acetate during catabolism or utilized in the synthesis of cell carbon during anabolism (Fig. 11). The Gibb's free energy for the overall reduction of 2 moles of CO_2 to 1 mole of acetate is approximately –95 kJ mol^{-1}.

The general properties of the enzymes involved in the acetyl-CoA pathway are outlined in Table 5. Many of the enzymes from acetogens are extremely susceptible to inactivation by oxidation. For example, formate dehydrogenase and acetyl-CoA synthase are among the most oxygen-sensitive enzymes known. Thus, the use of O_2-free chambers has become routine for studying the enzymes central to acetogenesis. Although all of the enzymes of the acetyl-CoA pathway are important to the functionality of the pathway, several of the enzymes are worthy of special note:

a) Acetyl-CoA synthase catalyzes two reactions and is often referred to by two names (Diekert and Thauer, 1978; Drake et al., 1980; Ragsdale et al., 1983; Wood and Ljungdahl, 1991b; Ragsdale, 1994):

CO dehydrogenase:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \quad (10)$$


$$\text{Acetyl-CoA synthase: } [CH_3^-] + [CO] + \text{Coenzyme-A} \rightarrow \text{acetyl-CoA} \quad (11)$$

The discovery of the ability of this enzyme to catalyze reaction 10 (Diekert and Thauer, 1978) was paramount to later studies that resolved the physiological importance of the enzyme in reaction 11 (see Table 1 and the section on Historical Perspectives in this Chapter). In the acetyl-CoA pathway, acetyl-CoA synthase reduces CO_2 to CO and subsequently fixes this CO_2-derived carbon (i.e., CO) into an organic form (i.e., in acetyl-CoA). Acetyl-CoA synthase catalyzes the thermodynamically least favorable reaction in the pathway (Fig. 11), a fact that might partially explain why this enzyme can represent up to 2% of the soluble cell protein of an acetogen (Ragsdale et al., 1983). That acetyl-CoA synthase was discovered as CO dehydrogenase resulted in the

acetyl-CoA pathway being sometimes referred to as the "CO dehydrogenase pathway." This nomenclature is less than ideal as it does not accurately portray the physiological function of the enzyme in the pathway and also does not differentiate the enzyme (and thus the pathway) from the CO dehydrogenase used by aerobic carboxydotrophs that grow via the oxidation of CO to CO_2 (Meyer, 1988; Meyer et al., 1993; Meyer et al., 2000).

Recent studies on the crystal structure of the enzyme from *M. thermoacetica* have shown that the α subunits of acetyl-CoA synthase display both closed and open conformations, and that the active form of the enzyme has an Ni-Ni-[Fe_4-S_4] cluster at the active site (Darnault et al., 2003). Copper, once thought to be a part of this nickel-containing metal cluster (Doukov et al., 2002), is an inhibitor of acetyl-CoA synthase and not a component of the catalytically active enzyme (Bramlett et al., 2003; Darnault et al., 2003). A nickel insertase is involved in the biosynthesis of acetyl-CoA synthase (Loke and Lindahl, 2003).

b) Formate dehydrogenase (Yamamoto et al., 1983) and formyltetrahydrofolate synthetase (Lovell et al., 1990) are also centrally important because formate dehydrogenase "reductively fixes" CO_2 to formate, and formyltetrahydrofolate synthetase subsequently "covalently fixes" this CO_2-derived carbon (i.e., formate) into an organic form on the methyl branch of the pathway. A functional gene probe for the detection of acetogens is based on the gene sequence of formyltetrahydrofolate synthetase (Lovell and Hui, 1991; Lovell, 1994; Leaphart and Lovell, 2001; see the subsection on Detection of Acetogens in this Chapter).

c) Under chemolithoautotrophic conditions (e.g., during growth on H_2-CO_2), the pathway must not only fix carbon but also conserve energy. Reactions that appear to be associated with this conservation of energy are facilitated by methlylenetetrahydrofolate reductase and methyltransferase (Clark and Ljungdahl, 1984; Park et al., 1991; Wohlfarth and Diekert, 1991; Müller et al., 2001; Müller, 2003; see the subsection on Conservation of Energy and Bioenergetics in this Chapter).

d) H_2-dependent growth under chemolithotrophic conditions is considered to be a hallmark of acetogens, and this capability requires the activation of H_2-derived reductant via hydrogenase. Though seldom highlighted in the pathway, hydrogenase thus catalyzes the first step in the chemolithoautotrophic fixation of CO_2 (i.e., without utilizable reductant, CO_2 cannot be fixed). Though the activities and properties of hydrogenases from different acetogens have been documented (e.g., Braun and Gottschalk, 1981; Kellum and Drake, 1984; Ragsdale and Ljungdahl, 1984; Dobrindt and Blaut, 1996; Drake et al., 1997), relatively little information has been published on hydrogenases from autotrophically grown acetogens. Acetogens can contain multiple hydrogenases (Kellum and Drake, 1984), and levels of hydrogenase activity in the membrane can increase when acetogens are cultivated at the expense of H_2-CO_2 (Braus-Stromeyer and Drake, 1996), indicating that the function and intracellular localization of hydrogenases in acetogens are affected by cultivation conditions.

e) Carbonic anhydrase catalyzes the following reversible reaction (Lindskog et al., 1971; Karrasch et al., 1989; Albers and Ferry, 1994; Kisker et al., 1996; Vandenberg et al., 1996):

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \qquad (12)$$

This enzyme has been demonstrated in numerous acetogens, including *A. woodii* and *S. silvacetica* (Braus-Stromeyer et al., 1997). Carbonic anhydrase is widespread in nature, occurs in an extensive number of organisms, including humans, plants and prokaryotes, and has multiple functions, including pH homeostasis, facilitated diffusion of CO_2, interconversion of CO_2 and HCO_3^-, and ion transport. Since CO_2 is important to the acetyl-CoA pathway, one physiological function of carbonic anhydrase in acetogens might be to increase intracellular levels of CO_2. Carbonic anhydrase has been purified approximately 300-fold from *A. woodii*; that specific activities of carbonic anhydrase in *A. woodii* are very high in both autotrophically and organotrophically grown cells indicate that this enzyme is physiologically important during acetogenesis (Braus-Stromeyer et al., 1997).

Conservation of Energy and Bioenergetics

Acetogens can conserve energy by substrate-level phosphorylation and chemiosmotic mechanisms. Under certain growth conditions, both processes can be utilized simultaneously. However, when acetogens grow chemolithoautotrophically (e.g., on H_2-CO_2), energy can only be conserved via a chemiosmotic mechanism.

SUBSTRATE-LEVEL PHOSPHORYLATION. Under homoacetogenic conditions, certain hexoses are converted stoichiometrically to acetate, and a net of 4 ATP (per hexose metabolized) are formed by substrate-level phosphorylation (Figs. 3 and 12). A point that is often overlooked when comparing acetogens to other anaerobes is that acetogens conserve more energy by substrate-level phosphorylation than do the more classic fermenters (Table 6). For example, twice as much energy is conserved via

Table 6. Amount of ATP formed via substrate-level phosphorylation (ATPSLP) during glucose-dependent acetogenesis and fermentative processes.

Process	Stoichiometry of the process	ATP_{SLP}
Acetogenesis	Glucose → 3 acetate	4
Butyrate fermentation	Glucose → butyrate + $2CO_2$ + $2H_2$	3
Bifidum fermentation	Glucose → 1.5 acetate + lactate	2.5
Ethanol fermentation	Glucose → 2 ethanol + $2CO_2$	2
Homolactate fermentation	Glucose → 2 lactate	2
Heterolactate fermentation	Glucose → lactate + ethanol + CO_2	1

substrate-level phosphorylation during the homoacetogenic metabolism of glucose than during glucose-dependent ethanol fermentation. It should be noted that when hexoses are metabolized, the net energy that is conserved via substrate-level phosphorylation is only indirectly linked to the acetyl-CoA pathway (see panels A and B in both Figs. 3 and 12). The ability of acetogens to conserve more energy via substrate-level phosphorylation than certain other anaerobes (i.e., on a per-mole-substrate-utilized basis) might make acetogens more competitive under certain in situ conditions.

MEMBRANOUS ELECTROCHEMICAL GRADIENTS AND ATPASES. Although the above comments suggest that the ability to conserve energy via substrate-level phosphorylation is important to the bioenergetics of acetogens, the net production of ATP via substrate-level phosphorylation does not directly occur in the acetyl-CoA pathway. During the reductive synthesis of acetate from CO_2, one ATP is consumed when formate is activated and one ATP is gained at the level of acetate kinase, thus yielding a break even relative to net ATP gain via substrate-level events (Fig. 11). In addition, the reduction of CO_2 to the carbonyl level on the carbonyl branch is thermodynamically unfavorable and requires energy (estimated at one-third ATP equivalent; Diekert, 1992). Thus, in the absence of chemiosmotic processes, the acetyl-CoA pathway does not directly yield net utilizable energy via substrate-level phosphorylation.

However, cell yields of acetogens are in excess of cell yields that can be explained from energy conserved via substrate-level phosphorylation. One mole of ATP yields approximately 10 g dry weight of microbial biomass, and cell yields of acetogens (e.g., *C. aceticum* and *A. woodii*) are 50–70 g cell dry weight per mole of glucose-equivalent (Andreesen et al., 1973; Tschech and Pfennig, 1984). Since a maximal cell yield of 40 g cell dry weight per mole of glucose is predicted from substrate-level phosphorylation (4 moles ATP_{SLP} per mole of glucose), the higher than expected cell yields indicate that acetogens conserve energy by chemiosmotic processes. The capacity of acetogens to grow chemolithoautotrophically on H_2-CO_2 likewise indicates that acetogens are capable of conserving energy via electron transport phosphorylation or chemiosmotic mechanisms (since there is no net ATP gain via substrate-level phoshorylation in the acetyl-CoA pathway).

Acetogens appear to use two different mechanisms for conserving energy by chemiosmotic processes. One process involves the generation of a membranous proton gradient and the synthesis of ATP by proton-dependent ATPase (Ljungdahl, 1994; Fig. 14A), and the other process involves the generation of a membranous sodium gradient and the synthesis of ATP by sodium-dependent ATPase (Müller and Gottschalk, 1994; Müller et al., 2001; Fig. 14B). Although the precise biochemical mechanisms for these two processes are not known, several generalizations can be made as to how these processes occur:

a) Acetogens are rich in electron carriers (e.g., ferredoxins, rubredoxins, quinones and cytochromes), and certain acetogens have membranous electron transport systems that can generate proton gradients across the membrane which can subsequently be used by proton-dependent ATP synthases (ATPases) to synthesize ATP (Das et al., 1989; Das et al., 1997; Kamlage and Blaut, 1993a; Kamlage et al., 1993b; Ljungdahl, 1994; Das and Ljungdahl, 2003). Likewise, certain oxidoreductases (e.g., hydrogenase) may be membrane-associated and also involved in generating proton gradients across the membrane (Ljungdahl, 1994; Drake et. al., 1997; Fig. 14A). The large, negative Gibb's free energy for the methylenetetrahydrofolate reductase-mediated step in the acetyl-CoA pathway (Table 5) suggests that this enzyme is associated with energy conservation (Wohlfarth and Diekert, 1991). This enzyme can be membrane-bound (Hugenholtz et al., 1987) and might function in close association with the other catalysts responsible for the synthesis of acetyl-CoA that might also be loosely associated with the membrane (e.g., acetyl-CoA synthase activity occurs in the cell membrane of *M. thermoautotrophica* [Hugenholtz et al., 1987; Hugenholtz and

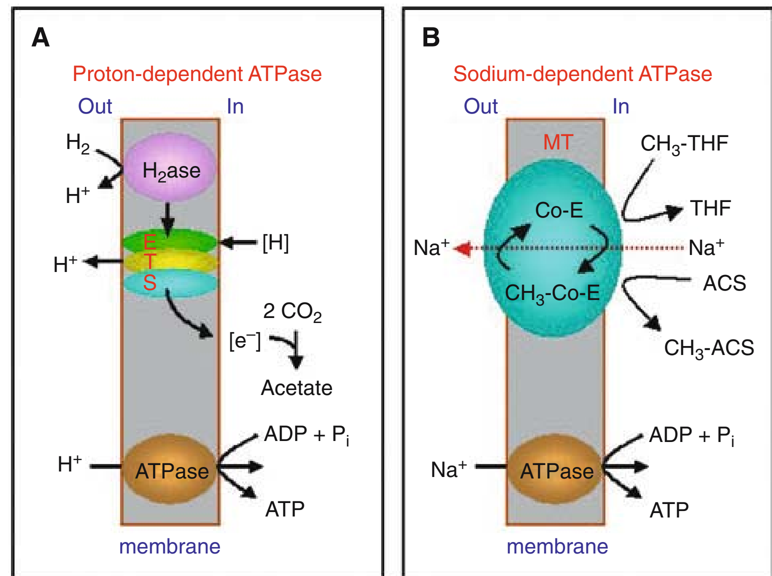


Fig. 14. Proton- (A) and sodium-dependent (B) mechanisms for the chemiosmotic conservation of energy by acetogens. Panel A is based on the work of Ljungdahl and coworkers, and Panel B is based on the work of Müller and coworkers. Abbreviations: H_2ase, hydrogenase; ETS, membranous electron transport system that is composed of various electron carriers (the system facilitates both the transport of electrons and translocation of protons); ATPase, ATP synthase; MT, methyltransferase, Co-E, corrinoid enzyme; THF, tetrahydrofolate, e^-, reducing equivalent; and ACS, acetyl-CoA synthase.

Ljungdahl, 1989] and *T. kivui* [Braus-Stromeyer et al., 1996; Drake et al., 1997]). The membranous *b*-type cytochrome that is linked to the activity of methylenetetrahydrofolate reductase and repressed during nitrate dissimilation (see the subsection on Regulation of the Acetyl-CoA Pathway and Other Metabolic Abilities in this Chapter) is closely interfaced to proton translocation by membranous menaquinone during the transport of electrons (Das and Ljungdahl, 2003). Examples of acetogens that have membranous electron-transport systems and proton-dependent ATPases include *M. thermoacetica*, *M. thermoautotrophica* and *S. sphaeroides*.

b) Some acetogens require sodium for growth, motility, and the optimal synthesis of acetate under certain conditions (Geerligs et al., 1989; Heise et al., 1989; Yang and Drake, 1990; Müller and Bowien, 1995). For example, *T. kivui* requires sodium for chemolithoautotrophic growth but not for organotrophic growth (Yang and Drake, 1990). In contrast, *M. thermoacetica* does not require sodium for either chemolithoautotrophic or organotrophic growth (Yang and Drake, 1990). Although the biochemical mechanism is not fully resolved, a membrane-associated complex that is interfaced to the methyl branch of the acetyl-CoA pathway appears to facilitate the translocation of sodium ions across the cell membrane (Müller and Gottschalk, 1994; Müller et al., 2001; Müller, 2003). The joint reaction catalyzed by methyltransferase and the corrinoid enzyme appears to be centrally important in the translocation of sodium ions, and the model proposed in Fig. 14B illustrates how the generation of a sodium gradient is interfaced to the synthesis of ATP via sodium-dependent ATPase (Reidlinger and Müller, 1994a; Spruth et al., 1995). Although this model is only hypothetical, numerous observations indicate that it has a sound theoretical basis. The reader is referred to Müller et al. (2001), Müller (2003) and Müller et al. (2004) for a more thorough treatment of this topic. *Acetobacterium woodii* and *T. kivui* are examples of acetogens that use sodium translocation and sodium-dependent ATPases for the conservation of energy.

c) Sodium-proton antiporters may facilitate specific changes in the type of electrochemical gradient that is used to conserve energy in acetogens (Terracciano et al., 1987; Yang and Drake, 1990; Müller and Gottschalk, 1994). For example, harmaline, an inhibitor of sodium-proton antiporters, uncouples the growth of *T. kivui* from the synthesis of acetate under chemolithoautotrophic conditions (i.e., the H_2-dependent production of acetate continues in the absence of growth when harmaline is added to the growth medium; Yang and Drake, 1990). Such observations suggest that sodium-proton antiporters are important to the ability of acetogens to conserve energy under certain conditions.

ADDITIONAL PERSPECTIVES ON THE BIOENERGETICS OF ACETOGENS. Pyrophosphate is a utilizable source of energy for certain anaerobes (Liu et al., 1982; Varma and Peck, 1983). *Moorella thermoacetica* forms and subsequently consumes intracellular pyrophosphate during growth (Heinonen and Drake, 1988). However, no direct correlation has been established between the intracellular turnover of pyrophosphate and cellular bioenergetics of acetogens.

Acetogens display different growth efficiencies with identical substrates. For example, the cell yields of *T. kivui* on H_2 and glucose are approximately twofold higher than those of *M. thermoacetica* (Daniel et al., 1990). The biochemical explanations for such differences have not been resolved. However, the different mechanisms by which acetogens conserve energy may in part be responsible for such differences in growth efficiencies. With *Sporomusa*, different cytochromes are utilized for H_2- and betaine-derived reductant (Kamlage and Blaut, 1993a; Kamlage et al., 1993b); thus, the engagement of different electron transport systems for different substrates might account for some of the differences in growth efficiencies (i.e., the bioenergetics of growth) of acetogens.

Occurrence of the Acetyl-CoA Pathway in Non-Acetogenic Microorganisms

Metabolic schemes that bear close biochemical resemblance to the acetyl-CoA pathway of acetogens are utilized by non-acetogenic bacteria (e.g., sulfate-reducing bacteria) and members of the domain Archaea (e.g., methanogens) for either the assimilation of CO_2 (i.e., carbon) into biomass or the oxidation of acetate (Table 7). Thus, many of the biochemical and physiological features of the acetyl-CoA pathway are widely distributed in the prokaryotes. It must be remembered that these metabolic processes are not exactly the same, and that different metabolic co-factors and enzymes are involved. Nonetheless, the general features of these different forms of the acetyl-CoA pathway are very similar. Different species of the sulfate-reducing bacteria have been observed to have acetogenic capabilities (Klemps et al., 1985; Min and Zinder, 1990; Madsen and Licht, 1992; Tasaki et al., 1992; Tasaki et al., 1993; Kuever et al., 1993; Kuever et al., 1999; Christiansen and Ahring, 1996; Sanford et al., 1996); however, definitive, enzymological information on this metabolic capability is scant. The acetyl-CoA pathway is the dominant biological mechanism for the anaerobic oxidation of acetate (Fuchs, 1990).

Diverse Physiological Talents of Acetogens

The standard redox potential of the CO_2/acetate, CO_2/methane, and sulfate/sulfide half-cell reactions approximates –290, –240 and –220 mV, respectively. Thus, under standard conditions, the reductive synthesis of acetate from CO_2 is thermodynamically less favorable than methanogenesis or the reduction of sulfate to sulfide. Such thermodynamic limitations are often cited to explain why acetogens are physiologically less competitive for available reductant than methanogens and sulfate-reducing bacteria. However, as outlined in this section, acetogens have very diverse metabolic abilities that would theoretically increase their in situ competitiveness.

Diverse Electron Donors

Diverse substrates can be oxidized and deliver reductant to the acetyl-CoA pathway and the reductive synthesis of acetate (Table 8). Oxidizable substrates include CO, H_2, carbohydrates, alcohols, carboxylic acids, dicarboxylic acids, aldehydes, substituent groups of aromatic compounds, and numerous other organic and halogenated substrates. By virtue of their ability to use a wide range of substrates, acetogens have

Table 7. Functions of acetyl-CoA synthase and the acetyl-CoA pathway in obligate anaerobes.[a]

Function	Group	Process	References[b]
Acetyl-CoA forming	Acetogens	CO_2 + [H] → [acetyl-CoA] → acetate + biomass	Drake, 1994
Acetyl-CoA forming	Autotrophic methanogens e.g., *Methanobacterium thermoautotrophicum*	CO_2 + [H] → [acetyl-CoA] → biomass	Whitman, 1994
Acetyl-CoA forming	Autotrophic S-reducers e.g., *Desulfobacterium autotrophicum*	CO_2 + [H] → [acetyl-CoA] → biomass	Fuchs, 1994
Acetyl-CoA degrading	Acetate-oxidizing methanogens e.g., *Methanosarcina barkert*	Acetate → [acetyl-CoA] → CO_2 + CH_4	Ferry, 1994
Acetyl-CoA degrading	Acetate-oxidizing S-reducers e.g., *Desulfotomaculum acetoxidans*	Acetate → [acetyl-CoA] → CO_2 + [H]	Fuchs, 1994
Bidirectional	Bidirectional acetogens e.g., "Reversibacterium strain AOR"	Acetate ⇆ [acetyl-CoA] ⇆ CO_2 + [H]	Zinder, 1994

[a][H] is reductant. Acetyl-CoA is bracketed to indicate that it is an intracellular intermediate.
[b]See also Fuchs (1986, 1989) and Wood and Ljungdahl (1991).
Modified from Drake (1992, 1994).

Table 8. Representative growth-supportive substrates and overall substrate-product stoichiometries of acetogens that can form acetate as their predominant reduced product.[a]

Substrate	Overall stoichiometry for acetate production	Representative acetogen
Acetoin	$2\ CH_3COCHOHCH_3 + 2\ CO_2 + 2\ H_2O \rightarrow 5\ CH_3COOH$	*Acetobacterium carbinolicum*
Alcoxyethanols	$4\ RO\text{-}CH_2CH_2OH + 2\ CO_2\ 2\ H_2O \rightarrow 4\ ROH + 5\ CH_3COOH$	*Acetobacterium malicum*
e.g., 2-methoxyethanol	$4\ CH_3OCH_2CH_2OH + 2\ CO_2 + 2\ H_2O \rightarrow 4\ CH_3OH + 5\ CH_3COOH$	*Acetobacterium malicum*
e.g., 2-ethoxyethanol	$4\ C_2H_5OCH_2CH_2OH + 2\ CO_2 + 2\ H_2O \rightarrow 4\ C_2H_5OH + 5\ CH_3COOH$	*Acetobacterium malicum*
2,3-butanediol	$4\ CH_3CHOHCHOHCH_3 + 6\ CO_2 + 2\ H_2O \rightarrow 11\ CH_3COOH$	*Clostridium magnum*
Cellobiose	$C_{12}H_{22}O_{11} + H_2O \rightarrow 6\ CH_3COOH$	*Ruminococcus productus*
Citrate	$4\ C_6H_8O_7 + 2\ H_2O \rightarrow 9\ CH_3COOH + 6\ CO_2$	*Clostridium magnum*
CO	$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2$	*Moorella thermoacetica*
Ethanol	$2\ CH_3CH_2OH + 2\ CO_2 \rightarrow 3\ CH_3COOH$	*Clostridium formicoaceticum*
Formate	$4\ HCOOH \rightarrow CH_3COOH + 2\ CO_2 + 2\ H_2O$	*Moorella thermoacetica*
Fructose	$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$	*Clostridium formicoaceticum*
Glucose	$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH$	*Clostridium thermoaceticum*
Glycerol	$4\ HOCH_2CHOHCH_2OH + 2\ CO_2 \rightarrow 7\ CH_3COOH + 2\ H_2O$	*Acetobacterium carbinolicum*
Glycolate	$4\ CH_2OHCOOH \rightarrow 3\ CH_3COOH + 2\ CO_2 + 2\ H_2O$	*Moorella thermoacetica*
Glyoxylate	$2\ HOCCOOH \rightarrow CH_3COOH + 2\ CO_2$	*Moorella thermoacetica*
$H_2 + CO_2$	$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$	*Clostridium aceticum*
$H_2 + CO$	$2\ H_2 + 2\ CO \rightarrow CH_3COOH$	*Moorella thermoacetica*
H_2 + formate	$2\ H_2 + 2\ HCOOH \rightarrow CH_3COOH + 2\ H_2O$	*Moorella thermoacetica*
4-hydroxybenzaldehyde	4, 4-hydroxybenzaldehyde + $2\ CO_2 + 2\ H_2O \rightarrow CH_3COOH$ + 4-hydroxybenzoate	*Clostridium formicoaceticum*
Malate	$2\ HOOCCHOHCH_2COOH \rightarrow 3\ CH_3COOH + 2\ CO_2$	*Clostridium magnum*
Methanol	$4\ CH_3OH + 2\ CO_2 \rightarrow 3\ CH_3COOH + 2\ H_2O$	*Moorella thermoacetica*
Methoxyacetate	$4\ CH_3OCH_2COOH + 2\ CO_2 + 2\ H_2O \rightarrow 3\ CH_3COOH + 4\ HOCH_2COOH$	*Acetobacterium* sp. RMMac1
Methoxylated aromatics	4 aromatic-$[OCH_3] + 2\ CO_2 + 2\ H_2O \rightarrow$ 4 aromatic-$[OH] + 3\ CH_3COOH$	(Many acetogens)
e.g., syringate	2 syringate$[\text{-}OCH_3]_2 + 2\ CO_2 + 2\ H_2O \rightarrow$ 2 gallate$[\text{-}OH]_2 + 3\ CH_3COOH$	*Acetobacterium woodii*
e.g., vanillate	4 vanillate$[\text{-}OCH_3] + 2\ CO_2 + 2\ H_2O \rightarrow$ 4 protocatechuate$[\text{-}OH] + 3\ CH_3COOH$	*Acetobacterium woodii*
e.g., syringate + H_2-CO_2	syringate$[\text{-}OCH_3]_2 + 2\ CO_2 + 2\ H_2 \rightarrow$ gallate$[\text{-}OH]_2 + 2\ CH_3COOH$	Strain SS1
Methyl chloride	$4\ CH_3Cl + 2\ CO_2 + 2\ H_2O \rightarrow 3\ CH_3COOH + 4\ HCl$	*"Acetobacterium dehalogenans"*
Oxalate	$4\ HOOCCOOH \rightarrow CH_3COOH + 6\ CO_2 + 2\ H_2O$	*Moorella thermoacetica*
Pyruvate	$4\ CH_3COCOOH + 2\ H_2O \rightarrow 5\ CH_3COOH + 2\ CO_2$	*Moorella thermoacetica*
Xylose	$2\ C_5H_{10}O_5 \rightarrow 5\ CH_3COOH$	*Moorella thermoacetica*

[a]No distinction is made (a) between acids and their dissociated salt forms or (b) between CO_2 and its carbonate or bicarbonate forms. Although only one representative acetogen is listed per substrate, many acetogens may be capable of utilizing each substrate (e.g., almost all acetogens can synthesize acetate from H_2/CO_2 or methoxylated aromatic compounds).

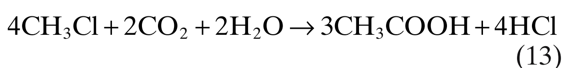
numerous trophic links to other organisms under in situ conditions.

Most acetogens have not been observed to degrade complex polymers, such as lignin or cellulose. However, little effort has gone into finding such acetogenic isolates. The recently described acetogen "*B. formatexigens*," isolated from human feces, utilized amorphous (cabbage) cellulose and carboxymethylcellulose when isolated, but lost this ability upon storage under frozen conditions (Wolin and Miller, 1994; Wolin et al., 2003). Another acetogen phylogenetically closely related to *M. thermoacetica*, strain F21 (isolated in a roll tube containing Avicel® [crystalline cellulose]), is cellulolytic and has carboxymethylcellulase and xylanase activities (Karita et al., 2003). The cellulolytic capabilities of these two organisms suggest that certain acetogens may be able to degrade certain polymers.

An organism referred to as "*M. thermoautotrophica*" (*C. thermoautotrophicum* at the time of publication) was reported to degrade inulin, a large (M_r ca. 5,000; Budavari, 1989) storage polysaccharide of plants (Drent and Gottschal, 1991). However, the product profile (1.0 hexose → 0.4 formate + 0.7 acetate + 1.3 ethanol + 1.0 H_2 + 1.0 CO_2 + 0.6 cell carbon; Drent and Gottschal, 1991) of this isolate is very inconsistent with that of *M. thermoautotrophica* (Wiegel et al., 1981), and also of acetogens. In the absence of additional information, it cannot be assumed that this organism is either *M. thermoautotrophica* or an acetogen.

The ability to utilize methoxyl groups of aromatic compounds (e.g., vanillate) is a widespread metabolic potential of acetogens (Bache and Pfennig, 1981; Frazer and Young, 1985; Daniel et al., 1991; Frazer, 1994). Figure 15 illustrates how methyl groups can be metabolized by acetogens. This metabolic scheme is based on the work of several laboratories (see below), and it must be remembered that the demethylase system varies, depending on the substrate that is demethylated. Furthermore, there may be secondary ATP-dependent activation steps that maintain corrinoid proteins (which may be involved in demethylation and transfer of methyl groups) in a reduced, active form (Kaufmann et al., 1997; Kaufmann et al., 1998).

The methylotrophic potential of acetogens to utilize methyl groups makes physiological sense, given the importance of methyl-level intermediates in the acetyl-CoA pathway. An unusual and apparently highly specialized methylotrophic metabolism is seen in the ability of "*A. dehalogenans*" to utilize methyl chloride according to the following reaction (Traunecker et al., 1991):



$$4CH_3Cl + 2CO_2 + 2H_2O \rightarrow 3CH_3COOH + 4HCl \quad (13)$$

Methyl chloride is dehalogenated by methyl chloride dehalogenase, which yields chloride and methyltetrahydrofolate (Meßner et al., 1993; Meßner et al., 1996). The methyl unit of methyltetrahydrofolate is oxidized to CO_2 by reversal of the methyl branch of the acetyl-CoA pathway, the reductant being subsequently used for the reduction of CO_2 to CO on the carbonyl branch of the pathway (Fig. 15). "*Acetobacterium dehalogenans*" also utilizes methoxylated aromatic compounds, and the *O*-demethylase of "*A. dehalogenans*" consists of two distinct methyltransferases. Methyltransferase I *O*-demethylates the methoxylated aromatic compound and transfers the methyl group to a corrinoid protein. Methyltransferase II transfers the methyl group of the methylated corrinoid protein to tetrahydrofolate (Kaufmann et al., 1997; Kaufmann et al., 1998). The methyltetrahydrofolate is metabolized in the same manner as the methyltetrahydrofolate that is derived from methyl chloride, i.e., the methyl group of methyltetrahydrofolate 1) serves as a methyl donor at the terminal stage of the methyl branch of the acetyl-CoA pathway or 2) is oxidized, and thus serves as a source of reductant for the reductive formation of CO on the carbonyl branch of the acetyl-CoA pathway (Fig. 15). *Acetobacterium woodii* and *M. thermoacetica* appear to utilize *O*-demethylating systems that are similar to that of "*A. dehalogenans*" (Berman and Frazer, 1992; Frazer, 1994; Naidu and Ragsdale, 2001).

An *O*-demethylating methyltransferase system that is involved in the metabolism of methoxyl groups of aromatic compounds has also been characterized from *H. foetida* (Kreft and Schink, 1997). *Holophaga foetida* is an unusual acetogen that can 1) degrade aromatic rings (Bak et al., 1992; Kreft and Schink, 1993; Liesack et al., 1994) and 2) methylate sulfide to dimethylsulfide via a non-energy conserving, methyltransferase-mediated reaction (Kappler et al., 1997). The *O*-demethylating methyltransferase system of *H. foetida* does not appear to be identical to the methyltransferase system characterized from "*A. dehalogenans*" (Kaufmann et al., 1998). *O*-demethylating methyltransferases of acetogens are inducible and may be either specific or nonspecific for the methoxyl group that is *O*-demethylated (Wu et al., 1988; Daniel et al., 1991; Häggblom et al., 1993; Kreft and Schink, 1997). Certain acetogens can *O*-demethylate halogenated aromatic compounds; however, the residual aromatic compound may not be subject to dehalogenation (Häggblom et al., 1993).

The acetyl-CoA pathway can be considered a one-carbon pathway, in that each of the two branches of the pathway facilitate the reduction

Fig. 15. Pathway illustrating how methyl groups from methoxylated aromatic compounds (e.g., vanillate) or methyl chloride can be metabolized by acetogens. Abbreviations: THF, tetrahydrofolate; CoA, coenzyme A; e^-, reducing equivalent; and Co-Protein, corrinoid enzyme.

of one-carbon substrates. Thus, entry-level, one-carbon substrates are particularly well suited for use by acetogens, especially under co-substrate conditions. For example, CO and the methoxyl group of aromatic compounds can be simultaneously and readily metabolized via the carbonyl or methyl branches of the acetyl-CoA pathway, respectively. Methyl groups of osmolytes can also be metabolized by acetogens (Jansen and Hansen, 2001). Certain acetogens also utilize two-carbon substrates, such as oxalate, glyoxylate and glycolate; however, how these two-carbon molecules are metabolized is not well resolved (Daniel and Drake, 1993; Daniel et al., 2004; Seifritz et al., 1999; Seifritz et al., 2002).

Acetogens are specialized in the use of short-chain substituent groups of aromatic compounds, and the use of such substituent groups can have regulatory effects on the flow of carbon and reductant. For example, use of the aldehyde group of certain lignin-derived aromatic compounds by *C. formicoaceticum* inhibits the cell's ability to use fructose (i.e., in the presence of both fructose and 4-hydroxybenzaldehyde, reductant for the acetyl-CoA pathway and the reduction of CO_2 is preferentially derived from the aldehyde group of 4-hydroxybenzaldehyde; Gößer et al., 1994; Frank et al., 1998; Drake and Küsel, 2003, 2005).

Use of Diverse Terminal Electron Acceptors

The acetyl-CoA pathway is the hallmark of acetogens, and, as outlined in the previous subsection Diverse Electron Donors, diverse substrates can be oxidized by acetogens and thus utilized for the reductive synthesis of acetate from CO_2. However, the acetyl-CoA pathway is not the only terminal electron-accepting process utilized by acetogens (Table 9). Thus, the trophic relationships and adaptation strategies of acetogens under in situ conditions may not be solely determined by their acetogenic capabilities (i.e., their use of the acetyl-CoA pathway). A few generalizations can be made about the ability of acetogens to engage in these alternative metabolic processes:

a) Almost all known acetogens can use more than one terminal electron acceptor, and therefore can produce other reduced end products in addition to acetate (Table 9). The capacity to utilize a particular terminal electron acceptor is dependent on the availability of both reductant and terminal electron acceptor. Thus, homoacetogenesis is usually a conditional capacity of an acetogen, and referring to an acetogen as a homoacetogen is almost always a misnomer.

b) The availability of CO_2 can determine how a particular substrate is metabolized by an acetogen. Indeed, for acetogenic growth, exogenous

Table 9. Terminal electron acceptors used by acetogens.[a]

Electron acceptor	Reduced end product	Representative acetogen	References
Acetaldehyde	Ethanol	*Ruminococcus productus*	Misoph and Drake, 1996
Carbon dioxide	Acetate	All acetogens	Wood and Ljungdahl, 1991
Dimethylsulfoxide	Dimethylsulfide	*Moorella thermoacetica*	Beaty and Ljungdahl, 1991
Fumarate	Succinate	*Clostridium aceticum*	Matthies et al., 1993
Methoxylated phenylacrylates	Methoxylated phenylpropionates	*Acetobacterium woodii*	Bache and Pfennig, 1981[b]
Nitrate	Nitrite	*Moorella thermoacetica*	Seifritz et al., 1993[c]
Nitrite	Ammonium	*Moorella thermoacetica*	Seifritz et al., 2003
Protons	Molecular hydrogen	*Acetobacterium woodii*	Winter and Wolfe, 1980[d]
Pyruvate	Lactate	*Ruminococcus productus*	Misoph and Drake, 1996
Thiosulfate	Sulfide	*Moorella thermoautotrophica*	Beaty and Ljungdahl, 1990

[a]No known acetogen is able to use all of the electron acceptors listed. The ability to use a particular electron acceptor is conditional, and is dependent upon in situ conditions and the acetogen in question.
[b]See also Tschech and Pfennig (1984), Hansen et al. (1988), and Imkamp and Müller (2002).
[c]See also Fröstl et al. (1996) and Seifritz et al. (2002).
[d]The H_2 formed can be used by an H_2-utilizing methanogen for interspecies hydrogen transfer.

(i.e., supplemental) CO_2 is often essential for growth (see the subsection on CO_2 as Terminal Electron Acceptor and the Concept of Fermentation in this Chapter). However, even when exogenous CO_2 is readily available, an acetogen may utilize other terminal electron acceptors, either in preference to or simultaneously with CO_2. For example, *R. productus* simultaneously reduces CO_2 to acetate and phenylacrylates to phenylpropionates (Misoph et al., 1996b), and also forms lactate as a fermentation end product concomitant to the reductive synthesis of acetate from CO_2 during the metabolism of fructose (Misoph and Drake, 1996a).

c) H_2-dependent acetogenesis is thermodynamically difficult because of the thermodynamic constraints of the entry-level redox reactions for CO_2 in the acetyl-CoA pathway (Fig. 11). For example, the standard redox potential of the CO_2/CO half-cell (–520 mV) is approximately 100 mV more negative than that of the $2H^+ + 2e^-/H_2$ half-cell (–420 mV). However, the capacity of an acetogen to consume H_2 can be significantly improved when an alternative electron acceptor is utilized. The H_2 threshold of *A. woodii* is 520 parts per million (ppm) when CO_2 is utilized as the terminal electron acceptor but decreases to 3 ppm when aromatic acrylates (e.g., caffeate) are utilized as terminal acceptors (Cord-Ruwisch et al., 1988). Likewise, the amount of biomass synthesized per mole of H_2 consumed increases eightfold when nitrate instead of CO_2 is utilized as the terminal electron acceptor by *M. thermoautotrophicum* during growth on low concentrations of H_2 (Fröstl et al., 1996). Thus, the capacity of an acetogen to compete for H_2 can increase significantly when alternative electron acceptors are utilized.

d) The use of diverse electron acceptors by acetogens indicates that acetogens can accommodate a wide range of redox conditions. For example, nitrate is the preferred terminal electron acceptor of the classic acetogen *M. thermoacetica* (Seifritz et al., 1993; Seifritz et al., 2002; Fröstl et al., 1996), suggesting that this acetogen does not require stringently reduced conditions (i.e., the standard redox potential of the nitrate/ammonium half-cell approximates +360 mV while that of the CO_2/acetate half-cell approximates –290 mV). Indeed, *M. thermoacetica* is easily isolated from aerated soils that have fluctuating redox conditions (Gößner and Drake, 1997; Gößner et al., 1998; Gößner et al., 1999).

e) The use of an alternative electron acceptor can have regulatory effects on the acetyl-CoA pathway (see the subsection on Regulation of the Acetyl-CoA Pathway and Other Metabolic Abilities in this Chapter).

f) The use of an alternative electron acceptor can conserve energy and increase the general efficiency of growth. For example, when *A. woodii* is grown at the expense of methanol, growth yields are significantly greater when aromatic acrylates (e.g., caffeate or ferulate) are used as terminal electron acceptors rather than CO_2 (Tschech and Pfennig, 1984). The sodium-dependent reduction of aromatic acrylates by *A. woodii* is coupled to the synthesis of ATP (Hansen et al., 1988; Imkamp and Müller, 2002). Likewise, growth yields of *M. thermoacetica* and *M. thermoautotrophica* increase significantly when reductant flow is directed to the dissimilation of nitrate rather than the reduction of CO_2 to acetate (Seifritz et al., 1993; Fröstl et al., 1996). In fact, ethanol or propanol are not acetogenic substrates for these two acetogens, yet both alcohols are oxidized and growth supportive when nitrate is dissimilated (Fröstl et al., 1996). Thus, the use of alternative electron acceptors by an acetogen can increase the likelihood that certain

compounds can be oxidized and be growth supportive.

g) Energy might not always be conserved by the reduction of a given terminal electron acceptor. For example, H_2 is produced as an end product by *M. thermoacetica* even though the production of H_2 is very likely not directly linked to the conservation of energy (Martin et al., 1983; Martin et al., 1985; Fröstl et al., 1996). However, the ability of certain acetogens (e.g., *A. woodii*) to produce H_2 as a substrate for the interspecies transfer of H_2 indicates that the production of H_2 can be coupled to the conservation of energy under certain in situ conditions (Winter and Wolfe, 1980; Cord-Ruwisch and Olliver, 1986; Heijthuijsen and Hansen, 1986).

h) Certain acetogens have the ability to reductively dehalogenate small molecular weight halogenated compounds (e.g., carbon tetrachloride and tetrachloroethylene; Egli et al., 1988; Freedman and Gossett, 1991; Traunecker et al., 1991; Holliger and Schraa, 1994; Terzenbach and Blaut, 1994; Hashsham and Freedman, 1999). Thus, halogenated compounds can be an electron sink for acetogens. Reductive dehalogenation occurs concomitantly with acetogenesis. However, results to date indicate that reductive dehalogenation 1) is not directly linked to an enzymatic process, 2) is due to chemical reactions with reduced corrinoids that normally serve as cofactors during normal acetogenic metabolism, and 3) does not conserve energy.

Regulation of the Acetyl-CoA Pathway and Other Metabolic Abilities

Acetogenesis was initially thought of as a constitutive trait of the classic acetogen *M. thermoacetica*. Since our understanding of acetogens was largely influenced by the decades of work on this acetogen (see the section on Historical Perspectives in this Chapter), it has only been in more recent times that we have learned that this view is a misconception regarding not only *M. thermoacetica* but acetogens in general. Although the molecular details are not yet well understood, it is now clear that many of the diverse metabolic processes of acetogens are regulated. Indeed, because acetogens have so many diverse metabolic capabilities, it is essential that these capabilities can be regulated. Numerous examples with many acetogens can be cited to reinforce this fact: 1) hydrogenase, formate dehydrogenase, and acetyl-CoA synthase activities are significantly influenced by growth substrates (Braun and Gottschalk, 1981; Kellum and Drake, 1986; Daniel et al., 1990; Lux and Drake, 1992), 2) the capacity to utilize the carboxyl, methoxyl, and acrylate groups of certain aromatic compounds is inducible (Wu et al., 1988; DeWeerd et al., 1988; Hsu et al., 1990b; Imkamp and Müller, 2002), 3) the ability to dehalogenate methyl chloride is inducible (Meßner et al., 1993; Meßner et al., 1996), and 4) electron transport systems are regulated (Kamlage et al., 1993b; Fröstl et al., 1996; Seifritz et al., 2002; Seifritz et al., 2003). Several genes encoding enzymes of the acetyl-CoA pathway of *M. thermoacetica* have been cloned and successfully expressed in *Escherichia coli* (Morton et al., 1992); however, regulation is not well understood at the molecular level.

Relatively few studies have directly assessed the regulation of the acetyl-CoA pathway. The first evidence that the acetyl-CoA pathway is subject to regulation was obtained with *A. woodii*. The use of acrylate groups of certain aromatic compounds (e.g., caffeate) as an alternative electron acceptor when *A. woodii* (grown at the expense of methanol) results in the total shutdown in the cell's ability to produce acetate (Tschech and Pfennig, 1984). Thus, the use of an alternative electron acceptor can have regulatory effects on the acetyl-CoA pathway.

How the acetyl-CoA pathway can be regulated is best understood from information obtained with *M. thermoacetica*. The dissimilation of nitrate represses the function or engagement of the acetyl-CoA pathway by repressing the synthesis of a membranous *b*-type cytochrome that is essential for the formation of methyltetrahydrofolate on the methyl branch of the pathway (Nitrate Block Site 1; Fröstl et al., 1996; Drake et al., 2002; Fig. 16). The amount of certain enzymes of the acetyl-CoA pathway might be less under nitrate-dissimilating conditions than under acetogenic conditions (Arendsen et al., 1999). However, all of the enzymes responsible for the flow of carbon in the acetyl-CoA pathway are expressed and functionally present in the cell when the cell is dissimilating nitrate but not reductively synthesizing acetate from CO_2 (Fröstl et al., 1996). Thus, the membranous *b*-type cytochrome is a key element in regulating the flow of both reductant and carbon in the acetyl-CoA pathway. Hydrogenase activity is strongly repressed when cells dissimilate nitrate, and a second metabolic block occurs at the level of acetyl-CoA synthase on the carbonyl branch of the pathway by an unknown mechanism that might also involve the flow of reductant to CO_2 (Nitrate Block Site 2; Fig. 16). Since acetyl-CoA synthase and hydrogenase can be associated with the cell membrane of certain acetogens (Hugenholtz et al., 1987; Hugenholtz and Ljungdahl, 1989; Braus-Stromeyer et al., 1996; Dobrindt and Blaut, 1996; Drake et al., 1997), it could be that the membranous *b*-type cytochrome and/or membranous hydrogenase is important for electron flow on the carbonyl

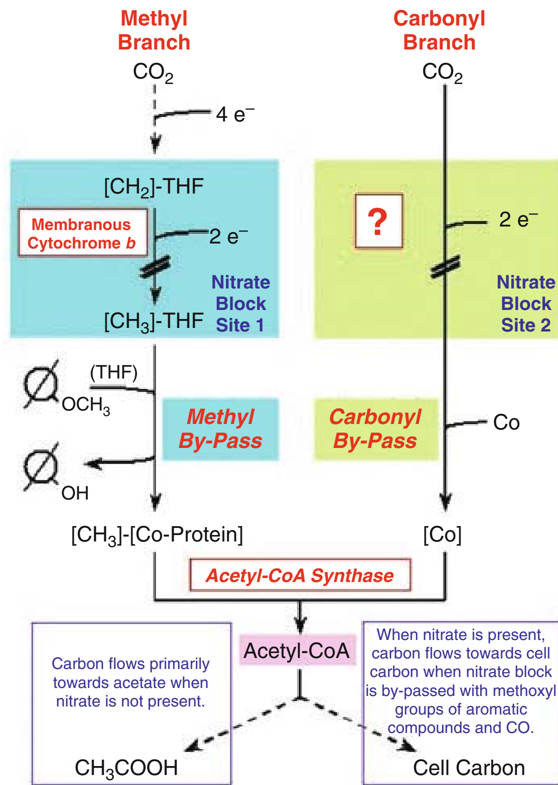


Fig. 16. Pathway illustrating where nitrate blocks the flow of carbon in the acetyl-CoA pathway when nitrate is dissimilated to ammonium by *Moorella thermoacetica*. Abbreviations: THF, tetrahydrofolate; CoA, coenzyme-A; and Co-Protein, corrinoid enzyme. The dissimilation of nitrite to ammonium appears to have the same effect (Seifritz et al., 2002a). Modified from Drake et al. (2003).

branch of the pathway. Although the different functions of hydrogenase(s) during growth of acetogens on sugars are unknown, this oxidoreductase might be essential for the proper flow of sugar-derived reductant during the reduction of CO_2 to acetate (Drake and Küsel, 2003).

Tolerance to Oxic Conditions and Metabolism of O_2

Acetogens have been mostly isolated from habitats that are anoxic (Table 2) and are routinely cultivated under anoxic conditions. Furthermore, many enzymes of the acetyl-CoA pathway are extremely sensitive to O_2. Thus, acetogens have been classically referred to as strict anaerobes. However, acetogens are also present in aerated soils and colonize habitats with fluctuating redox conditions (e.g., the rhizosphere of sea grass). It should therefore come as no surprise that acetogens, like other so-called "strict anaerobes" (e.g., sulfate-reducing bacteria [Marschall et al., 1993; Johnson et al., 1997; Teske et al., 1998; Cypionka, 2000]), are able to cope with oxidative stress. Indeed, acetogens isolated from aerated soils and the rhizosphere of sea grass have the ability to tolerate and consume O_2 (Küsel et al., 2001; Karnholz et al., 2002). Acetogens isolated from the termite gut can also tolerate transient, moderately oxic conditions (Boga and Brune, 2003; Graber and Breznak, 2004a). Although acetogens such as *A. woodii*, *C. magnum*, *C. glycolicum* RD-1, *M. thermoacetica* and *S. silvacetica* can tolerate 0.5–6% O_2 (amount is dependent upon the acetogen and the conditions of incubation) in the headspace of culture tubes, the ability of acetogens to metabolize O_2 does not appear to be directly coupled to the conservation of energy. The capacity of acetogens to metabolize O_2 is likely used as a means to remove trace amounts of O_2 (or toxic products derived from O_2 [e.g., superoxides and peroxides]) from acetogen-colonized microniches that are subject to transient oxic conditions.

Information to date suggests that there are three basic ways that acetogens can cope with oxic conditions:

1) When certain acetogens are challenged with oxic conditions, they make use of alternative electron acceptors and metabolically bypass the need to use the acetyl-CoA pathway. For example, the ability of *M. thermoacetica* to dissimilate nitrate negates the need of the cell to use CO_2 as a terminal electron acceptor when conditions become more oxic. Another example is *C. glycolicum* RD-1, an aerotolerant acetogen that was isolated from sea grass roots and can tolerate up to 4% O_2 in the headspace of shaken culture tubes (Küsel et al., 2001). *Clostridium glycolicum* RD-1 is unusual in that it simultaneously utilizes acetogenesis and ethanol fermentation under anoxic conditions (Fig. 17). However, when conditions become more oxic, acetaldehyde, pyruvate, and protons are exclusively used as terminal electron acceptors, and ethanol, lactate and H_2 become the reduced end products (Fig. 17). Thus, the ability of certain acetogens to cope with oxidative stress appears to be maximized when sugars or other fermentable substrates are available. Such findings indicate that certain acetogens can shift the flow of reductant towards catabolic processes that are less sensitive to O_2 when conditions become more oxic.

2) Several acetogens have been examined at either the enzyme-activity level or gene level for the presence of enzymes that might be involved in the removal (i.e., metabolism) of O_2 or its toxic by-products (Table 10; it should be noted that, thus far, not very many acetogens have been examined for these enzymes). The enzymes that have been detected at either the activity or gene level include NADH-oxidase, peroxidase, super-

oxide dismutase, rubredoxin oxidoreductase, and rubrerythrin. Unlike aerobes that utilize superoxide dismutase, an enzyme that forms O_2 (Table 10), only one acetogen (*C. glycolicum* RD1, an acetogen that displays a very high tolerance to O_2 [above]) has been found to have this enzyme (Küsel et al., 2001). *Clostridium glycolicum* RD1 also contains peroxidase and NADH-oxidase. To date, catalase, which also forms O_2, has only been detected in the termite isolates of *A. longum*, *S. termitida* and *S. aerivorans* (Kane and Breznak, 1991a; Boga and Brune, 2003; Boga et al., 2003). *Clostridium magnum*, *M. thermoacetica* and *S. silvacetica* contain peroxidase and NADH-oxidase activities, but lack catalase and superoxide dismutase (Karnholz et al., 2002). Rubredoxin oxidoreductase and rubrerythrin are oxidative stress enzymes in certain sulfate-reducing bacteria (Lumppio et al., 2001; Kurtz, 2003), and genes for similar proteins occur in *M. thermoacetica* (Das and Ljungdahl, 2001). A flavoprotein/rubredoxin combination (derived from *M. thermoacetica*) functions as an NADH:O_2 oxidoreductase (Silaghi-Dumitrescu et al., 2003). Although these enzymatic capabilities vary from one acetogen to another, it can be concluded that acetogens have the capacity to reductively remove O_2, peroxide and superoxide.

3) Close trophic relationships between acetogens and microaerophiles that consume trace amounts of oxygen is also a means by which acetogens might cope with transient oxic conditions (Gößner et al., 1999). This trophic relationship is discussed in the subsection on Metabolic Interactions in Pure Culture and Complex Ecosystems in this Chapter.

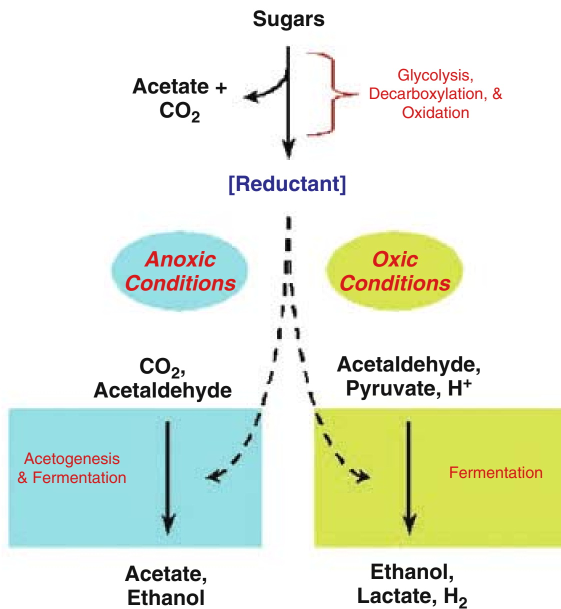


Fig. 17. Flow of sugar-derived reductant during the metabolism of *Clostridium glycolicum* RD-1. Under anoxic conditions, intracellular CO_2 (taken up from exogenous sources or produced from the decarboxylation of pyruvate) and acetaldehyde (produced during catabolism) serve as terminal electron acceptors and are reduced to acetate and ethanol, respectively. Under oxic conditions, intracellular acetaldehyde, pyruvate, and protons (all produced during catabolism) serve as terminal electron acceptors and are reduced to ethanol, lactate and H_2, respectively. Modified from Küsel et al. (2001).

Ecology of Acetogens

There is a general paradox regarding the ecology of acetogens: although theoretical considerations suggest that acetogenesis should not be a highly competitive microbial process, acetogens occur in highly diverse habitats and their activity can sometimes compete with and even overshadow that of other anaerobes. This section will evaluate both sides of this paradox and will focus on the metabolic interactions of acetogens in certain ecosystems.

Table 10. Possible oxidative stress enzymes in acetogens.

Enzyme	Reaction	Detected	References
NADH-oxidase	$O_2 + 2\ NADH + 2\ H^+ \rightarrow 2\ NAD^+ + 2\ H_2O$	Activity	Küsel et al., 2001 Karnholz et al., 2002
Peroxidase	$H_2O_2 + XH_2 \rightarrow X + 2\ H_2O$	Activity	Küsel et al., 2001 Karnholz et al., 2002
Superoxide dismutase	$2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$	Activity	Küsel et al., 2001
Catalase	$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$	Activity	Kane and Breznak, 1991 Boga et al., 2003
FprA/Hrb[a]	$O_2 + 2\ NADH + 2\ H^+ \rightarrow 2\ NAD^+ + 2\ H_2O$	Activity	Silaghi-Dumitrescu et al., 2003
Rubredoxin oxidoreductase	$e^- + O_2^- + 2\ H^+ \rightarrow H_2O_2$	Gene	Das et al., 2001
Rubrerythrin	$H_2O_2 + XH_2 \rightarrow X + 2\ H_2O$	Gene	Das et al., 2001

Abbreviations: NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; FprA, type A flavoprotein; and Hrb, rubredoxin.
[a]This combination also displays NO reductase activity.

Metabolic Interactions of Acetogens in Pure Cultures and Complex Ecosystems

In anoxic environments, acetogens compete with primary fermenters for monomeric compounds that are derived from the degradation of polymers and with secondary fermenters for typical fermentation products such as lactate, ethanol or H_2 (McInerney and Bryant, 1981). Acetogenesis yields more ATP per mole of sugar than classic fermentations do, which might increase the competitiveness of acetogens under certain conditions (see the subsection on Conservation of Energy and Bioenergetics and Table 6 in this Chapter). However, in pure culture, acetogens tend to grow slower on sugars than do classic fermenters (e.g., *Clostridium butyricum*; Schink, 1994). Nonetheless, acetogenesis is the most important anaerobic glucose-consuming process in anoxic paddy soils and lake sediments (Krumböck and Conrad, 1991). When H_2 is utilized as a substrate, acetogens must compete with Fe(III) reducers, sulfate reducers, and methanogens. In most anoxic habitats with low amounts of Fe(III) and sulfate, CO_2-dependent methanogenesis dominates as the terminal reductive process during the oxidation of organic matter. H_2-CO_2-dependent methanogenesis ($\Delta G'_o = -130$ kJ per mole reaction) is more exergonic than H_2-CO_2-dependent acetogenesis ($\Delta G'_o = -95$ kJ per mole reaction; Diekert and Wohlfarth, 1994b; Fuchs, 1994). At the in situ concentrations of reactants and products found in many anoxic environments, methanogenesis may be energetically more favorable than acetogenesis (Dolfing, 1988). H_2-thresholds (i.e., the minimum concentration of H_2 required for the uptake of H_2 by a cell) decrease with increasing redox potential of the energy-yielding reaction (Conrad, 1996). The redox potential of the CO_2/acetate half-cell reaction is –290 mV and is more negative than that of most other terminal electron-accepting processes. Thus, under pure culture conditions, the H_2 threshold of acetogens is higher than that of other hydrogenotrophic anaerobic bacteria (Cord-Ruwisch et al., 1988). For example, the H_2 threshold for acetogens when CO_2 is utilized as a terminal electron acceptor (362–4660 ppm) is 10- to 100-fold higher than that of methanogens. Thus, on these theoretical grounds, H_2-dependent acetogenesis is not a competitive process at low concentrations of H_2.

The capacity of acetogens to utilize various substrates simultaneously might contribute to their competitiveness in nature. Aromatic methoxyl groups are more readily utilized by deep subsurface acetogens in the presence of H_2 (Liu and Suflita, 1993). During mixotrophic growth on H_2 and lactate, *A. woodii* can utilize lower concentrations of H_2; however, the residual partial pressure is still too high for a successful competition with H_2-utilizing methanogens (Peters et al., 1998).

In certain complex microbial habitats (e.g., soils and sediments), the metabolic interactions of anaerobic populations are influenced by unstable physical and chemical parameters such as pH, temperature, periodic oxygenation, spatial arrangements, and different sizes of microbial populations. In certain cases, these complex factors might be favorable for the theoretically disadvantaged acetogens. For example, in freshwater sediments with low pH and low temperature, acetogens can outcompete methanogens for H_2 (Phelps and Zeikus, 1984; Conrad et al., 1989; Nozhevnikova et al., 1994; Zavarzin et al., 1994; see the subsection on Diverse Habitats in this Chapter). In contrast to laboratory cultures, most in situ microbial habitats are not composed of homogeneous or well-mixed microbial populations. Thus, the success of acetogens to compete for H_2 in situ could be enhanced by being spatially associated with H_2-producing cells in a heterogenic system. However, information about the in situ spatial distribution of acetogens at the microscale level is scant, mainly because broad-based, group-specific 16S rRNA-based probes cannot be developed for the phylogenetically diverse acetogens (see the subsection on Detection of Acetogens in this Chapter). Furthermore, the functional gene probe that has been developed for acetogens appears to be restricted to a subgroup of acetogens and, likewise, is not absolutely specific for acetogens (Lovell and Hui, 1991).

Methanogens appear to be more sensitive to O_2 than acetogens, and acetogens can occur in higher cultured numbers than methanogens in habitats that are subject to fluctuating concentrations of O_2 (e.g., soils). In the hindgut of termites, a microbial habitat that can have volumes of 1 μl or less, only the inner portion of the microbe-packed paunch is completely anoxic; the epithelial surface of the paunch is characterized by a diminishing O_2 gradient (Brune et al., 1995; Brune et al., 2000). H_2-utilizing acetogens from the rumen are more competitive for H_2 than H_2-utilizing methanogens in certain termite guts (Brauman et al., 1992). The extent to which H_2-derived reductant flows towards the reduction of CO_2 to acetate rather than the reduction of CO_2 to methane varies with the feeding guild of the termite (Brauman et al., 1992). Although acetogens are usually obligate anaerobes, acetogens are relatively tolerant to fluxes of O_2 in terrestrial soils (Wagner et al., 1996). The enumerability of acetogenic bacteria in well drained, oxic soils and freshly fallen litter (Peters and Conrad, 1995; Küsel et al., 1999c; Reith et al., 2002), as well as the isolation of *S. silvacetica* and *M. ther-*

moacetica from different soils (Gößer and Drake, 1997; Kuhner et al., 1997; Gößer et al., 1999; Karita et al., 2003), attests to the ability of acetogens to cope with fluxes of aeration and to withstand drying under oxic conditions (Wagner et al., 1996). In contrast, the cultured numbers of other so-called "obligate anaerobes," like sulfate reducers or methanogens, are negligible in well-drained soils and litter (Peters and Conrad, 1995; Küsel et al., 1999c).

The ability of acetogens to survive in habitats that are subject to fluctuations in O_2 is due in part to their ability to reductively remove traces of O_2 (see the subsection on Tolerance to Oxic Conditions and Metabolism of O_2 in this Chapter). Their survival in such habitats can also be enhanced by trophic interactions with facultative anaerobes. The classic acetogen *M. thermoacetica* and the fermentative microaerophile *Thermicanus aegyptius* were co-isolated as a commensal pair from an oxic high-temperature soil (Gößer et al., 1999). The two organisms grow commensally on oligosaccharides (Fig. 18). In addition to the production of fermentation products by *T. aegyptius* that can be used by the juxtaposed acetogen, the fermentative microaerophile also minimizes the level of incoming O_2 in microzones inhabited by acetogens. Collectively, these findings demonstrate that the ecological roles of acetogens are not restricted to anoxic and water-saturated habitats.

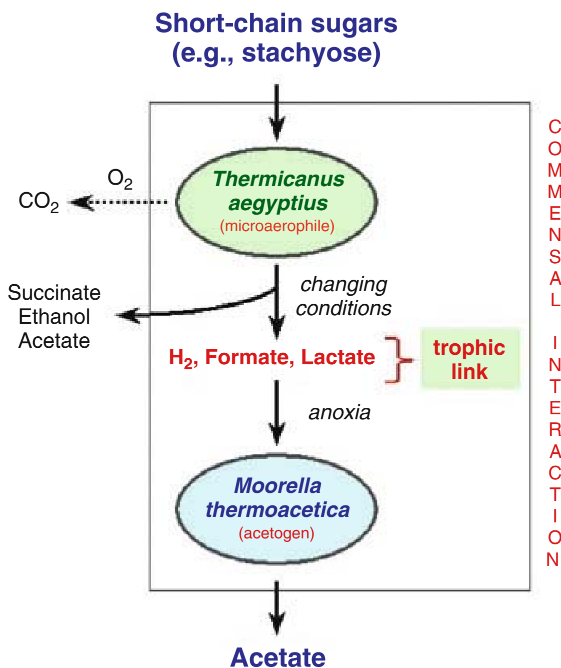


Fig. 18. Scheme illustrating the hypothetical trophic interaction of *Thermicanus aegyptius* ET-5b and *Moorella thermoacetica* ET-5a. Modified from Gößer et al. (1999).

Diverse Habitats

Acetogens have been mostly obtained from habitats that are permanently anoxic, such as freshwater or marine sediments, sewage sludge, and gastrointestinal tracts (see the subsection on Description of Species in this Chapter). However, during more recent years, the presence and ecological role of acetogens has also been investigated in other habitats, such as hypersaline sediments, deep aquifers, oxic soils, and plant roots. Although the magnitude and consequence of acetogenesis in these diverse habitats is often unclear, the occurrence of acetogens in such diverse habitats underscores the potential importance of acetogenesis at a more global level.

HUMAN COLON. The gastrointestinal tracts of mammals are colonized by acetogens (Prins and Lankhorst, 1977; Breznak and Kane, 1990; Mackie and Bryant, 1994; Wolin and Miller, 1994; Leedle et al., 1995). In humans, dietary components (cellulose, hemicellulose, pectin and starch) not absorbed in the upper digestive tract reach the colon where they are fermented by the cooperative metabolism of a great variety of bacterial species (Wolin and Miller, 1983). Short-chain fatty acids (acetate, propionate and butyrate) and gases (CO_2 and H_2) are the main fermentation products in the colon, and it is estimated that 95% of the short-chain fatty acids are absorbed and utilized by the host. Part of the daily production of acetate in the intestine (10–30 g of acetate; Royall et al., 1990) can be attributed to the activity of acetogens, as evidenced by the formation of [1,2-^{14}C]acetate from [3,4-^{14}C] glucose in fecal incubations (Miller and Wolin, 1996).

Part of the H_2 formed during colonic fermentation is exhaled in breath and vented in flatus. A large amount of the H_2 that is produced in the colon is consumed via the interspecies transfer of H_2. Humans (30–50% of the European population) who harbor large populations of methanogens (10^8–10^{10} cultured methanogens per gram dry wt. of feces) exhale CH_4 in detectable concentrations (i.e., >1 ppm of CH_4; Miller and Wolin, 1982; Pochart et al., 1992). In the colon of these humans, methanogenesis is probably the main hydrogenotrophic process. H_2-utilizing *Methanobrevibacter smithii* is believed to be primarily responsible for producing almost all colonic CH_4 (Wolin and Miller, 1983). Depending of the human sampled, the cultured number of methanogens in human feces range from undetectable to 10^{10} per gram dry wt. of feces. In contrast, the cultured numbers of H_2-utilizing sulfate reducers in the feces of both CH_4-excreting and non-CH_4-excreting humans are similar (10^7 per gram dry wt. of feces; Doré et al., 1995). However, cultured numbers of H_2-utilizing fecal

acetogens of non-CH_4-excreting humans are higher than those of CH_4-excreting humans (10^7 versus 10^5 H_2-utilizing acetogens per gram dry wt. of feces, respectively). A negative correlation between the numbers of acetogens and methanogens also exist in the rumens of lambs and in the feces of rats (Prins and Lankhorst, 1977). These correlations in the relative distributions of H_2-dependent anaerobes suggest that H_2-dependent acetogenesis is important in gastrointestinal ecosystems which have low numbers of methanogens (Doré et al., 1995; Bernalier et al., 1996a). The synthesis of [^{13}C]acetate from $^{13}CO_2$ by human fecal suspensions is supportive evidence of the hypothesis that reductive acetogenesis is a major colonic process of non-CH_4 excreting humans (Lajoie et al., 1988). Numerous H_2-utilizing acetogens have been isolated from human feces (Wolin and Miller, 1993; Bernalier et al., 1996b; Bernalier et al., 1996c; Kamlage et al., 1997; Leclerc et al., 1997a), and some of these isolates (i.e., *R. hydrogenotrophicus* and *C. coccoides* 1410) form a separate subgroup within the clostridial cluster XIVa (Collins et al., 1994).

RUMEN ECOSYSTEMS. The rumen is arguably the most completely described, intensively investigated gastrointestinal ecosystem, and the classic studies of Hungate (1966) and coworkers were formative to our general understanding of gut ecosystems and the various symbiotic relationships of gut microbiota. CH_4 is the main reduced end product that is produced in the rumen (Mackie and Bryant, 1994).

The major metabolic groups of microorganisms involved in the overall decomposition of organic matter in the rumen are described in the frequently cited three-stage model of McInerney and Bryant (1981). The molar proportions of short-chain fatty acids produced in the rumen approximate 63% acetic, 21% propionic, 14% butyric, and 2% higher acids. Most (60–80%) of the daily energy needs of the ruminant are provided by absorption of short-chain fatty acids from the rumen. The loss of energy in the end product CH_4 approximates 5–15% of the feed energy consumed by the host animal (Mackie and Bryant, 1994). It would theoretically be beneficial to the host animal if the H_2 and CO_2 that otherwise are consumed in the production of CH_4 were instead converted to acetate by acetogens (Wood and Ljungdahl, 1991b). CH_4 is a greenhouse gas, and, since ruminants produce approximately 50% of the global biogenic emission of CH_4 (Mackie and Bryant, 1994), a reduction in the emission of methane by ruminants would likewise have theoretically positive effects on the global greenhouse gas budget.

In general, methanogens are the dominant H_2-utilizers in the rumen (Hungate, 1976; Bryant, 1979). Thus, the addition of a specific inhibitor for methanogens (e.g., bromoethanesulfonic acid) is usually necessary to enrich or isolate H_2-utilizing acetogens. The addition of rumen fluid may be stimulatory to the growth of acetogens found in the rumen (Rieu-Lesme et al., 1995). The capacity of acetogens to utilize methanol, sugars or methoxylated aromatic compounds has been used to isolate or enumerate ruminal acetogens. Depending on the host animal and substrate used, the number of cultured acetogenic bacteria in the rumen range from 10^6 to 10^9 acetogens per gram of ruminal content (Sharak-Genthner et al., 1981; Krumholz and Bryant, 1986; Leedle and Greening, 1988). The number of cultured methanogens in the rumen approximates 10^8–10^9 methanogens per gram of ruminal content (Leedle and Greening, 1988). Postprandial changes in the population profiles of ruminal acetogens and methanogens occur in steers fed either high- or low-forage diets. After a shift from a low-forage diet to a higher input of readily available carbohydrate, a twofold increase in the cultured numbers of both H_2-oxidizing methanogens and acetogens occurs after 1–2 hours of feeding. The cultured numbers of ruminal acetogens obtained from steers maintained on a high-forage diet are higher than the cultured numbers of ruminal methanogens, suggesting that acetogens capable of utilizing H_2 grow preferentially on organic substrates in the rumen (Leedle and Greening, 1988). Numerous acetogens have been isolated from the rumen (Sharak-Genthner et al., 1981; Krumholz and Bryant, 1986; Greening and Leedle, 1989; Rieu-Lesme et al., 1995; Rieu-Lesme et al., 1996a; Rieu-Lesme et al., 1996b; Rieu-Lesme et al., 1998; see the subsection on Description of Species). However, the diversity and ecology of acetogens and the competition between acetogens and methanogens in ruminal ecosystems remain poorly resolved, and a substantial amount of information in these areas will be needed before the methanogenic nature of the rumen can be reasonably and successfully manipulated. In this regard, inhibitors (i.e., N-substituted derivatives of *p*-aminobenzoic acid) of the enzyme responsible for the synthesis of methanopterin (an intermediate in methanogenesis) can block the growth of certain methanogens but not interfere with the growth of acetogens (Dumitru et al., 2003).

TERMITE GUTS. About 4% of the plant material synthesized annually in terrestrial ecosystems is consumed by termites (Ljungdahl and Eriksson, 1985). Termites can be divided into four different feeding guilds: the wood-, grass-, and soil-feeding, and the fungus-growing termites (Brauman et al., 1992). The digestion of lignocellulose by wood-feeding termites has

been studied for over seven decades (e.g., Cleveland, 1925; Hungate, 1943; Varma et al., 1994). The wood-feeding termites include the so-called "lower" termites, like the well-studied *Reticulitermes flavipes*, and also some "higher" termites like *Nasutitermes nigriceps*. All termites harbor a diverse and dense hindgut microbial community that aids in digestion and is the source of fermentation products such as acetate, H_2 and CH_4.

The hindgut microbiota of the wood-feeding lower termites is composed of cellulolytic protozoa and bacteria that symbiotically affect an essentially acetogenic decomposition of wood polysaccharides (Brauman et al., 1992). Acetate constitutes 94–99% of the short-chain fatty acid pool in the extracellular hindgut fluid of *R. flavipes*. Protozoa initially convert cellulose to acetate, H_2 and CO_2 in a 1:2:1 ratio, and acetogenic bacteria subsequently convert the H_2 and CO_2 to acetate (Breznak and Switzer, 1986). Acetate 1) is absorbed in the hindgut, 2) is oxidized by the termite, and 3) can support up to 100% of the insect's energy requirement (Breznak, 1994). Thus, H_2-utilizing acetogens outcompete H_2-utilizing methanogens in wood-feeding termites; similar patterns occur for grass-feeding termites (Breznak, 1994). In contrast, H_2-dependent acetogenesis is of little significance in fungus-growing and soil-feeding termites, both of which evolve more methane than do wood- and grass-feeding termites (Brauman et al., 1992). It is not known whether the nature of the food consumed or other parameters, like a modified gut anatomy or digestive physiology, affect the terminal electron flow in the hindgut microbiota.

The core of dissected hindguts in *R. flavipes* is anoxic, whereas the peripheral lumen of dissected hindguts exhibits high oxygen uptake rates, suggesting that the hindgut has an oxic periphery and an anoxic center under in situ conditions (Brune et al., 1995; Ebert and Brune, 1997). The influx of oxygen via the gut epithelium and its reduction in the hindgut periphery appears to have a significant impact on the flow of carbon and reductant within the hindgut microbial community (Tholen and Brune, 2000). Although acetogenic bacteria are obligate anaerobes, it is now well known that some of them are not only quite tolerant to oxygen exposure, but can also reduce oxygen (Küsel et al., 2001; Karnholz et al., 2002; Drake et al., 2002; Boga and Brune, 2003; Drake and Küsel, 2003). The highest concentration of H_2 in the hindgut occurs in the central region in which H_2-producing protozoa also occur. The central region of the hindgut is also the major zone of H_2 consumption. Excess H_2 diffuses radially outward to the gut epithelium where it seems to be consumed by methanogens, which, for unknown reasons, appear to preferentially colonize the region near the gut wall (Leadbetter and Breznak, 1996; Ebert and Brune, 1997). Spirochetes occur in the central region of the hindgut and are among and often attached to the H_2-producing protozoa. Pure cultures of termite gut spirochetes (e.g., "*Treponema primitia*") catalyze the synthesis of acetate from H_2 and CO_2 (Leadbetter et al., 1999; Graber and Breznak 2004a; Graber et al., 2004b). Attachment of acetogenic spirochetes to termite gut protozoa yields a syntrophy that is based on the interspecies transfer of H_2 to the acetogen; this symbiosis provides H_2 concentrations well above the known H_2-threshold values for acetogens.

In situ activity measurements of acetogenic bacteria in combination with axial H_2 profiles in the highly compartmentalized hindgut of soil-feeding higher termites (*Cubitermes* spp.) revealed that acetogenesis might have a larger impact on the overall carbon flow than expected from previous observations. Acetogens in the posterior hindgut seem to be supported by either substrates other than H_2 or by a cross-epithelial H_2 transfer from anterior gut regions, which may create microniches favorable for H_2-dependent acetogenesis (Schmitt-Wagner and Brune, 1999; Tholen and Brune, 1999). Thus, the in situ spatial distribution of acetogens and their orientation in metabolic gradients contributes to their ability to successfully compete for H_2 in the termite gut.

MARINE, ESTUARINE, AND FRESHWATER SEDIMENTS. Acetogens are ubiquitous and plentiful in the sediments of aquatic habitats. The first species of the genus *Acetobacterium* was isolated from an estuarine sediment (Balch et al., 1977), but other *Acetobacterium* species have been isolated from freshwater and marine sediments, and from waterlogged terrestrial soils, such as subsurface sandstone, tundra wetland soils, and fens (see the subsection on Description of Species in this Chapter). In such habitats, acetogens might have to compete for H_2. Theoretically, sulfate reducers and methanogens can maintain H_2 concentrations at levels lower that that needed for the acetogenic reduction of CO_2 (Cord-Ruwisch et al., 1988). However, in certain sediments, mildly acidic conditions or low temperatures appear to favor acetogenesis.

Acetogens can outcompete methanogens for H_2 at the in situ pH of 6.2 and also at more acidic pH values (Phelps and Zeikus, 1984). *Clostridium scatologenes* SL1 is an H_2-utilizing acetogen isolated from acidic freshwater sediments and is capable of growing at pH 4 (Küsel et al., 2000). Sodium-proton antiporters (see the subsection on Conservation of Energy and Bioenergetics in this Chapter) might enable certain acetogens to cope with broad variations in pH (Schink, 1994).

Acetogens successfully compete with methanogens for H_2 at an in situ temperature of 4°C in

sediments of Lake Constanz (Conrad et al., 1989). Although the partial pressure of H_2 measured in the pore water is too low for the utilization of H_2 by pure cultures of acetogens, the actual in situ partial pressure of H_2 might be higher for acetogens living in close proximity to H_2-producing organisms. Acetogenic bacteria are important during the degradation of organic matter in permanently cold sediments, and acetate serves as the primary substrate for methanogenesis. Hydrogenotrophic methanogens from sediments of Lake Constanz can be enumerated and activated only at incubation temperatures of 20°C or higher (Schulz and Conrad, 1996). In tundra wetland soils or sediments polluted with paper-mill wastewater, acetogens can successfully compete with methanogens for H_2 and methanol at temperatures below 15°C (Nozhevnikova et al., 1994; Zavarzin et al., 1994). Acetate accumulates during the first phase of activity and is followed by a slow acetoclastic methanogenesis. The isolation of psychrophilic or psychrotrophic acetogens from these habitats and from cold lake sediments (Conrad et al., 1989; Kotsyurbenko et al., 1995; Kotsyurbenko et al., 1996; Simankova et al., 2000; Nozhevnikova et al., 2001) underscores the potential importance of acetogenesis in low temperature ecosystems.

Very little information is available on the occurrence and activity of acetogens in marine habitats. Cultured numbers of H_2-utilizing acetogens and acetate-utilizing sulfate reducers from a marine sediment approximated 10^5 and 10^7 cells per g wet wt. of sediment, respectively (Küsel et al., 1999b). The concentration of sulfate in the pore-water of marine sediments can vary owing to seasonal thermal stratification. Owing to the depletion of sulfate in the sediment, the flow of carbon and reductant shifts from sulfate reduction to methanogenesis (Hoehler et al., 1999). During this transition period, the concentration of acetate increases, which might be due to a temporary decoupling of acetate-producing and acetate-consuming processes (Sansone and Martens, 1982). The concentration of H_2 in the sediment is elevated at the beginning of this transition period, thus making the acetogenic reduction of CO_2 more favorable. The production of [^{14}C]-acetate from $^{14}CO_2$ occurs at rates comparable to those of methanogenesis or sulfate reduction during their respective period of dominance (Hoehler et al., 1999). Thus, the transient acetogenic reduction of CO_2 may be important in marine sediments and also in other ecosytems that experience geochemical fluctuations.

HYPERSALINE ENVIRONMENTS. Little is known about the presence of halotolerant or halophilic anaerobes or the anaerobic breakdown of organic matter in hypersaline ecosystems (e.g., inland lake and marine salterns). In hypersaline ecosystems, the salinity ranges from 9 to over 20% (wt./vol.) and the pH ranges from 7 to 10 (Ollivier et al., 1994). The concentration of sulfate, which can be an important electron acceptor, varies from 0.5 g per liter (Dead Sea, Israel) to 21 g per liter (Soap Lake, Washington State, United States). High salt concentrations prevent the growth of vertebrates, and only invertebrates, algae and prokaryotes are present when the salinity exceeds 10% (wt./vol.; Ollivier et al., 1994).

The concentrations of volatile fatty acids and H_2 present in sediments increase when the concentration of salt is high (Oren, 1988), indicating that the anaerobic mineralization of organic matter is inhibited by salinity. Bacteria maintain high intracellular salt concentrations or accumulate organic osmolytes (e.g., betaine) to maintain cell turgor at high salt concentrations (Oren, 1999). Betaine does not appear to be a substrate for methanogens or sulfate reducers. However, halophilic acetogens (e.g., *A. arabaticum*) decompose betaine to trimethylamine and acetate (Zhilina and Zavarzin, 1990). Trimethylamine is subsequently utilized by methylotrophic methanogens and, thus, is a trophic link between acetogens and methanogens. Since halophilic acetoclastic methanogens are unknown, and since acetate-consuming sulfate reducers are inhibited at high salinity (Widdel, 1988), the anoxic fate of acetate in these habitats is unresolved (Zavarzin et al., 1994). The haloalkaliphilic acetogens *N. acetigena* (Zhilina et al., 1996) and *N. histidinovorans* (Zhilina et al., 1998) were isolated from soda-depositing lakes and utilize glutamate, which can also be accumulated as an osmolyte by moderate halophiles. The anaerobic decomposition of osmoregulatory compounds in halophilic communities might therefore be coupled to the activity of *Acetohalobium*-type and *Natroniella*-type acetogens. Species of the acetogenic genera *Eubacterium*, *Sporomusa* and *Acetobacterium* demethylate the osmolytes dimethylsulfoniopropionate and glycine-betaine to methylthiopropionate and dimethylglycine, respectively; however, only the demethylation of glycine-betaine supported growth of the organism (Jansen and Hansen, 2001), indicating that certain in situ transformations of osmolytes by acetogens are not coupled to growth.

WATER-LOGGED SOILS. The flow of carbon and reductant in flooded rice paddy soils occurs mainly under anoxic conditions, and CH_4 is the major reduced end product of organic matter breakdown (Conrad, 1993). Root exudates and rice straw are important sources of energy and carbon for microbial activity (Chidthaisong et al., 1999). Approximately 80% of the CH_4 formed is derived from acetoclastic methanogen-

esis, which is proportionally more than the amount of CH_4 formed in a normal methanogenic food web (Chin and Conrad, 1995). Thus, acetogens may be important to the formation of acetate in such water-logged habitats. Hydrogenotrophic methanogenesis in flooded paddy soils and tundra wetland soils is inhibited by low temperatures (Conrad et al., 1989; Nozhevnikova et al., 1994; Chin and Conrad, 1995), and low incubation temperatures have been used to select against hydrogenotrophic methanogens during the enrichment of H_2-utilizing acetogens from paddy soils (Conrad et al., 1989). Cultured numbers of acetogens in the soil of rice plant microcosms range from 10^3 to 10^5 cells per g dry wt. of soil, and most dominant cultured acetogens belong to the genus *Sporomusa* (Rosencrantz et al., 1999). However, the occurrence of acetogens in the rhizosphere of rice plants is not resolved. Fluorescent in situ hybridization analysis has revealed the occurrence of Archaea on the surfaces of rice roots (Großkopf et al., 1998).

PLANT ROOTS. The rhizosphere is an important microhabitat where complex biogeochemical processes occur at accelerated rates. Plant roots exude easily degradable organic compounds that can chemotactically attract microorganisms (Waisel and Agami, 1996). Most rhizosphere bacteria are thought to live near the root tips or in the rhizoplane, which is defined as the root surface and outermost cells of the root. However, little is known about the colonization of the rhizosphere and plant roots by anaerobic bacteria (Großkopf et al., 1998; Hines et al., 1999). Although the rhizospheres of salt marsh vegetation and rice plants might be thought of as anoxic, a gradient of O_2 is generated around roots via the transport of O_2 that is produced by leaf photosynthesis during the day (Gilbert and Frenzel, 1995; Revsbech et al., 1999). Thus, obligate anaerobes colonizing such rhizospheres experience periods of elevated O_2 tension.

Sea grass rhizosphere has higher numbers of acetogenic bacteria than unvegetated soil, and acetogenic *O*-demethylation activity is tightly associated with sea grass roots (Küsel et al., 1999b). Hybridization of root thin sections with ^{33}P-labeled probes specific for *Acetobacterium* revealed the intercellular colonization of sea grass roots by *Acetobacterium*-like bacteria (Küsel et al., 1999b). The *Acetobacterium*-like bacteria occur mostly in the rhizoplane and outermost cell layers of the cortex. An H_2-utilizing acetogen, RD1, was obtained from the highest, growth-positive dilution of a sea grass root most-probable number series, and analysis of the 16S rRNA gene sequence indicated that the acetogen was closely related to a bacterium (*Clostridium glycolicum*; 99.7% gene sequence similarity) not previously known to be an acetogen (Küsel

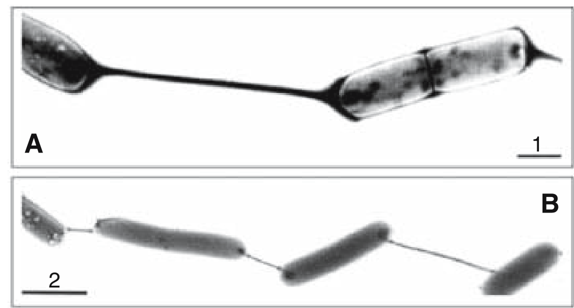


Fig. 19. Electron micrographs of (A) the acetogen *Clostridium glycolicum* RD-1 that was isolated from a sea grass root (used with permission from Küsel et al., 2001) and (B) the nitrogen-fixing soil bacterium *Clostridium akagii* (Kuhner et al., 2000). These organisms have connecting filaments that might provide cells with a means of remaining in close proximity to one another for either structural or communication purposes under certain in situ conditions. The structural nature of the filaments are not fully resolved, but recent ultra structural analyses suggest that the outer portion of the filament is an extension of the outer surface layer of the cell. Bars are in micrometers.

et al., 2001; Fig. 19). Retrieval of formyltetrahydrofolate synthetase sequences from salt marsh plant roots indicates that such roots are colonized by diverse acetogens most closely related to the genera *Sporomusa*, *Acetobacterium*, *Clostridium* and *Eubacterium* (Leaphart et al., 2003). Acetogens associated with salt marsh plant roots might also display a high tolerance to O_2 (Küsel et al., 2003). These results indicate that the biogeochemistry of the sea grass rhizosphere fosters the growth of acetogens in a habitat classically considered to be sulfate-reducing.

DEEP SUBSURFACE. Many subterranean environments are anoxic and habitats for anaerobic microorganisms. In the past, deep subsurface microbial communities have been thought to be supported by organic matter deposited with the formation of sediments, or by organic matter that migrated from the surface along different flowpaths (Krumholz, 2000). Thus, most studies have focused on the occurrence and activity of microorganisms within recently deposited or highly permeable sediments rather than from consolidated subterranean rock. However, diverse microbial communities also occur in subsurface fractured granitic rock, in suboceanic basalts, and in deep sediments of oceans (Krumholz, 2000; Kotelnikova, 2002). It is estimated that the global carbon content in subsurface prokaryota is comparable to the carbon content stored in terrestrial plants (Whitman et al., 1998). The density of a microbial community is limited by nutrient availability, which decreases in general with increasing depth. In deep, low organic carbon sediments in the Wood-

land Basin of the Pacific Ocean, numbers of cultured H_2-utilizing acetogens decrease with increasing depth from a surface maximum of approximately 10^6 cells per ml of sediment to negligible numbers at a depth of 800 m below the seafloor (Wellsbury et al., 1997; Wellsbury et al., 2002). However, microbial life might also be supported by other mechanisms, e.g., by the use of rock- or sediment-bound organic material previously thought to be unavailable.

Organic matter trapped in shales during deposition in the Cretaceous period (about 100 million years ago) can fuel heterotrophic microbial communities and the formation of acetate in adjacent permeable sandstones (Krumholz et al., 1997). From these sandstones, the acetogen *A. psammolithicum* was isolated with H_2-CO_2 as a substrate (Krumholz et al., 1999). However, the reduction of sulfate might be the dominant sink for H_2-derived reductant, and organic compounds might be the main substrates for acetogens in such terrestrial subsurface habitats (Krumholz et al., 1999).

In suboceanic sediments, bacterial populations and their activities can increase even in deeper layers near gas hydrate zones. Pore water concentrations of acetate can reach surprisingly high concentrations of approximately 15 mM at 700 meters below the seafloor, approximately 100 times higher than average near surface concentrations (Wellsbury et al., 1997; Wellsbury et al., 2000). Acetate seems to be the principal energy source for methane formation, and high turnover rates of acetate indicate an upward migration of high concentrations of dissolved organic carbon into the sediments. Apparently, the bioavailability of sedimentary organic matter appears to be enhanced by low-temperature heating during burial (Wellsbury et al., 1997). In addition to this biological enhanced formation of acetate, acetate can also be formed by thermogenic alteration of organic matter at temperatures above 80°C. The potential role of mesophilic and/or themophilic acetogens in the formation of acetate in these deep sediment layers is unclear.

Dissolved organic carbon may serve as a source of energy and carbon for microorganisms in deep subterranean groundwater of thin granitic fractures (Kotelnikova, 2002). In addition, concentrations of dissolved H_2 in these deep aquifers can be significantly higher (20–100 μM) than in other aquatic surface habitats (Krumholz, 2000). Acetogenic and acetotrophic methanogenic bacteria dominate the viable cell counts of different physiological groups in deep granitic groundwater that contains H_2 and CH_4 (Kotelnikova and Petersen, 1997; Kotelnikova and Petersen, 1998). The cultured numbers of H_2-utilizing acetogens approximate up to 10^4 cells per ml of groundwater. In microcosms containing granitic groundwater, $^{14}CH_4$ and ^{14}C-labeled acetate are formed from $^{14}CO_2$, and ^{14}C-labeled acetate is converted to $^{14}CH_4$. Thus, chemolithoautotrophic microorganisms that can grow on H_2-CO_2 might act as primary producers of organic carbon, initiating heterotrophic food chains in deep subterranean habitats. Acetogenesis appears to be involved in these H_2-based autotrophic biospheres (Haveman and Pedersen, 2002; Kotelnikova, 2002).

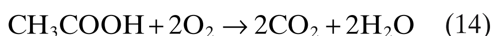
The origin of H_2 in subsurface habitats appears to be diverse. H_2 may result from mixed geochemical and biogenic reactions including the hydrolysis of water under strongly reduced conditions by ferrous iron present in basalt (Kotelnikova, 2002). In controlled laboratory experiments, basalts as well as granitic rock samples incubated with buffered water produce H_2 gas (Stevens and McKinley, 1995). However, whether those levels of H_2 could be produced at environmentally relevant pH values or sustained over geological time has been questioned (Anderson et al., 1998). Nonetheless, deep basalt aquifers can contain up to 60 μM dissolved H_2, and autotrophs outnumber heterotrophs in such habitats. Stable isotope measurements suggest that autotrophic methanogenesis dominates this lithoautotrophic microbial ecosystem in deep basalt aquifers (Stevens and McKinley, 1995).

OXIC SOILS. Anoxic microzones can occur in oxic soils and litter when the consumption of O_2 exceeds its supply (Tiedje et al., 1984; Sexstone et al., 1985; Smith and Arah, 1986; Van der Lee, 1999). Forest, agricultural, and grassland soils have a tremendous capacity to form acetate from endogenous organic matter under anoxic conditions (up to 15 g of C-acetate per kg dry wt. of soil; Küsel and Drake, 1994; Peters and Conrad, 1996; Wagner et al., 1996). Beech leaf litter and spruce litter display a spontaneous capacity to form aliphatic acids (mainly acetate), alcohols, H_2, and CO_2 under anoxic conditions, indicating that a subcommunity of the microbiota can respond rapidly to anoxic conditions (Küsel and Drake, 1996; Reith et al., 2002). Supplemental H_2, CO or ethanol are converted to acetate by soils under anoxic conditions in stoichiometries that approximate those associated with H_2-, CO-, or ethanol-dependent acetogenesis. The acetogen *S. silvacetica* was isolated from forest soil and utilizes H_2 and ethanol (Kuhner et al., 1997). Acetogenic activities of soils are relatively stable when soils are subjected to oxic drying or fluxes of O_2 (Wagner et al., 1996). At in situ temperatures and independent of moisture content and the concentrations of acetate that are formed, acetate is a stable end product in anoxic soil and litter microcosms. After extended incubation periods (1–3 months), acetoclastic methanogenesis is induced (Küsel and Drake, 1995), which is

consisitent with 1) the view that oxic soils are not a significant source of methane (Boone, 1991; Tyler, 1991) and 2) the fact that the number of cultured methanogens is negligible in oxic soils (Peters and Conrad, 1995; Küsel et al., 1999c).

The cultured number of anaerobes from both forest mineral soil and litter is identical with the cultured number of acetate-producing anaerobes (Küsel et al., 1999c). H_2-utilizing acetogens are a dominant group of the cultured anaerobes and approximate 10^4 to 10^5 cells per g of dry soil or litter (Peters and Conrad, 1995; Küsel et al., 1999c). Because H_2 is thermodynamically less than an ideal substrate for acetogens, i.e., for reducing CO_2 to acetate (see the subsection on Use of Diverse Electron Acceptors in this Chapter), soil acetogens capable of utilizing organic molecules are likely to occur in greater numbers than those that respond to H_2 under laboratory conditions; thus, H_2 likely reveals only a small subset of the total acetogenic population. In addition to acetogens, other acetate-forming microorganisms (e.g., facultative members of the Enterobacteriacae) are plentiful in soils and are likely responsible for the majority of acetate formed anaerobically in soils (Küsel, 1999c). Independent of these considerations, only 1% of the cultured H_2-utilizing soil acetogens are detected after pasteurization (Küsel et al., 1999c), indicating that 1) a large percentage of the spore-forming soil acetogens are in a vegetative active state or 2) the dominant acetogens present in soil and litter are not spore-formers. The capacity of soils to form acetate from H_2-CO_2 is enhanced by elevated temperatures (e.g., 30 and 55°C; Küsel and Drake, 1995; Wagner et al., 1996), suggesting that high temperature soils harbor thermophilic acetogens. The isolation of different strains of *M. thermoacetica* from soils from Kansas, Egypt and Japan demonstrate that this classic acetogenic thermophile has a wide geographical distribution in oxic soils (Gößer and Drake, 1997; Gößer et al., 1998; Gößer et al., 1999; Karita et al., 2003).

Because of the apparent stability of acetate under anoxic conditions, and because of the temporal and spatial variability of O_2 in soils, the consumption of acetate that is formed anaerobically might be linked to oxidative processes. O_2 is rapidly consumed when it is added to litter or soil that has been incubated under anoxic conditions (Küsel and Drake, 1995; Wagner et al., 1996; Küsel et al., 1999c), and the consumption of O_2 is concomitant to an increase in CO_2 and the disappearance of anaerobically formed acetate according to the following stoichiometry:



$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O \quad (14)$$

The rate at which acetate is consumed exceeds the rate at which acetate is formed, indicating that acetate undergoes a rapid in situ turnover. The turnover of acetate can also be linked to other oxidative processes in soils, such as denitrification (Küsel and Drake, 1995; Wagner et al., 1996) or Fe(III) reduction (Küsel et al., 2002). Thus, under in situ conditions, the acetate formed in anoxic microzones of oxic soils is likely subject to rapid consumption via 1) the diffusion of O_2 into formerly anoxic zones or 2) the transport of acetate with the soil solution into zones where electron acceptors like O_2, nitrate or Fe(III) are present (Fig. 20). These findings indicate that acetate is a trophic link between the different anaerobic and aerobic microbial populations that collectively decompose organic matter in oxic soils.

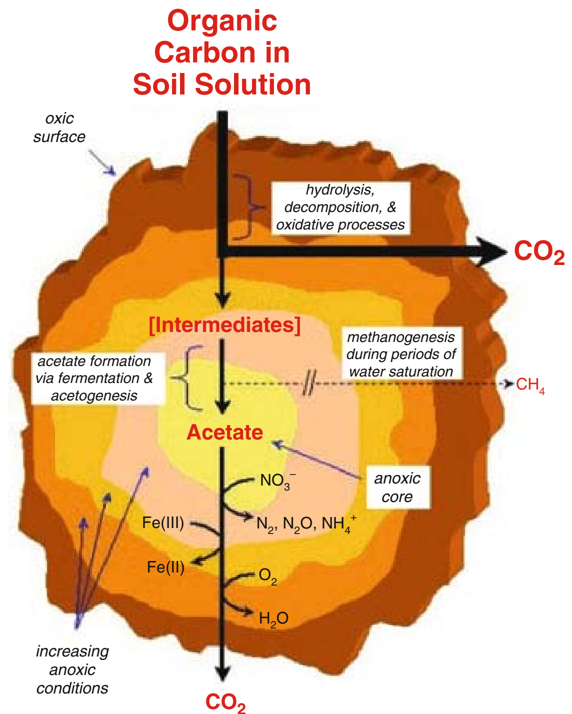


Fig. 20. Cross-section of a soil aggregate showing a hypothetical anoxic core and possible trophic links between acetate and other redox processes during the oxidation of soil organic carbon to CO_2. Modified from Drake et al. (1997).

Biotechnological Applications of Acetogens

The biotechnological application of acetogens has been the subject of numerous investigations. However, to date, a commercial-scale application of an acetogen or acetogenesis has not been reported. It is beyond the scope of this chapter to evaluate this topic in detail, and the reader is

directed to reviews for further information (Wiegel, 1990; Wiegel, 1994; Lowe et al., 1993).

Commercial Production of Acetic Acid from Sugars

Acetogens convert sugars stoichiometrically to acetate. This metabolic capacity has been the main focal point of studies designed to evaluate the commercial application of acetogens. Acetic acid is produced commercially from feedstock compounds (e.g., methanol), and global production in 2001 approximated 10^{10} kg (Anonymous, 2002; Causey et al., 2003). Acetic acid can be produced microbiologically from sugars. In the two-stage vinegar process, a hexose is converted to two molecules of acetic acid by the sequential activities of a yeast (e.g., *Saccharomyces cerevisiae*) that anaerobically produces two molecules of ethanol per hexose and of an aerobe (e.g., *A. aceti*) that only partially oxidizes ethanol to acetic acid. In contrast, the production of acetic acid by acetogenic bacteria is a single-stage process. Although acetogenesis conserves all of the carbon of glucose in the product acetic acid and might therefore be considered the ideal microbial process for the commercial production of acetic acid, the commercialization of the process has thus far not been realized. Furthermore, both microbiological processes are about 35% more expensive than the cost of the synthetic process from feedstock chemicals (estimated at $0.30/lb of acetic acid in 1991; Busche, 1991). *Escherichia coli* has been genetically modified to produce 2 moles of acetate per mole glucose fermented (Causey et al., 2003); such an organism might be competitive with the two-stage vinegar process.

The two main unsolved problems for the commercialization of the acetogenic process are: 1) acetogens are inhibited by high concentrations of acetate and 2) acetogens do not grow under acidic conditions. No known acetogen can adequately produce acetic acid at the concentrations required (i.e., 50 g of acetic acid per liter) for the process to be commercially feasible (Wiegel, 1994). These problems have been addressed in numerous studies, but significant breakthroughs in overcoming these problems have not been reported (Schwartz and Keller, 1982; Wang and Wang, 1983; Wang and Wang, 1984; Ljungdahl et al., 1985; Ljungdahl et al., 1989; Sugaya et al., 1986; Klemps et al., 1987; Brumm, 1988; Von Eysmondt et al., 1990; Ibba and Fynn, 1991; Parekh and Cheryan, 1991; Cheryan and Parekh, 1992). Commercialization of acetogenesis would theoretically become cost-competitive with the synthetic process if an acetogenic bacterium that could tolerate acidic conditions and produce high concentrations of acetic acid were either discovered or engineered (Brusche, 1991). Although mutants have been obtained that have increased tolerance to acidic acid and acidic conditions, such mutants grow poorly (Schwartz and Keller, 1982; Wiegel, 1994). The strong uncoupling effect of acetic acid and protons on the proton motive force (ΔpH) and transmembrane electrical potentials ($\Delta\Psi$) of acetogens results in a collapse of the cell's ability to conserve energy, and thus makes it unlikely that simple mutations could circumvent these problems and yield a mutant with tolerance to acetic acid and acidic conditions that is grossly different from the parent strain's tolerance (Baronofsky et al., 1984).

Although sugar dimers can be utilized by certain acetogens (e.g., cellobiose is a substrate for *R. productus*; Lorowitz and Bryant, 1984), a somewhat serious disadvantage of commercializing acetogenesis is that acetogens do not degrade large sugar polymers (e.g., cellulose). However, two acetogens, "*B. formatexigens*" (Wolin et al., 2003) and *M. thermoacetica* strain F21 (Karita et al., 2003), with the capacity to degrade cellulose have recently been found. In addition, cocultures of cellulolytic *Clostridium thermocellum* and the thermophilic acetogen *T. kivui* convert cellulose to acetate, with near full recovery of cellulose-derived carbon in acetate (Le Ruyet et al., 1984). Similar results were obtained with a coculture of a cellulolytic strain of *Ruminococcus albus* and the unclassified acetogen HA (Miller and Wolin, 1995). *Ruminococcus albus* forms ethanol and H_2 as reduced end products in the absence of the acetogen HA, but in coculture with HA, the reducing equivalents derived from the oxidation of cellulose-derived hexoses are utilized by the acetogen via the interspecies transfer of H_2. These findings suggest that acetogens can in fact be utilized for the conversion of cellulolytic material to acetate. *Clostridium lentocellum* strain SG6 forms high amounts of acetate from cellulose (Ravinder et al., 2001). Although the metabolism of this strain was described as acetogenic, significant amounts of ethanol are also produced and the engagement of the acetyl-CoA pathway during the degradation of cellulose is therefore uncertain.

Despite the limitations and cost barriers outlined above, the potential use of calcium-magnesium acetate as an environmentally safe road de-icer and in controlling sulfur emissions during the combustion of high sulfur coal have continued to foster interest in the commercial production of acetic acid by acetogens (Ljungdahl et al., 1985; Ljungdahl et al., 1989; Wiegel et al., 1990; Parekh and Cheryan, 1991; Cheryan and Parekh, 1992; Wiegel, 1994; Cheryan et al., 1997). Thermophilic species of acetogens (e.g., *M. thermoacetica* and *T. kivuii*) offer several theoretical advantages for the commercial production of environmentally safe

calcium-magnesium acetate: 1) thermophilic acetogenesis bypasses the need to use sterilized medium, the production of which is costly, 2) the dispersal of thermophiles or their spores in low temperature climates does not constitute an environmental threat, and 3) the growth of pathogens and their subsequent dispersal with the calcium-magnesium acetate would be highly unlikely owing to the thermophilic production conditions (Wiegel, 1994). Although *T. kivuii* displays one of the shortest doubling times of all known acetogens (approx. 2.5 h on either H_2-CO_2 or glucose [Leigh et al., 1981; Daniel et al., 1990]), it is more sensitive to acetic acid than certain strains of *M. thermoacetica* or *M. thermoautotrophica*, and therefore does not appear to be a model of choice for commercial purposes (Wiegel, 1994). *Moorella thermoacetica* and *Clostridium thermolacticum* can convert lactose to acetic acid when the two organisms are immobilized together in a fibrous-bed reactor (Talabardon et al., 2000). Under these conditions, *C. thermolacticum* forms lactate, which is subsequently used for lactate-dependent acetogenesis by *M. thermoacetica*. This trophic association mimics that exemplified by *M. thermoacetica* and *T. aegyptius* (Gößner et al., 1999; Fig. 20). Immobilization of acetogens with metabolic partners might offer certain advantages for commercializing the use of acetogens.

One approach to increasing the amount of acetate produced by acetogens is to uncouple growth from acetogenesis. Harmaline, a putative inhibitor of Na^+/H^+ antiporters, uncouples acetogenesis from the growth of *A. kivuii* (Yang and Drake, 1990). In the presence of harmaline, the acetate-to-biomass ratio during H_2-dependent acetogenesis increased 13-fold. Thus, use of agents that uncouple growth from the production of acetate might be of value in making acetogenesis more commercially feasible.

Bioconversion of Synthesis Gas to Acetic Acid, Ethanol, and Other Chemicals

Synthesis gas is obtained by the indirect liquefaction of coal and mainly consists of H_2, CO, and CO_2. These gaseous molecules can be converted to acetate by acetogenic bacteria, and the potential use of acetogens for the bioconversion of synthesis gas to acetic acid has been evaluated (Grethlein and Jain, 1992). Butyrate and *n*-butanol are additional products that can be produced from synthesis gas by "*B. methylotrophicum*" (Worden et al., 1989; Grethlein et al., 1991). Likewise, metabolically altered or unique strains of acetogens (e.g., *C. ljungdahlii*) can produce ethanol from the components of synthesis gas (Buschhorn et al., 1989; Grethlein and Jain, 1992; Tanner et al., 1993; Phillips et al., 1994). Electrochemical processes for converting CO_2 to acetate with enzymes from *M. thermoacetica* have been reported (Shin et al., 2001).

Bioremediation, Bioreactors, and Landfills

Although acetogens have robust metabolic capabilities and might be thought of as having significant bioremediation potentials, such potentials have not been extensively examined. Certain acetogens (e.g., "*A. dehalogenans*") can dehalogenate toxic compounds (Egli et al., 1988; Freedman and Gossett, 1991; Traunecker et al., 1991; Meßner et al., 1996). However, few studies have addressed this potential. On the basis of information to date, the ability to degrade aromatic rings is not a widespread metabolic potential of acetogens. A noted exception is *H. foetida* (Liesack et al., 1994). Acetyl-CoA synthase can transform 2,4,6-trinitrotoluene (TNT), a highly explosive anthropogenic compound that contaminates certain soils (Preuss et al., 1993; Huang et al., 2000); however, a commercial (or environmental) application of the potential of acetogens to transform TNT has not been reported.

Moorella thermoacetica has been shown to be effective in sequestering (i.e., precipitating) the heavy metal cadmium, suggesting that acetogens might be of applied value in the cleanup of environments or materials contaminated with heavy metals (Cunningham and Lundie, 1993). By virtue of their ability to oxidize and consume CO, acetogens have been cited as being significant in the detoxification of environmental CO (Ragsdale, 1991). However, the detoxification of environmental CO occurs mainly by abiotic processes in the atmosphere or aerobic CO-oxidizers (e.g., soil carboxydotrophs; Meyer, 1988; Meyer et al., 1993). The acetyl-CoA synthase of acetogens transforms 2,4,6-trinitrotoluene (TNT; Preuss et al., 1993; Huang et al., 2000); application of this catalytic potential has not been reported.

In methanogenic bioreactors and landfills, acetate that is produced by acetogens is a substrate for acetoclastic methanogens. Thus, acetogens contribute significantly to the turnover of organic matter in methanogenic bioreactors and landfills (McInerney and Bryant, 1981; Ibba and Fynn, 1991; Wiegel, 1994; Barlaz, 1997).

Other Potential Applications

The commercial production of corrinoids (i.e., vitamin B_{12}) and cysteine by acetogens has been evaluated; however, commercial-scale production has not been reported (Koesnandar et al., 1991; Inoue et al., 1992; Lebloas et al., 1994).

Acetate kinase from *M. thermoacetica* is very stable, and its industrial use in the immobilized form has been patented in Japan (Wiegel, 1994). A variety of fine chemicals (e.g., enantiomers of malic acid) have been produced with *C. formicoaceticum* (Eck and Simon, 1994a; Eck and Simon, 1994b). *Moorella thermoacetica* has been utilized for the electromicrobial regeneration of pyridine nucleotides (Schulz et al., 1995; see also Günther et al., 2000). Enhancement of acetogenesis in the rumen might enhance the cost-efficiency of ruminant husbandry and also decrease the emission of the greenhouse gas methane by ruminants; this topic is discussed in the subsection on Diverse Habitats.

Summary and Conclusions

The following items summarize the main characteristics of acetogens and the acetyl-CoA pathway:

1) Acetogens belong to the domain Bacteria and use the acetyl-CoA "Wood Ljungdahl" pathway as a terminal electron accepting process.

2) The acetyl-CoA pathway fixes CO_2, conserves energy, and produces acetyl-CoA that is utilized in the synthesis of either acetate or biomass.

3) Pathways that are biochemically very similar to the acetyl-CoA pathway are used by methanogens and sulfate reducers for the oxidation of acetate or the autotrophic fixation of CO_2 and the synthesis of biomass. The collective use of acetyl-CoA synthase-dependent pathways by acetogens, methanogens, and sulfate reducers facilitates an enormous turnover of carbon in the global carbon cycle. An acetyl-CoA synthase-dependent process may have been the first autotrophic process on earth.

4) Acetogens can grow autotrophically and heterotrophically, and can oxidize a wide range of carbonaceous substrates, including aromatic compounds and small-molecular-weight halogenated compounds. Two recent isolates are cellulolytic.

5) Twenty-one genera of acetogens have been isolated from very diverse habitats, ranging from the gastrointestinal tracts of mammals and insects to sea grass rhizospheres. Their closest 16S rRNA-phylogenetic neighbor is very often not an acetogen, making it impossible to develop a broad-based, 16S rRNA-acetogen probe.

6) Although acetogens have been classically regarded as obligate anaerobes, they can tolerate and reduce small quantities of O_2 and exist in habitats subject to transient fluxes of O_2.

7) Acetogens utilize substrate-level phosphorylation, membranous electron transport systems, and ATPases to conserve energy.

8) Although the acetyl-CoA pathway is the hallmark of acetogens, they can also utilize other terminal electron-accepting, energy-conserving processes, including the dissimilation of aromatic acrylates and nitrate. Thus, acetogens are not strictly dependent upon acetogenesis.

9) The acetyl-CoA pathway can be repressed when alternative terminal electron acceptors are utilized. Thus, acetogens do not always produce acetate.

10) Acetogens form different types of trophic interactions for other microorganisms, including syntrophic relationships with other anaerobes and commensal relationships with fermentative microaerophiles.

11) Numerous theoretical commercial applications for acetogens have been evaluated; however, to date, no commercial-scale application has been reported.

Numerous unresolved questions might be considered worthy of further investigation: What is the biochemical nature of the diverse catabolic processes of acetogens? How are these processes regulated at the gene level? Are acetogens more capable of degrading cellulose and other large biopolymers than previously thought? Can acetogens oxidize inorganic compounds, or is their catabolism restricted to the oxidation of carbonaceous substrates? What is the in situ impact of acetogens in complex habitats, such as the endorhizosphere of plants, gastrointestinal ecosystems, and the terrestrial subsurface? Can the metabolic capabilities of acetogens be successfully harnessed and put to commercial use?

The body of published information on acetogens has reached enormous proportions, and it has not been possible to provide adequate coverage to all of these works in this chapter. The authors invite individuals working in this area to send their latest results to the corresponding author (H. L. Drake) so that this information can be included when this chapter is updated at a future date.

Dedication and Acknowledgment

This tapestry is dedicated to Harland G. Wood and Lars G. Ljungdahl, the two individuals who carried the ball when no one else could. The authors express their appreciation to Anita Gößer for her many years of excellence in culturing and analyzing acetogens, to Marcus Horn for assistance with the phylogenetic analyses, to Georg Acker for electron microscopy of isolates, to Millie Wood for permission to publish the photo of Harland Wood, to Volker Müller for helpful discussions on bioenergetics, and to John Breznak, Paul Lindahl, Terry Miller, Steve Ragsdale, and Meyer Wolin for providing

unpublished information and helpful suggestions. Current support for the authors' laboratory is derived in part from funds from the German Research Society (DFG) and the German Ministry of Education, Research, and Technology (BMBF), and is gratefully acknowledged.

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Prokaryotes (2006) 2:421–440
DOI: 10.1007/0-387-30742-7_14

CHAPTER 1.14

Virulence Strategies of Plant Pathogenic Bacteria

BARBARA N. KUNKEL AND ZHONGYING CHEN

Introduction

Plant pathogenic bacteria, like bacterial pathogens that infect animals, must be able to evade or suppress general antimicrobial defenses and acquire nutrients and water from their hosts to successfully colonize and grow within host tissue. Plant pathogenic bacteria have adapted well to their hosts, which are structurally and physiologically quite different from animals. Since successful infection relies to a great extent on the ability of a pathogen to modulate the physiology of its host, plant pathogenic bacteria have evolved several unique virulence strategies in addition to virulence mechanisms also utilized by bacterial pathogens of animals.

One current area of intense research in the field of plant-pathogen interactions is the identification and characterization of pathogen virulence factors and the elucidation of their mode of action within the host. This chapter summarizes recent progress in this area of research, focusing on four Gram-negative bacterial pathogens that grow on living tissue and cause primarily leaf spotting or wilt diseases of plants: *Pseudomonas syringae*, *Xanthomonas campestris*, *Ralstonia solanacearum* and *Erwinia amylovora*, the causal agents of leaf spots, leaf blights, vascular wilts, and fire blights, respectively (Schroth, 1981; Chan and Goodwin, 1999; Eastgate, 2000; Genin and Boucher, 2002). The focus is on these pathogens because in recent years significant progress has been made towards elucidating the molecular mechanisms underlying their virulence (Staskawicz et al., 2001; da Silva et al., 2002; Salanoubat et al., 2002; Buell et al., 2003; Buttner and Bonas, 2003). The recent genome sequence data made available for representative strains of several of these pathogens (*P. syringae*, *X. campestris* and *R. solanacearum*) have also begun to provide additional insight into their virulence strategies (da Silva et al., 2002; Salanoubat et al., 2002; Buell et al., 2003). Further, since several of these pathogens can infect *Arabidopsis thaliana*, a widely studied model plant, use of molecular and genetic approaches to investigate the mode of action of pathogen virulence factors within this host has begun to contribute to our understanding of the virulence strategies of these plant pathogenic bacteria (Kunkel, 1996; Glazebrook, 2001; Quirino and Bent, 2003). For reviews on several other fascinating groups of plant-associated microbes, the tumor-inducing *Agrobacterium* spp., the soft rot *Erwinia* species, and the root-nodulating Rhizobia, refer to several recent articles (Broughton et al., 2000; Gelvin, 2003; Toth et al., 2003) and the relevant chapters in this volume (The Genus Agrobacterium in Volume 5; Erwinia and Related Genera in Volume 6; and Root and Stem Nodule Bacteria of Legumes in this Volume).

The Biology of Bacterial Plant Pathogens and Their Hosts

The Plant Apoplast as a Unique Niche for Bacterial Pathogens

Phytopathogenic bacteria are extracellular pathogens. Depending on the specific pathogen, they can grow epiphytically on plant surfaces, in between plant cells within host tissues, in what is referred to as the "apoplast," or within the vasculatory system (Beattie and Lindow, 1994; Alfano and Collmer, 1996; Agrios, 1997). The apoplast is considered to be an unfavorable environment for most microorganisms, as it contains several antimicrobial compounds (Dangl and Jones, 2001; Dixon, 2001; Glazebrook, 2001; Pignocchi and Foyer, 2003) and is believed to be relatively nutrient-poor. Additionally, plant defenses that are induced upon microbial attack are often targeted to the extracellular space (Hammond-Kosack and Jones, 1996). Therefore, to successfully colonize plant tissue, pathogens must both tolerate existing antimicrobial defenses and modulate the apoplastic environment to render it suitable for pathogen growth. This includes evading or suppressing antimicrobial host defenses and eliciting the release of nutrients and water from plant cells (Fig. 1).

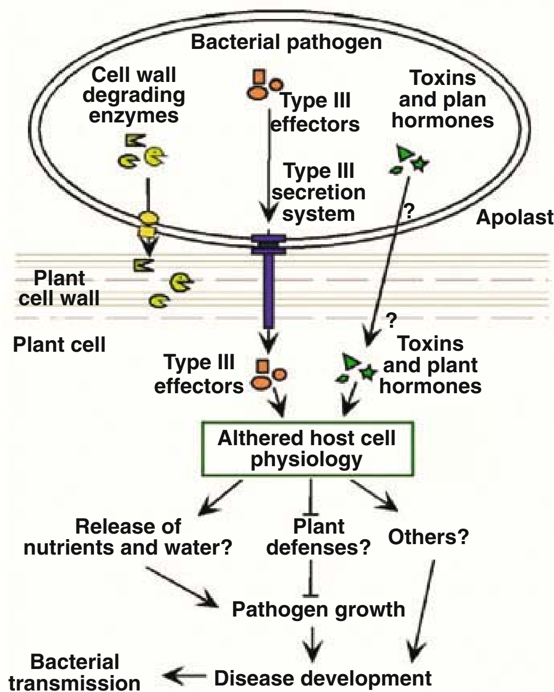

Fig. 1. Secreted virulence factors deployed by bacterial plant pathogens. Bacterial plant pathogens colonize the apoplastic space between plant cells. As extracellular pathogens, these organisms deploy an arsenal of secreted virulence factors to modulate host cell processes from outside plant cells. These factors include: 1) low molecular weight phytotoxins, plant hormones, and hormone analogs that are secreted into the apoplast, many of which presumably enter or are taken up by plant cells; 2) protein virulence factors (or "effectors") that are delivered directly into the plant cell cytosol via a specialized type III secretion system (TTSS), and 3) plant cell wall degrading enzymes that are secreted through a sec-dependent type II secretion system (Alfano and Collmer, 1996; Sandkvist, 2001) and function to degrade or remodel the plant cell wall. Type III-delivered effector molecules are proposed to function inside plant cells to modulate host cell physiology, thus rendering host tissue suitable for pathogen growth and disease development. The activities of these molecules may include: suppression of plant defense responses, stimulating the release of nutrients and water into the apoplast, promoting disease symptom development, and facilitating pathogen release from infected tissue, and hence pathogen transmission.

Plant cell walls also play an important role in plant-pathogen interactions. Unlike animal cells, plant cells are surrounded by a semi-rigid cell wall that provides structural support, maintains cell shape, cements adjacent plant cells together, and serves as a barrier to pathogen invasion and spread within infected tissue. Plant cell walls are composed of several complex carbohydrate polymers, the most abundant of which are cellulose and pectin (Carpita and McCann, 2000). Several of these carbohydrates may also serve as carbon sources for bacteria that can degrade cell wall polymers and take up and metabolize the resulting polysaccharide fragments.

Plant Defense Against Microbial Attack

Plants have evolved multiple basal defense mechanisms to protect themselves against microbial attack (Heath, 2000; Thordal-Christensen, 2003). For example, plants constitutively produce an array of nonspecific, antimicrobial compounds that serve as biochemical barriers to microbial colonization (Hammond-Kosack and Jones, 2000; Dixon, 2001). Additionally, in response to microbial attack, plants activate a complex series of general defense responses that are believed to inhibit colonization by microbial organisms. These inducible defenses include a rapid oxidative burst, accumulation of elevated levels of one or more endogenous signaling molecules, such as salicylic acid (SA), jasmonic acid (JA) and ethylene, induction of several defense-related genes (e.g., pathogenesis related or *PR* genes), the production of antimicrobial phytoalexins and lytic enzymes, and the reinforcement of plant cell walls surrounding the site of infection (Hammond-Kosack and Jones, 1996; Glazebrook et al., 1997; Felix et al., 1999; Gomez-Gomez and Boller, 2002).

Many of these defenses are also induced in response to infection by successful pathogens. However, in susceptible interactions (i.e., those resulting in disease) induction is weaker and occurs at a relatively late stage of infection and does not prevent disease development (Jakobek and Lindgren, 1993; Glazebrook et al., 1997; Tao et al., 2003). Rather, the expression of these defenses appears to limit spread of the pathogen and the severity of disease. Thus, it is widely believed that successful plant pathogens must be able to evade or actively inhibit induction of these general antimicrobial defenses to facilitate colonization of plant tissues (Fig. 1). Presumably, the ability to tolerate host defenses or to avoid and suppress activation of defenses normally induced upon microbial attack are traits that distinguish successful plant pathogens from non-pathogenic organisms (Alfano and Collmer, 1996; Felix et al., 1999; Jin et al., 2003). Recently, much interest has been focused on identifying the pathogen virulence factors involved in these processes and elucidating how they function to modify host defense mechanisms.

In addition to the basal defense mechanisms described above, plants have evolved a unique mechanism allowing them to detect and fend off invading pathogens (Dangl and Jones, 2001; Staskawicz et al., 2001). In many instances, even though a pathogen is able to colonize and initiate growth within host tissue, further multiplication and spread of the pathogen is curtailed by the

rapid and strong activation of host defense responses (Dangl and Jones, 2001; Glazebrook, 2001). The end result is disease resistance and little-to-no visible sign of infection.

In such resistant interactions, the rapid activation of host defenses is triggered by specific recognition of one or more elicitor molecules produced by the invading pathogen and is dependent on the expression of specific plant disease resistance (*R*) genes within the host (Dangl and Jones, 2001; Staskawicz et al., 2001; Bonas and Lahaye, 2002). Thus, pathogen recognition is controlled at the genetic level and is governed by plant *R* genes that confer on the plant the ability to recognize pathogen strains expressing specific elicitors, which are often referred to as "avirulence factors" (Dangl and Jones, 2001; Nimchuk et al., 2001; Staskawicz et al., 2001; Bonas and Lahaye, 2002). In many cases the pathogen avirulence (*avr*) genes directing production of these elicitors have been identified. Many pathogen *avr* genes and the corresponding plant *R* genes have been cloned, and much research has been focused on elucidating the mechanisms by which they mediate pathogen recognition and disease resistance (reviewed in Dangl and Jones [2001] and Martin et al. [2003]).

The existence of *avr* factors seems paradoxical, as it is unclear how elicitation of host defense responses would be of benefit to the pathogen. However, the prevalence of *avr* genes among pathogenic strains indicates that they must provide some selective advantage for the pathogen. Thus it has been proposed that the primary function of bacterial Avr proteins is to promote pathogen growth and disease development on susceptible host plants (Alfano and Collmer, 1996; Nimchuk et al., 2001; Staskawicz et al., 2001; Ponciano et al., 2003). Accordingly, several bacterial *avr* genes have been shown to contribute to virulence on susceptible plant lines lacking the corresponding resistance gene (Nimchuk et al., 2001; Ponciano et al., 2003; and see the section Type III Secretion in this Chapter). However, the function of Avr proteins and the mechanisms by which they promote parasitism and disease are not well understood. Given that most Avr proteins appear to be secreted directly into the plant cell (see the section Type III Secretion in this Chapter), perhaps it is not surprising that plants have evolved the ability to recognize these molecules as signs of pathogen attack (Dangl and Jones, 2001).

Virulence Strategies of Extracellular Pathogens

Given the physiology of plants and the nature of the antimicrobial defense responses they deploy, plant pathogens have evolved a variety of specialized virulence strategies to facilitate colonization of plant tissue. These include: tolerating pre-existing antimicrobial compounds; evading, overcoming or suppressing antimicrobial host defenses; eliciting the release of nutrients and water from plant cells; and in many cases, interfering with the integrity of plant cell walls surrounding the initial site of infection.

The achievement of these goals relies to a great extent on the ability of plant pathogens to modulate host physiology. As these pathogens are extracellular, they deploy an arsenal of secreted virulence factors to modulate host cell processes from outside plant cells (Fig. 1). These virulence factors include: 1) low molecular weight phytotoxins that are secreted into the apoplast (Bender et al., 1999), 2) protein virulence factors (or "effectors") that are delivered directly into the plant cell cytosol via a specialized, type III secretion system (TTSS; Galan and Collmer, 1999; Jin et al., 2003), 3) degradative enzymes that target the plant cell wall, and 4) extracellular polysaccharides (EPS). Additionally, in some interactions plant pathogens may directly modulate hormone physiology within their hosts through the production of plant hormones or hormone analogs (Alfano and Collmer, 1996).

Plant pathogens also express genes believed to help them adapt to the stressful conditions that are constitutively present or that are generated by the host in response to microbial attack. These include the production of proteins and enzymes to counter oxidative stress (e.g., glutathione S-transferase, superoxide dismutase, and catalase), as well as enzymes that may detoxify antimicrobial compounds (Boch et al., 2002; Salanoubat et al., 2002; Buell et al., 2003).

In the majority of pathogenic interactions, disease ensues only after the pathogen has colonized and grown to high levels in the infected tissue. In many cases, disease symptom production is believed to facilitate pathogen release from infected tissue and spread to uninfected tissues and neighboring plants (Agrios, 1997). Therefore, the elicitation of disease symptoms is also often considered an important virulence strategy.

Low Molecular Weight Phytotoxins

Many plant pathogens produce low molecular weight, non-host specific phytotoxins that contribute to virulence, either by directly damaging plant cells or by modulating host cellular metabolism or physiology to promote symptom development (Alfano and Collmer, 1996; Bender et al., 1999; Birch, 2001). The most well character-

ized of these phytotoxins are those produced by *P. syringae* species, and include lipodepsipeptide toxins (e.g., syringomycins and syringopeptins), modified peptides (e.g., tabtoxin and phaseolotoxin), and polyketides (e.g., coronatine).

Lipodepsipeptide Toxins

Syringomycins and syringopeptins are examples of the two classes of lipodepsipeptide toxins produced by *P. syringae* pv. *syringae* during infection. The syringomycins are cyclic lipodepsinonapeptide phytotoxins that consist of a polar cyclic peptide head containing nine amino acids attached to a hydrophobic 3-hydroxy carboxylic acid tail (Bender and Scholz-Schroeder, 2004). Several structurally similar syringomycins are produced by different *P. syringae* pv. *syringae* strains that contain different amino acid residues in the nine peptide ring. Syringopeptins are somewhat larger than the syringomycins and contain a peptide moiety of 22 or 25 amino acids attached to either a 3-hydroxydecanoic or a 3-hydroxydodecdanoic acid (Bender and Scholz-Schroeder, 2004). As in syringomycins, the amino acid chain is cyclized to form a nine-peptide ring. Many of the amino acids present in the syringopeptins are hydrophobic, and thus contribute to the amphipathic nature of these toxins.

The syringomycins and syringopeptins induce necrosis in plant tissues by inserting into plant cell plasma membranes, causing increased transmembrane flux of ions, the disruption of electrical potential across the cell membrane, and eventual plant cell death (Hutchison and Gross, 1997). The amphipathic nature of these toxins is likely to facilitate their insertion into plant cell membranes. Both classes of toxins have been shown to form channels in lipid membranes through a mechanism believed to involve initial insertion of toxin monomers into the membrane, followed by aggregation of multiple monomers to form a pore (Bender and Scholz-Schroeder, 2004).

Lipodepsipeptide phytotoxins are likely to play an important role in interactions between *P. syringae* pv. *syringae* and its hosts, as all strains of *P. syringae* pv. *syringae* analyzed to date produce both syringomycins and syringopeptins. However, these toxins appear to contribute quantitatively to *P. syringae* pv. *syringae* virulence, and the relative importance of these toxins appears to vary among different pathogen-host interactions (Bender et al., 1999; Scholz-Schroeder et al., 2001).

Although much progress has been made toward understanding the biosynthesis and pore-forming activities of the lipodepsipeptide phytotoxins (Bender and Scholz-Schroeder, 2004), their actual role in pathogenesis is not understood. They may contribute to pathogen virulence by stimulating plant cell necrosis and disease lesion development, or by modulating host cell physiology or signaling by altering the flux of ions across plant cell membranes. Additionally, as both syringomycins and syringopeptins exhibit biosurfactant activities, they could potentially contribute to virulence by reducing the surface tension of water and thus facilitate the spread of bacteria across plant surfaces (Bender et al., 1999), thereby promoting tissue colonization and spread of the pathogen within infected plant tissue.

Interestingly, the recent completion of the genomic sequence of *R. solanacearum* has revealed two large open reading frames predicted to encode proteins with high similarity to syringomycin synthase (Salanoubat et al., 2002), suggesting that this pathogen may also produce syringomycin.

Modified Peptide Toxins

The structure and mode of action of two modified peptide toxins, tabtoxin and phaseolotoxin, produced by *P. syringae* pathovars *tabaci* and *phaseolicola*, respectively, are especially well understood. Tabtoxin is a dipeptide toxin that contains tabtoxinine-β-lactam (TβL), linked by a peptide bond to threonine (Bender et al., 1999). TβL is the toxic moiety of tabtoxin and is released from the intact toxin upon hydrolysis of the peptide bond by the action of aminopeptidases within the plant (Levi and Durbin, 1986). TβL, which induces the degradation of chlorophyll in plant cells (thus causing yellowing or "chlorosis" of normally green plant tissue), irreversibly inhibits the enzyme glutamine synthetase (Thomas et al., 1983). As glutamine synthetase is required for efficient detoxification of ammonia in plant cells, inactivation of this enzyme results in accumulation of high levels of ammonia and the disruption of thylakoid membranes within the chloroplast.

Phaseolotoxin is a tripeptide consisting of ornithine, alanine and a homoarginine linked to a sulfo-diaminophosphinyl moiety (Moore et al., 1984). When taken up by plant cells, phaseolotoxin is hydrolyzed to produce octicidine, an irreversible inhibitor of ornithine carbamoyl transferase (OCTase; Mitchell and Bieleski, 1977). OCTase is a key enzyme in the urea cycle that converts ornithine and carbamoyl phosphate to citrulline. Inhibition of OCTase by phaseolotoxin results in accumulation of ornithine and reduction in arginine levels, and leads to the production of severe chlorosis within plant tissue (Bender et al., 1999). Both tabtoxin and phaseolotoxin contribute significantly to

pathogen virulence, presumably by inhibiting photosynthesis, and thus limiting available resources within the plant for mounting a successful defense response and by contributing to the severe yellowing of plant tissues associated with disease (Agrios, 1997).

Coronatine

The polyketide phytotoxin coronatine is produced by many different *P. syringae* strains (Bender et al., 1999). Coronatine is of interest to both plant biologists and plant pathologists, as it appears to function as a molecular mimic of two different endogenous plant hormones, JA and ethylene, both of which are known to play important roles in plant defense (Bender et al., 1999; Kunkel and Brooks, 2002). Coronatine is comprised of two distinct chemical moieties, coronafacic acid (CFA; a polyketide) and coronamic acid (CMA; an ethylcyclopropyl amino acid) that are joined by an amide linkage (Bender et al., 1999). The CFA moiety shares structural and functional relatedness with several jasmonates (e.g., MeJA), a group of plant growth regulators and defense signaling molecules that are produced under conditions of biological stress (Wasternack and Parthier, 1997; Weber, 2002). The CMA moiety also has biological activity and resembles aminocyclopropyl carboxylic acid (ACC), the immediate precursor of ethylene (Toshima et al., 1993). Ethylene is involved in many aspects of plant biology, including fruit ripening, senescence and defense (Bleecker and Kende, 2000; and see the section Ethylene in this Chapter).

Coronatine contributes to the virulence of *P. syringae* by promoting both pathogen growth and lesion formation in several host plants (Bender et al., 1999; Brooks et al., 2004). The biological effects of coronatine closely resemble those induced by jasmonates and include induction of chlorosis (i.e., yellowing of green tissue due to degradation of chlorophyll), production of the protective pigment anthocyanin, inhibition of root growth, promotion of plant cell growth and enlargement, and the induction of several JA-responsive genes (Feys et al., 1994; Bender et al., 1999; Zhao et al., 2003).

The mechanisms underlying the virulence activity of coronatine are not well understood. Results from genetic studies utilizing both *P. syringae* mutants impaired in coronatine biosynthesis and *A. thaliana* and tomato mutants that are impaired in JA signaling, suggest that coronatine promotes pathogen virulence by stimulating JA signaling within the plant (Feys, 1994; Kloek, 2001; Zhao, 2003; D. Brooks et al., manuscript submitted). However, how this leads to increased susceptibility to *P. syringae* is unclear. One hypothesis, based on mounting evidence that the SA and JA-dependent defense signaling pathways are mutually antagonistic, is that coronatine-induced activation of JA signaling results in inhibition of SA-dependent defense responses, which are effective in limiting *P. syringae* infection and disease (Kunkel and Brooks, 2002). The finding that reduced susceptibility to *P. syringae* in *A. thaliana* coronatine insensitive (*coi1*) mutant plants is associated with increased signaling through the SA-dependent defense pathway (Kloek et al., 2001), and the recent observation that coronatine suppresses induction of several SA-dependent defense-related genes in tomato (Zhao et al., 2003) are consistent with this hypothesis. Thus, production of coronatine appears to result in suppression of host defenses, thereby providing *P. syringae* with a window of opportunity during which it can colonize and grow within host tissue.

Coronatine may also be directly involved in disease symptom development (Kloek et al., 2001). The in planta growth defect of *P. syringae* coronatine biosynthetic mutants is restored in *A. thaliana* plant mutants in which SA-dependent defenses are compromised. However, although the coronatine mutants grow to wildtype levels in these plants, disease symptom development is not fully restored (D. Brooks et al., manuscript submitted). The mechanism by which coronatine contributes to lesion formation is not understood.

Type III Secretion

Like most Gram-negative bacterial pathogens of animals studied to date, the bacterial plant pathogens discussed in this chapter require a functional type III secretion system (TTSS) for pathogenesis (Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000; Staskawicz et al., 2001; Buttner and Bonas, 2003; Jin et al., 2003). Type III secretion systems mediate the transfer of bacterial proteins (also referred to as "effectors") directly into the cytosol of the host cell, where they interfere with or modulate normal host cell processes to facilitate bacterial invasion, growth and disease production (Fig. 1). In animal systems, many of these effectors induce changes in the host cell cytoskeleton, while others modify eukaryotic signal transduction pathways (Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000; Cornelis, 2002; Buttner and Bonas, 2003).

In the case of plant pathogenic bacteria, mutants defective in TTSS are usually unable to grow or cause disease on normally susceptible hosts, indicating that the integrity of the TTSS is essential for pathogenesis (Lindgren et al., 1986). Much progress has been made towards

elucidating the structure and components of the TTSS, and in identifying the effector proteins secreted through this apparatus. Insights into the function of several type III effectors and how they contribute to the virulence of plant pathogens have also been recently obtained.

Structure and Components of TTSS of Bacterial Plant Pathogens

The structural components of the TTSS of Gram-negative bacterial pathogens of animal and plants are highly conserved. As these systems have been extensively described elsewhere (Galan and Collmer, 1999; Collmer et al., 2000; Cornelis and Van Gijsegem, 2000; Buttner and Bonas, 2002; Jin et al., 2003), the TTSS apparatus will not be discussed in detail. However, note that structurally the TTSSs of plant pathogenic bacteria are slightly different from those described for the animal pathogens. For example, the TTSS of several mammalian pathogens, including *Salmonella typhimurium* and *Shigella flexneri*, are associated with protruding, needle-like surface structures that are approximately 80 nm in length (Kubori et al., 1998; Blocker et al., 1999). The TTSS of several plant pathogenic bacteria are associated with relatively longer, pilus-like structures (referred to as "Hrp pili"; He and Jin, 2003). The Hrp pilus of *P. syringae* pv *tomato* strain DC3000 is approximately 8 nm in diameter and has been observed to be up to 200 nm in length, which is presumably long enough to span the plant cell wall (Brown et al., 2001; Jin and He, 2001). Several studies suggest (but do not directly demonstrate) that the Hrp pilus serves as the conduit through which bacterial proteins are secreted (Brown et al., 2001; Jin and He, 2001).

Identification of Type III Effectors

The importance of TTSS for pathogenesis has prompted many research groups to direct a significant amount of effort towards identifying and characterizing proteins that are secreted through the TTSS. An inventory of effector proteins secreted by plant pathogenic bacteria has been recently compiled in several excellent reviews (Collmer et al., 2002; Buttner and Bonas, 2003; Buttner et al., 2003; Greenberg and Vinatzer, 2003; Jin et al., 2003).

A variety of approaches have been used to identify these effectors. The first type III-secreted proteins studied were those identified on the basis of their ability to elicit TTSS-dependent host defense responses on resistant plant genotypes (Staskawicz, 2001). This may not be surprising, given the eagerness of plant pathologists to elucidate the mechanisms underlying pathogen recognition and disease resistance. Further, given that type III effectors are secreted directly into host cells, and thus may serve as "easy targets" for recognition during the evolution of host surveillance systems, it may not be surprising that many effector molecules serve as elicitors of plant defense.

Recently, more comprehensive approaches for identifying genes encoding type III effectors have been employed. These approaches include: 1) utilizing information regarding gene location and gene regulation, 2) direct functional assays to screen for secreted proteins, and 3) taking advantage of common structural features of known TTSS-secreted proteins to carry out "genomic mining" experiments. For instance, in *P. syringae* and *X. campestris*, several genes encoding effector proteins are located within or adjacent to the gene clusters encoding the structural components of the TTSS (Alfano et al., 2000; Noel et al., 2002; Charity et al., 2003). Further, the expression of many *P. syringae* genes encoding either structural TTSS components or TTSS effector proteins depends on HrpL, an alternative RNA polymerase σ factor required for pathogenesis (Xiao et al., 1994b). The HrpL σ factor directs transcription of TTSS-associated genes by recognizing a consensus "*hrp* box" in the promoter regions of these genes (Innes et al., 1993; Xiao and Hutcheson, 1994a). This information has been used as the basis of genetic screens to identify genes potentially encoding TTSS effector proteins (Fouts et al., 2002; Zwiesler-Vollick et al., 2002; Bretz et al., 2003). Likewise, a molecular genetic strategy has been used to identify *X. campestris* genes whose expression is dependent on HrpX, an AraC-like transcriptional activator that is essential for induction of TTSS-related genes in this organism (Noel et al., 2001).

Although type III effectors do not have an obvious signal sequence targeting them for secretion, the proteins appear to be modular in structure, with the amino terminal region carrying information required for secretion (Guttman et al., 2002). Analyses of the primary amino acid sequences of several known *P. syringae* type III effectors has revealed a strikingly well-conserved pattern of amino acid biases within the first 50 residues that appear to be essential for secretion (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). As the genomes of several plant pathogenic bacteria have been sequenced, the above features have facilitated genomic mining experiments to identify the entire repertoire of type III effectors secreted by these pathogens (Collmer et al., 2002; Greenberg and Vinatzer, 2003).

Interestingly, plant pathogenic bacteria appear to have larger inventories of type III effectors than do animal pathogenic bacteria. For instance,

at last count, *P. syringae* pv. *tomato* strain DC3000 has 38 predicted effector proteins (Buell et al., 2003), whereas only six effector proteins have been characterized for *Yersinia* spp. and 10 effector proteins (secreted from two different TTSS apparatuses) have been identified for *Salmonella* (Cornelis and Van Gijsegem, 2000). The fact that plant pathogenic bacteria appear to secrete more type III effectors may be an adaptive feature of plant pathogens. This also suggests that more functional redundancy may exist among the effectors deployed by plant pathogens (Buttner and Bonas, 2003). Consistent with this hypothesis, although the collective importance of type III effectors in pathogenesis is obvious, mutations in single effector genes usually do not dramatically alter bacterial virulence, at least when assayed under laboratory conditions (Ponciano et al., 2003).

In contrast to TTSS structural components, which are highly conserved between various plant and animal pathogens (Cornelis and Van Gijsegem, 2000; Buttner and Bonas, 2003; Jin et al., 2003), the sequences and inventories of type III effectors vary considerably among different plant pathogens (and even among different strains of the same species; Collmer et al., 2002; Greenberg and Vinatzer, 2003). This variation suggests that different strains have evolved different repertoires of virulence factors to infect and cause disease on specific host plants. Thus, characterizing type III effectors and elucidating the mechanisms through which they contribute to pathogenesis is of great interest and may eventually provide insight into the molecular basis of host-specificity.

Elucidating the Function of Type III Effectors

The majority of TTSS effectors secreted by bacterial plant pathogens are predicted to function inside plant cells, and secretion into the host cell has been demonstrated for several effector proteins (Casper-Lindley et al., 2002; Szurek et al., 2002; Hotson et al., 2003). Additionally, several type III effectors, including AvrB, AvrRpt2 and AvrPto from *P. syringae* and AvrBs3 from *X. campestris*, have been shown to function inside plant cells in experiments monitoring their elicitor and virulence activities when expressed in plant cells (Leister et al., 1996; Chen et al., 2000; Marois, 2002; Hauck et al., 2003; Jamir, 2004).

Consistent with their proposed site of activity within plant cells, despite their prokaryotic-origin, many type III effectors have features typical of eukaryotic proteins. For example, the *P. syringae* effector proteins AvrRpm1, AvrB, AvrPto, and AvrPphB, have consensus N-terminal myristoylation sites. Several of these proteins have been shown to be myristoylated inside host cells (Nimchuk et al., 2000), and this modification appears to be required for the proper localization of these type III effectors to the host plasma membrane (Nimchuk et al., 2000; Shan et al., 2000). All members of the AvrBs3/PthA family of effectors found in the genus *Xanthomonas* carry functional nuclear localization signals (NLSs). An NLS at the carboxy-terminus of AvrBs3 from *X. campestris* has been shown to be required for interaction with importin α, which is part of the host nuclear import machinery (Szurek et al., 2001). Thus, type III effectors of prokaryotic origin appear to take advantage of eukaryote-specific post-translational modification and targeting mechanisms to access specific subcellular compartments within the host cell.

Given that type III effector proteins are translocated into the host during infection, these molecules (including those that elicit host defenses) are believed to modulate various aspects of host cell biology and physiology to promote disease. The proposed mode of action of these proteins include suppressing plant defenses, eliciting release of water and nutrients from host cells into the apoplast, promoting disease symptom development, and facilitating bacterial transmission (Alfano and Collmer, 1996; Greenberg and Vinatzer, 2003; Jin et al., 2003; Ponciano et al., 2003; Fig. 1). However, in the majority of cases, neither the mode of action nor the targets of these effector proteins within the plant are known.

Various strategies to elucidate the activities of these effectors have been employed. These strategies include protein sequence and structural analyses, biochemical approaches to identify interacting proteins, and the analysis of transgenic plants expressing effector proteins. Such studies have revealed several different potential roles for TTSS effectors, including facilitating type III secretion, proteolysis of host proteins, suppressing plant defense, and modulating endogenous host signaling processes.

FACILITATORS OF TYPE III SECRETION. Several type III effectors, including HrpZ and HrpW from *P. syringae*, and HrpF from *X. campestris* pv. *vesicatoria*, are believed to be secreted into the apoplastic space and are proposed to function as "helper proteins" to facilitate type III secretion during pathogenesis. The predicted structural properties of the *P. syringae* HrpZ protein resemble those of other bacterial proteins believed to interact with host cell membranes, such as YopB from *Y. enterocolitica* (Lee et al., 2001). Likewise, HrpF from *X. campestris* has been found to contain two putative transmembrane regions, suggesting its association with membranes (Buttner et al., 2002). Consistent with these hypotheses, both proteins have

been shown to have lipid-binding activity and to form ion-conducting pores in vitro when associated with lipid bilayers (Lee et al., 2001; Buttner et al., 2002). The pore forming activity of these proteins suggests that they function either in assisting delivery of virulence factors into the plant cell cytoplasm or by mediating nutrient and water release from host cells. Since HrpF is dispensable for protein secretion in vitro but is required in vivo for the recognition of an effector with elicitor activity by resistant plants, it has been proposed that HrpF may facilitate translocation of one or more effector proteins into the host cell (Rossier et al., 2000).

PROTEOLYSIS OF HOST PROTEINS. Amino acid sequence alignment and structural analyses have suggested that several type III effectors have proteolytic activity. AvrPphB from *P. syringae* pv. *phaseolicola* has similarity to *Yersinia* YopT, a cysteine proteinase (Shao et al., 2002). Consistent with the hypothesis that AvrPphB is a protease, AvrPphB has been shown to proteolytically cleave both itself and PBS1, an *A. thaliana* protein kinase required for AvrPphB avirulence activity (Shao et al., 2003).

AvrRpt2 from *P. syringae* pv. *tomato* is also predicted to encode a cysteine protease. Although AvrRpt2 protease activity has not been demonstrated biochemically, the amino acid residues predicted to make up the catalytic core of this protease are required for the defense-inducing activity of AvrRpt2 (Axtell et al., 2003). Two possible substrates for AvrRpt2 have been identified: AvrRpt2 itself (Axtell et al., 2003) and the *A. thaliana* RIN4 protein, which is required for *RPM1*-mediated resistance (Axtell and Staskawicz, 2003; Mackey et al., 2003; and see the section Suppression of Host Defenses in this Chapter).

The C-terminal portion of the XopD protein from *X. campestris* pv. *vesicatoria* has a high degree of similarity with the C-terminal catalytic domain of the Ulp1 ubiquitin-like protease protein family and has been shown to have cysteine protease activity specific for small ubiquitin-like modifier (SUMO)-lated substrates found specifically in plants (Hotson et al., 2003). On the basis of amino acid sequence similarity, three additional effectors from *X. campestris* pv. *vesicatoria*, AvrRxv, AvrBsT and AvrXv4, as well as PopP1 from *R. solanacearum* appear to belong to the YopJ family of ubiquitin-like protein proteases. Interestingly, like XopD, YopJ exhibits specificity for SUMO-lated proteins (Orth et al., 2000; Lavie et al., 2002).

Although the importance of the above demonstrated or predicted proteolytic activities in pathogen virulence has not yet been explored, these findings suggest that the use of type III effectors to cleave specific host signaling molecules is a common strategy of bacterial pathogens.

MODULATION OF HOST SIGNALING. Primary amino acid sequence analysis has also revealed a potential role for HopPtoD2 from *P. syringae.* The carboxyl-terminal domain of HopPtoD2 appears to encode a protein tyrosine phosphatase. Consistent with this finding, HopPtoD2 has protein tyrosine phosphatase activity and appears to contribute to virulence by modulating host defense responses (Petnicki-Ocwieja et al., 2002; Bretz et al., 2003; and see the section Suppression of Host Defenses in this Chapter).

SUPPRESSION OF HOST DEFENSES. Many type III effectors have been shown to modulate host defense responses. For example, several effectors, including AvrRpt2, AvrPphC and VirPphA, can inhibit host recognition of bacterial strains expressing specific avirulence factors (Ritter and Dangl, 1996; Jackson et al., 1999; Chen et al., 2000; Tsiamis et al., 2000). One of the best-studied cases of this type of defense suppression involves the *P. syringae* effector AvrRpt2. AvrRpt2 can interfere with the recognition of *P. syringae* strains carrying *avrRpm1* or *avrB* by plants carrying the corresponding resistance gene, *RPM1* (Ritter and Dangl, 1996; Chen et al., 2000). The mechanism underlying this interference activity of AvrRpt2 has been recently revealed by studies demonstrating that the TTSS-mediated delivery of AvrRpt2 leads to the disappearance of RIN4, a plant protein required for the stability of RPM1 (Mackey et al., 2002; Mackey et al., 2003; Axtell and Staskawicz, 2003). This finding is consistent with the hypothesis that AvrRpt2 is a sequence-divergent cysteine protease whose activity is required for elimination of RIN4 during infection (Axtell et al., 2003).

The suppression of host defenses by type III effector proteins can also occur downstream of pathogen recognition. For instance, the type III effectors AvrPtoB and HopPtoD2 suppress programmed cell death (e.g., the Hypersensitive Response or HR). HopPtoD2 also suppresses production of reactive oxygen species, and AvrPto suppresses cell wall-based extracellular defenses that are normally induced following pathogen recognition (Abramovitch et al., 2003; Bretz et al., 2003; Espinosa et al., 2003; Hauck et al., 2003; Jamir et al., 2004). These observations indicate that plant pathogenic bacteria can use type III effectors to suppress plant defenses at multiple steps in the plant defense signaling pathway.

ALTERATION OF OTHER ASPECTS OF HOST PHYSIOLOGY. Several type III effectors may promote pathogen virulence by affecting other aspects of host biology, for example by

modulating plant hormone physiology. The expression of the *P. syringae* effector protein AvrRpt2 in susceptible transgenic *A. thaliana* plants (i.e., lacking the corresponding resistance gene *RPS2*) promotes pathogen growth and disease development (Chen et al., 2000). Interestingly, three independent transgenic lines expressing *avrRpt2* exhibited seedling phenotypes reminiscent of signaling mutants defective in responding to the plant hormone auxin. These transgenic seedlings also exhibited increased sensitivity to exogenous application of auxin (Kunkel et al., 2004; Z. Chen et al., manuscript in preparation). These findings suggest that AvrRpt2 modulates host auxin physiology . Consistent with this hypothesis, AvrRpt2 appears to modulate free auxin levels, both in uninfected transgenic seedlings and during infection with *P. syringae* (Kunkel et al., 2004; Z. Chen et al., manuscript in preparation). The mechanism by which AvrRpt2 modulates auxin physiology within the host is presently not understood, nor is it clear whether this activity is related to the virulence activity of AvrRpt2. However, as is discussed in more detail in the section Auxin, it is reasonable to speculate that *P. syringae* may utilize virulence factors such as AvrRpt2 to modulate endogenous free auxin levels within the plant as a strategy to promote pathogen growth and disease formation.

AvrBs3 from *X. campestris* may also modulate host auxin biology. AvrBs3 stimulates host cell enlargement, a process associated with auxin, and induces expression of a group of auxin-regulated genes (Marois et al., 2002). However, whether these physiological changes induced by AvrBs3 contribute to pathogen virulence has not been demonstrated. Additional examples of modulation of hormone physiology by pathogens are discussed in the section Modulation of Plant Hormone Physiology in this Chapter.

Type III effectors can also function to promote several other aspects of pathogenesis, including formation of bacterial colonies in plant tissue, production of necrotic disease lesions, and facilitating pathogen transmission (Guttman and Greenberg, 2001; Badel et al., 2002; Badel et al., 2003; Wichmann and Bergelson, 2004).

ANALYSIS OF TYPE III EFFECTOR FUNCTION. One of the major challenges in understanding TTSS effector function is to identify the targets of these virulence factors within the host and to elucidate the roles of these molecules in pathogenesis. Thus the possibility that individual host molecules may be targeted by multiple type III effectors is important to keep in mind. For instance, in addition to being targeted for elimination by AvrRpt2, the *A. thaliana* RIN4 protein is phosphorylated by two other *P. syringae* effectors, AvrRpm1 and AvrB (Mackey et al., 2002). The fact that RIN4 is modified by multiple type III effectors suggests that this host protein may play an important role in *P. syringae/A. thaliana* pathogenesis.

Note also that individual type III effectors may target more than one host molecule or process. For instance, the *P. syringae* effector AvrRpt2 interferes with *RPM1*-mediated pathogen recognition, a process that is tightly correlated with the ability of AvrRpt2 to trigger the disappearance of RIN4 (Mackey et al., 2002; Mackey et al., 2003; Axtell and Staskawicz, 2003). In addition, AvrRpt2 promotes bacterial virulence during susceptible interactions (Chen et al., 2000; Guttman and Greenberg, 2001), and modulates host auxin physiology (Kunkel, 2004; Z. Chen et al., manuscript in preparation). Importantly, since the virulence activity of AvrRpt2 is maintained on *A. thaliana* plants lacking a functional *RIN4* gene, AvrRpt2 virulence activity is not dependent on RIN4 (Lim and Kunkel, 2004; Belkhadir et al., 2004). Thus, AvrRpt2 must have virulence targets other than RIN4.

Although a tremendous amount of progress towards identifying and characterizing plant pathogenic type III effectors has been recently made, the mode of action of most of these effectors is still unknown. The finding that the primary amino acid sequences of most effector proteins do not provide much insight regarding function, and that in most cases mutation of the effector genes does not result in pronounced virulence phenotypes have not helped to hasten our understanding of these proteins. Future studies involving host gene expression profiling and proteomic approaches to reveal the potential effects of these pathogen molecules on plant defense and other aspects of host physiology may help elucidate the function of some of these effectors. Likewise, localization of effector proteins within host cells, as well as identification of plant proteins that interact with the effectors, will also contribute to our understanding of effector function within plant cells.

Plant Cell Wall Degrading Enzymes

Plant cell walls play an important role in plant-pathogen interactions. As extracellular pathogens, phytopathogenic bacteria encounter plant cell walls as barriers preventing access to the cytoplasmic contents of host cells, as deterrents to pathogen spread within infected tissue, as physical substrates on which to grow, and as a potentially rich source of carbon (Agrios, 1997). Thus, not surprisingly, many plant pathogens include a battery of cell wall degrading enzymes in their repertoire of virulence factors.

Plant cell walls are composed primarily of complex carbohydrate polymers, the most

abundant of which are pectins, hemicelluloses and celluloses (Carpita and McCann, 2000). These polymers are arranged into a highly organized structure consisting of a meshwork of cellulose microfibrils imbedded in a gel-like matrix of pectins and hemicelluloses. Plant pathogens synthesize and secrete a variety of cell wall-degrading enzymes, including pectinases (e.g., polygalacturonases, pectate lyases, and pectin methyl esterases), cellulases and proteases. These enzymes work collectively to soften or break down plant cell walls, thereby facilitating pathogen entry and the release of nutrients for pathogen growth (Barras et al., 1994). The secretion of these exoenzymes may also result in the loosening of the middle lamellae that hold together adjacent plant cell walls, thus promoting the spread of pathogens between host cells and beyond the initial infection site.

The soft-rot pathogens, such as *Erwinia chrysanthemi* and *E. carotovora*, which make their living by macerating the plants' tissue, secrete multiple cell wall degrading enzymes. The importance of these enzymes in the virulence of these pathogens is well established (reviewed in Toth [2003] and Erwinia and Related Genera in Volume 6). *Xanthomonas campestris* pv. *campestris* also has an extensive collection of genes encoding putative cell wall degrading enzymes, including several pectic enzymes and cellulases (da Silva et al., 2002). Presumably, these enzymes contribute to the massive degeneration of plant tissue that occurs during development of black rot disease in plants infected with *X. campestris* pv. *campestris* (Agrios, 1997). However, as *Erwinia* and *X. campestris* pathogens secrete complex mixtures of degradative enzymes and possess multiple genes encoding functionally redundant isoenzymes, the precise role of any one of these enzymes in pathogenesis has been difficult to determine (Chan and Goodwin, 1999; Toth et al., 2003).

The roles of plant cell wall degrading enzymes during pathogenesis of vascular wilt and leaf spotting pathogens such as *R. solanacearun* and *P. syringae* are less clear. *Ralstonia solanacearum* encodes multiple known or predicted pectolytic enzymes, including endoglucanases, polygalacturonases, and a pectin methyl esterase (Genin and Boucher, 2002; Salanoubat et al., 2002). Genetic studies have revealed that several of these pectolytic enzymes contribute quantitatively to bacterial wilt disease development by facilitating invasion, colonization, and systemic spread of the pathogen within host tissue (Schell et al., 1988; Huang and Allen, 1997; Huang and Allen, 2000).

Recent sequence analysis has revealed that *P. syringae* pv. *tomato* strain DC3000 also encodes several potential cell wall degrading enzymes, including a polygalacturonase, a pectin lyase, and three enzymes predicted to have cellulytic activity (Buell et al., 2003). The role of these enzymes in DC3000 virulence is not known, and no cell wall degrading activity has been reported for this strain. However, pectolytic enzymes have been reported to contribute to symptom development during infection by *P. syringae* pv. *lachrymans* (Bauer and Collmer, 1997). Interestingly, three TTSS effector proteins (HopPmaH$_{Pto}$, HrpW and HopPtoP) classified as "helper proteins" that may assist in delivery of TTSS secreted proteins, possess carboxy-terminal domains with similarity to pectolytic enzymes (Charkowski et al., 1998; Boch et al., 2002; Collmer et al., 2002). The secretion of these potential pectolytic enzymes (either through sec-dependent or TTSS-dependent processes) could possibly facilitate the assembly of functional type III secretion complexes at the bacteria-plant cell wall interface.

Extracellular Polysaccharides

Many plant pathogens produce large amounts of exopolysaccharides (EPS). EPSs are carbohydrate polymers that are secreted by bacteria and form either a closely attached capsule layer surrounding the bacterial cell, or a loosely associated extracellular slime (Denny, 1995). The virulence of several phytopathogenic bacteria, including *R. solanacearum*, *E. amylovora*, *X. campestris* and *P. syringae* is associated with their ability to produce various EPS polymers during growth in plant tissue (Denny, 1995). EPSs are believed to provide a selective advantage to phytopathogenic bacteria through multiple functions including: 1) facilitating absorption of water, minerals and nutrients; 2) providing protection from abiotic stresses encountered during epiphytic or saprophytic growth, as well as from toxic molecules encountered during growth in plant tissue; 3) promoting colonization and spread within host tissue; and 4) contributing to the production of disease symptoms such as water-soaking and wilting (Denny, 1995).

One of the most important virulence-associated characteristics of the wilt pathogen *R. solanacearum* is the ability to produce large amounts of a viscous, high molecular mass, acidic EPS (EPS1) in planta. Production of large amounts of EPS1 by bacteria colonizing vascular tissue appears to interfere with transduction of water and nutrients within infected plants, resulting in wilting and, in some cases, the ultimate death of aerial portions of the plant (Denny and Baek, 1991; Kao et al., 1992). Consistent with these observations, infection with *R. solanacearum* strains bearing mutations in the EPS1 biosynthetic loci resulted in reduced wilting

(Denny and Baek, 1991). A study involving detailed microscopic analysis of the infection process revealed that EPS1-deficient mutants of *R. solanacearum* are less invasive than wildtype strains, suggesting that EPS1 may also be required for efficient colonization and movement within plant roots (Saile et al., 1997; Araud-Razou et al., 1998). Further, the accumulation of electron-dense material in plant tissue infected with *eps1* mutants raises the possibility that these mutants elicit nonspecific defenses within the host. Thus, EPS1 may also contribute to pathogen virulence by evading or suppressing host defenses (Araud-Razou et al., 1998).

Erwinia amylovora, well-known as the causal agent of fire blight of pear, produces two major EPSs, levan and amylovoran, that may contribute to this pathogen's ability to also cause wilting diseases on young plants (Denny, 1995). However, only amylovoran, a viscous, acidic heteropolysaccharide containing primarily galactose and glucuronic acid (Eastgate, 2000), has been clearly demonstrated to contribute to virulence of *E. amylovora*, and amylovoran-negative mutants exhibit reduced in planta bacterial growth and symptom development (Bellemann and Geider, 1992; Bernhard et al., 1993). Amylovoran is proposed to promote virulence by suppressing pathogen recognition by the host (Metzger et al., 1994), promoting tissue invasion and causing water-soaking and tissue collapse (Eastgate, 2000).

Xanthomonas campestris strains produce large amounts of the EPS known as xanthan gum that can accumulate to very high levels in infected plant tissues (Denny, 1995). Xanthan gum is a high molecular weight EPS composed of a cellulose backbone to which trisaccharide side chains are attached. Xanthan exhibits several unique properties in solution that have rendered it useful in industrial applications (Becker et al., 1998). However, despite being one of the most well-studied polysaccharides produced by phytopathogenic bacteria, the role of xanthan in pathogenesis is not understood. Xanthan clearly contributes to pathogen aggressiveness, as *X. campestris* strains carrying mutations that specifically disrupt EPS production exhibit reduced virulence (Katzen et al., 1998). It has been proposed that xanthan contributes to *X. campestris* fitness by providing protection against desiccation and hydrophobic molecules, and through facilitating tissue colonization by promoting adhesion of bacteria to biological surfaces (Chan and Goodwin, 1999). The recent discovery that xanthan is involved in formation of aggregates of *X. campestris* pv. *campestris* in culture suggests that this EPS may be involved in biofilm formation (Dow et al., 2003). Biofilm formation may be important during early stages of tissue colonization, for example, by promoting epiphytic survival or by providing protection against antimicrobial compounds encountered within plant tissues. Interestingly, dispersal of bacteria from such a biofilm at later stages of infection may be required to facilitate colonization of the vascular system (Dow et al., 2003).

The major EPS produced by *P. syringae* growing in planta is alginate, a copolymer of *O*-acetylated β-1,4-linked D-mannuronic acid and its C-5 epimer, L-glucuronic acid (Osman et al., 1986). Studies have associated *P. syringae* virulence with the amount of alginate produced in culture (Osman et al., 1986; Denny, 1995). Although the role of alginate in promoting virulence of *P. syringae* is not fully understood, alginate contributes to the virulence of the human pathogen *P. aeruginosa* by providing protection from host defenses and antibiotic treatment (Boyd and Chakrabarty, 1995). In studies designed to assess the role of alginate in *P. syringae* pv. *syringae* virulence, a *P. syringae* pv. *syringae* alginate lyase (*algL*) mutant impaired in alginate production exhibited reduced epiphytic fitness, grew to lower levels in plant tissue and elicited less severe disease symptoms on bean leaves (Yu et al., 1999). These findings indicate that production of alginate by this *P. syringae* strain is associated with increased epiphytic fitness on leaf surfaces and may also contribute to pathogen virulence by facilitating colonization or dissemination of the bacterium in planta.

Modulation of Plant Hormone Physiology

Several plant pathogens have evolved the ability to modulate signaling processes mediated by plant hormones as a strategy for manipulating host physiology (Agrios, 1997). Plant hormones, which are also often referred to as "plant growth regulators," are endogenous signaling molecules important for many aspects of plant growth and development. The most well-known growth regulators are auxin, ethylene, cytokinins, abscisic acid, gibberellins, jasmonates (JA) and salicylic acid (SA; Davies, 1995). Three of these hormones in particular, SA, JA and ethylene, are important in mediating plant defenses in response to pathogen or herbivore attack (Hammond-Kosack and Jones, 2000; Kunkel and Brooks, 2002). Not surprisingly, there is mounting evidence that the SA, JA and ethylene defense signaling pathways are modulated by plant pathogens. However, the specific hormone signaling pathway targeted by a given pathogen seems to depend on the virulence strategy employed by the pathogen (e.g., whether it is a necrotroph that rapidly kills plant cells to obtain nutrients, or a biotroph that colonizes living

plant tissue; Reymond and Farmer, 1998; Thomma et al., 2001).

SALICYLIC ACID SA plays a central role in defense against pathogen attack. During infection, plants often accumulate SA, and exogenous application of SA or SA analogs results in enhanced resistance to a wide variety of pathogens (Ryals et al., 1996). Plant mutants that are impaired in their ability to accumulate SA exhibit enhanced susceptibility to many pathogens (Nawrath and Metraux, 1999; Wildermuth et al., 2001). Thus, to successfully colonize host tissue, virulent bacterial pathogens presumably have evolved mechanisms for interfering with SA-mediated defense responses, for instance by delaying or preventing the accumulation of high levels of SA within host tissue or by suppressing SA-dependent signaling downstream of SA accumulation.

For example, the deployment of several *P. syringae* type III effectors, including AvrRpt2, AvrPphC, VirPphA and AvrPphF, delays the accumulation of SA within the infected plant by inhibiting host recognition of bacteria expressing specific avirulence factors (Ritter and Dangl, 1996; Jackson et al., 1999; Chen et al., 2000; Tsiamis et al., 2000; and the section Suppression of Host Defenses in this Chapter). Another potential strategy for interfering with induction of SA-dependent defenses is degradation of SA. This mechanism may be deployed by *R. solanacearum*, whose genome includes several genes encoding putative SA-degrading enzymes (Salanoubat et al., 2002).

Pseudomonas syringae, and presumably other pathogens as well, may also facilitate colonization of host tissue by suppressing SA-dependent signaling. As discussed in the section on the phytotoxin Coronatine, coronatine may be utilized by *P. syringae* to downregulate SA-dependent defense responses (Kunkel and Brooks, 2002). Likewise, the *P. syringae* type III effectors AvrRpt2 and HopPtoD2 suppress the expression of SA-regulated defense-related genes during infection on susceptible plants (Bretz et al., 2003; Chen et al., 2004). In the case of AvrRpt2, this appears to occur without altering SA levels (Chen et al., 2004). Therefore, plant pathogenic bacteria appear to deploy several different strategies to interfere with various aspects of SA-dependent defenses within the host.

JASMONATES A group of biochemically related plant growth regulators collectively referred to as "jasmonates" ("JAs") are involved in defense against both herbivorous insect pests and necrotrophic bacterial and fungal pathogens that colonize dead plant tissues. Thus, intact JA signaling processes are required for resistance to attack by these organisms (Kunkel and Brooks, 2002; Farmer et al., 2003). In contrast, JA signaling is required for disease susceptibility of *A. thaliana* and tomato plants to the biotrophic bacterial pathogen *P. syringae* (Feys et al., 1994; Kloek et al., 2001; Zhao et al., 2003; Laurie-Berry et al., manuscript in preparation). This may not be surprising, given that coronatine, an important virulence factor for *P. syringae*, is a molecular mimic of JAs (Feys et al., 1994; Weiler et al., 1994; Bender et al., 1999). Molecular and genetic studies suggest that coronatine modulates JA signaling within the plant to promote pathogenesis and disease development (Feys et al., 1994; Weiler et al., 1994; Kloek et al., 2001; Zhao et al., 2003). However, the mechanism(s) underlying this process is not well understood, nor is it clear why stimulation of JA signaling promotes *P. syringae* pathogenesis. One hypothesis is that stimulation of JA signaling results in antagonism of SA-dependent defenses (Kloek, 2001; Zhao, 2003; Brooks et al., manuscript submitted; and the section Coronatine). Alternatively (or additionally), stimulation of JA signaling within the plant could result in enhanced disease production, for example, by stimulating an increase in free auxin levels within the plant (Kunkel et al., 2004; see Auxin).

ETHYLENE The role of the gaseous plant hormone ethylene in plant-microbe interactions is complex, as it is required for resistance against some pathogens and for disease susceptibility in others (Kunkel and Brooks, 2002). *Ralstonia solanacearum* and *P. syringae*, two pathogens for which normal ethylene responsiveness in the host is important for disease development (Bent et al., 1992; Lund et al., 1998; Hoffman et al., 1999; Weingart et al., 2001; Hirsch et al., 2002), have been reported to produce ethylene, both in culture and in planta (Freebairn and Buddenhagen, 1964; Weingart and Volksch, 1997). These findings suggest that ethylene production by *R. solanacearum* and *P. syringae* plays an important role in disease development. Consistent with this hypothesis, it was found that ethylene synthesis mutants of some strains of *P. syringae* pv. *glycinea* grew to significantly reduced levels in bean and soybean plants (Weingart et al., 2001). Interestingly, in addition to encoding the ethylene biosynthetic gene ACC oxidase, the *R. solanacearum* genome contains a gene encoding ACC deaminase (Salanoubat et al., 2002), an enzyme involved in ethylene degradation. This finding suggests that *R. solanacearum* may carefully modulate ethylene levels within the plant for a maximal virulence effect.

AUXIN Although the roles of auxin in promoting plant cell division and growth in diseases caused by tumorigenic plant pathogens such as *A. tumefaciens* and *P. savastanoi* is well established (Yamada, 1993; Gelvin, 2003; and see The Genus Agrobacterium in Volume 5), the involvement of this plant growth regulator in disease caused by the nontumorigenic bacterial pathogens discussed in this chapter has not been carefully investigated.

Interestingly, a number of non gall-forming plant pathogens, including *R. solanacearum*, *X. oryzae* pv. *oryzae* and several *P. syringae* strains from different pathovar groups, have been reported to produce indole acetic acid (IAA), the predominant naturally occurring active form of auxin, when grown in culture (Phelps and Sequeira, 1968; Fett et al., 1987; Glickmann et al., 1998; Ansari and Sridhar, 2000). Further, recent sequence analysis has revealed that *R. solanacearum* strain GM1000 and *P. syringae* strain DC3000 possess genes predicted to be involved in auxin biosynthesis (Salanoubat et al., 2002; Buell et al., 2003). Thus, auxin production appears to be a common feature of many bacterial plant pathogens that are not reported to cause tumorigenic growth of plant tissues. Several *P. syringae* strains also harbor an *iaaL* gene that encodes an enzyme believed to catalyze the conversion of IAA to IAA-lysine, a conjugated form of IAA that is considered to be biologically less active than free IAA (Glickmann et al., 1998). Thus, presumably these bacteria are not only able to produce auxin, but are also able to adjust free IAA levels within the plant.

Pathogen infection can result in stimulation of auxin production by the host, and many biochemical studies to investigate the biosynthetic pathways responsible for this increase in IAA have been carried out (Phelps and Sequeira, 1968). However, the role that auxin plays in these interactions is not well understood. More recently IAA levels have been reported to increase in *A. thaliana* plants infected with virulent *X. campestris* or *P. syringae* strains (O'Donnell et al., 2003; Kunkel et al., 2004). The source of this increase in free IAA has not been established. However, the observation that several *A. thaliana* genes encoding enzymes involved either in IAA biosynthesis or in hydrolysis of IAA-amino-acid conjugates are upregulated upon infection with *P. syringae* (Niyogi et al., 1993; Bartel and Fink, 1994; Zhao and Last, 1996; Hull et al., 2000; Tao et al., 2003; Kunkel et al., 2004) suggests that the increase in free IAA in infected plants is generated, at least in part, by the plant.

It is presently unclear whether the increase in free IAA levels observed in infected plants is defense-related and is induced as a protective mechanism in response to pathogen attack, or alternatively, whether it is stimulated by the pathogen to render the plant more susceptible to infection. However, given that several pathogens are able to produce IAA in culture (and presumably in plant tissue as well), it is reasonable to speculate that auxin contributes to disease development and the virulence of these pathogens. Several pieces of evidence support this hypothesis. The observation that auxin downregulates expression of defense-related genes in cultured tobacco cells and plant tissues suggests that auxin may inhibit host defenses (Shinshi et al., 1987; Rezzonico et al., 1998). Additionally, injection of auxin-producing *A. tumefaciens* or *P. savastanoi* bacteria into tobacco leaves prior to injection of an avirulent *P. syrinage* strain inhibited the development of visible tissue collapse (e.g., the hypersensitive response or HR) associated with the host defenses response. The ability of the *A. tumefaciens* or *P. savastanoi* strains to suppress the *P. syringae*-induced HR was dependent on the presence of functional auxin biosynthetic genes, suggesting that auxin is directly involved in suppressing the HR (Robinette and Matthysse, 1990). Therefore, auxin may contribute to pathogen virulence by suppressing plant defenses. Our recent observation that exogenous application of the auxin analog 1-naphthalene-acetic acid (NAA) to *A. thaliana* plants resulted in increased disease susceptibility to *P. syringae* further supports the hypothesis that auxin promotes disease development during pathogenesis of *P. syringae* (Kunkel, 2004; Z. Chen et al., manuscript in preparation).

Taken together, the above observations suggest that some plant pathogens may modulate endogenous free auxin levels within the plant as a strategy to promote pathogen growth and disease formation. Three virulence factors that appear to contribute to this process have been recently identified: the *P. syringae* phytotoxin coronatine and two TTSS effector proteins, AvrRpt2 from *P. syringae* and AvrBs3 from *X. campestris*. Coronatine may play an important role in modulating host auxin physiology during infection by *P. syringae*. The expression of several genes involved in either producing IAA (e.g., *CYP79B2*) or releasing free IAA from conjugated pools within the plant (e.g., *IAR3*) are induced by JA treatment (Sasaki et al., 2001). Thus, induction of these genes upon infection by *P. syringae* may be stimulated by the production of coronatine, which is a molecular mimic of JA. The observation that normal induction of IAA-producing genes was not observed upon infection with coronatine biosynthetic mutants of *P. syringae* strain DC3000 indicates that coronatine is required for the induction of these genes

and suggests that *P. syringae* utilizes coronatine to actively modulate free IAA levels within the plant during infection (Kunkel et al., 2004).

Transgenic *A. thaliana* plants constitutively expressing the *P. syringae* type III effector AvrRpt2 (and lacking the corresponding resistance gene, *RPS2*) exhibit several phenotypes associated with altered auxin physiology. These include an increased number of lateral roots, altered gravitropic responses, and increased sensitivity to the auxin analogs 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA. Further, transgenic seedlings expressing AvrRpt2 accumulate slightly elevated levels of free IAA (Kunkel, 2004; Z. Chen et al., manuscript in preparation). The fact that these transgenic plants also exhibit enhanced susceptibility to *P. syringae* (Chen et al., 2000) suggests that AvrRpt2 may promote pathogen virulence by modulating host auxin physiology.

AvrBs3 of *X. campestris* pv. *vesicatoria* is another example of a type III effector that may modulate host auxin physiology. AvrBs3 contains nuclear localization signals and an acidic transcription activation domain, suggesting that it modulates host gene expression (Szurek et al., 2001). In susceptible pepper plants delivery of AvrBs3 specifically induces the expression of a group of auxin-induced *SAUR* genes (Marois et al., 2002). However, unlike AvrRpt2, the presence of AvrBs3 does not appear to affect free IAA levels of infected plants (Marois et al., 2002). Thus, AvrBs3 may alter host auxin physiology by altering IAA responses downstream of free IAA production and release from internal pools.

The Complexities of Hormone Signaling Networks in Plant-microbe Interactions

Note that the amount of crosstalk between the hormone signaling pathways discussed above is significant (Gazzarrini and McCourt, 2003). For instance, after reading this chapter it should be clear that a large amount of interplay exists between the SA and JA signaling pathways (see the sections Salicylic Acid and Jasmonates in this Chapter), and that pathogens such as *P. syringae* may take advantage of the mutually antagonistic crosstalk between these pathways to manipulate signaling within the plant (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; and see the section Coronatine in this Chapter). Likewise, auxin appears to upregulate the expression of ACC synthase 4 (ACS4; Abel et al., 1995), an enzyme that catalyzes a rate-limiting step in ethylene biosynthesis. Thus auxin may also induce ethylene biosynthesis. Moreover, as described in the sections on Auxin and Jasmonates, increasing evidence suggests that auxin and JA signaling pathways are interconnected. Therefore, the involvement of one plant hormone in a plant-pathogen interaction could be mediated, at least in part, through the action of one or more other plant hormones. Future studies aimed at untangling these complicated signaling networks will undoubtedly provide valuable insight into the virulence mechanisms used by bacterial plant pathogens.

Challenges

The recent use of a combination of genetic, molecular, and genomic approaches has led to major advances in the identification of numerous potential new virulence factors. The challenge that lies ahead is to develop experimental strategies that will facilitate the investigation of the mode of action of these factors and how they function collectively within the plant to promote pathogen virulence and disease. Given the potential functional redundancy of these factors, and the fact that their mode of action may not always be accurately predicted (so little is known about the plant processes that contribute to pathogenesis), it would be wise to utilize a variety of approaches in these future studies. Studies involving plant genetic, genomic and biochemical approaches, as well as physiological and gene expression analyses of transgenic plants expressing specific pathogen virulence factors (e.g., type III effector proteins) are already ongoing. In certain situations, advantage can also be taken of the observations that certain bacterial virulence factors are active in yeast (for examples, see Abramovitch et al. [2003] and Jamir et al. [2004]). Thus the power of yeast genetics is likely to facilitate the identification of host components that are important in mediating the activity of these virulence factors.

Collectively, these studies are likely to provide valuable information regarding both the molecular mechanisms underlying pathogen virulence and the host processes that are modulated during pathogenesis. The insight gained from these experiments may also lead to the development of new approaches for controlling virulence and disease development in agronomically important plant-pathogen interactions.

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Prokaryotes (2006) 2:441–456
DOI: 10.1007/0-387-30742-7_15

CHAPTER 1.15

The Chemolithotrophic Prokaryotes

DONOVAN P. KELLY AND ANN P. WOOD

Introduction

Ihre Lebensprozesse spielen sich nach einem viel einfacheren Schema ab; durch einen rein anorganischen chemischen Prozess . . . werden alle ihre Lebensbewegungen im Gange erhalten.

["Their life processes are played out in a very simple fashion; all their life activities are driven by a purely inorganic chemical process."]

—Winogradsky, 1887

Such was Winogradsky's (1887) description of the ability of certain bacteria to use energy from inorganic chemicals. Winogradsky's (1887) name for such organisms was "Anorgoxydanten" (literally "inorganic oxidizers"). Today the term chemolithotrophy is used to describe the energy metabolism of bacteria that use the oxidation of inorganic substances, in the absence of light, as a source of energy for cell biosynthesis and maintenance (Rittenberg, 1969; Brock and Schlegel, 1989; Kelly, 1990a). Chemolithotrophs exhibit extraordinary diversity of substrates, modes of carbon nutrition, morphology, and habitat. Grouping chemolithotrophs into some kind of homogeneous taxonomic unit is thus at least as artificial as grouping by most taxonomic devices in that virtually every possible morphology and physiology among bacteria (including the archaebacteria) are represented. Such taxonomic "lumping" does have value because some fundamental aspects of carbon and energy metabolism unify many of the chemolithotrophs into groups that are useful for physiological comparison.

The fundamental process in energy-conserving metabolism and in all respiratory processes is the transfer of hydrogen from a state more electronegative than that of the H^+/H_2O couple to that of water. Classically, "heterotrophs" or "chemoorganotrophs" obtain reducing potential from the dehydrogenation of organic compounds. Although a great variety of organic substrates is available and many are oxidized by heterotrophs, only a few principal metabolic processes exist whereby the hydrogen equivalents are fed into energy-conserving electron transport. Chief among these are processes that use the dehydrogenases of sugar phosphates and of organic acids, especially those of the tricarboxylic acid cycle. Diversity among substrates is thus merely a peripheral aspect of organism function; the central energy-generating and -conserving processes are fundamentally the same and involve the same kinds of components. This observation applies equally to the chemolithotrophs. Their electron-transporting and energy-trapping mechanisms are essentially the same as those of chemoorganotrophs (Kelly, 1978, 1982, 1989, 1990a).

From the time of its inception the concept of chemolithotrophy was linked with the autotrophic assimilation of carbon dioxide. Thus, Pfeffer (1897) coined "chemosynthesis," the term that was used for many years to describe the metabolism of bacteria that use inorganic oxidations to support autotrophic carbon dioxide assimilation (Kiesow, 1963; Jannasch and Wirsen, 1979; Brock and Schlegel, 1989). And Winogradsky's definition of the "Anorgoxydant" uncompromisingly coupled energy generation from inorganic oxidation with not only cell synthesis exclusively from carbon dioxide but also the concept of the general toxicity of organic nutrients (Winogradsky, 1922; Rittenberg, 1969, 1972; Schlegel, 1975; Whittenbury and Kelly, 1977). This concept is now known to be too restrictive because organisms (subsequently isolated) proved to be facultatively chemolithoautotrophs (growing on organic media as heterotrophs), mixotrophic (obtaining energy or carbon from both inorganic and organic sources), or chemolithotrophic heterotrophs (using inorganic energy substrates to effect assimilation of organic growth substrates).

The concepts of autotrophy (the assimilation of carbon dioxide as the major or sole source of biosynthetic carbon) and chemolithotrophy (growth with inorganic energy sources) thus were accepted as separate processes obligatorily linked in some specialized types of organisms. Paradoxically, this both clarified and blurred the boundaries between the "autotrophic" and "heterotrophic" bacteria (Rittenberg, 1972; Whittenbury and Kelly, 1977; Kelly, 1990a).

Clarification resulted from the fact that energy generated from an inorganic source does not have to be coupled exclusively to autotrophy (equally, the "organic oxidation" of formate or methanol can be coupled to autotrophic growth on carbon dioxide). Thus, physiological classification was in terms of either energy or carbon nutrition (Kelly, 1971). "Blurring" came from the possibility of extending the concept of autotrophy from a restricted definition. Thus, autotrophy could be defined in ever broader terms as metabolic processes that obtain (i) most carbon for biosynthesis from carbon dioxide by the action of ribulose bisphosphate carboxylase enzyme and derive energy chemolithotrophically; (ii) carbon from carbon dioxide by the Calvin-Bassham-Benson cycle (which we shall call the "Calvin cycle"); (iii) most carbon from carbon dioxide by any biochemical means; or (iv) one-carbon compounds for all biosynthesis by processes fundamentally akin to those involved in the autotrophic fixation of carbon dioxide (Smith and Hoare, 1977; Whittenbury and Kelly, 1977). It is clearly preferable to distinguish carbon and energy metabolism, especially among chemolithoautotrophs, but there is no merit in restricting the definition of autotrophy to those organisms using the Calvin cycle. Studies in recent years have established that distinct pathways, in addition to the Calvin cycle pathways from which they differ, operate in some groups of autotrophs. The term autotrophy should be applied without question to all organisms capable of basing biosynthesis on one-carbon compounds. In our view it is useful to separate "methylotrophy" from "autotrophy" when considering the pathways by which organisms growing on one-carbon compounds as sole source of energy convert those one-carbon units into biomass. This enables a clear distinction between methylotrophically-based energy- and carbon-metabolism to be made. There can be methylotrophic autotrophs, which use methanol or methylamine oxidation to drive carbon dioxide fixation by the Calvin cycle and non-autotrophic methylotrophs, which use from C1-compound oxidation to drive the serine pathway or the Quayle (ribulose monophosphate) cycle to assimilate formaldehyde.

Inorganic Oxidations as Sources of Energy

In principle, any inorganic exergonic oxidation reaction might be expected to be the basis of the energy-conserving metabolism of a chemolithotroph if (i) the reaction creates sufficient energy to support ATP synthesis and electron transport, enabling proton translocation and phosphorylation, (ii) conditions during geological time favor evolution and selection of suitable enzyme systems and organisms. The latter criterion was not always met, inasmuch as chemolithotrophs have not been shown to exploit some potentially energy-yielding processes. This absence probably reflects low concentrations or unavailability of the reaction components in the natural environment and no selective pressure or advantage leading to the survival of any organisms evolving such oxidation capacities.

The reactions unequivocally established as sources of chemolithotrophic energy are the oxidation of hydrogen, ammonia, nitrite, sulfur and its reduced compounds, ferrous iron, and possibly cuprous copper, antimony, and uranium (IV). Detailed consideration of the organisms involved, the mechanisms and types of reactions catalyzed, and the mechanisms of energy trapping are given in the specialist chapters in *The Prokaryotes* and in specialist reviews. This chapter attempts to present views of the chemolithotrophs from the perspective of energy yields of known chemolithotrophic oxidations, possible origins of chemolithotrophic processes, factors limiting the distribution and diversity of chemolithotrophs, and the potential for chemolithotrophy among known bacteria and those which still may remain to be discovered.

Energy Yields from Inorganic Oxidations

Estimates of the efficiency of energy production from inorganic oxidations in chemolithotrophs have been made from thermodynamic calculations and the measurement of growth and carbon dioxide assimilation (e.g., Baas Becking and Parks, 1927; Fromageot and Senez, 1960; Kelly, 1978, 1982, 1990a, 1999). Estimates of ATP production for oxidative phosphorylation in nitrifying bacteria and for sulfur, hydrogen, and iron oxidation are available and generally reflect P/O ratios of about 1.0, except for hydrogen where a normal complete electron transport chain, possibly allowing P/O = 3.0, is present. Some studies using chemostat culture with chemolithotrophs have been reported, and in some cases, maximum theoretical growth yields for the oxidation of different inorganic substrates can be compared and related to probable oxidation pathways and the theoretically available free energy. Chemostat culture methods with thiobacilli growing on sulfur compounds or iron have proved useful to the interpretation of oxidation and energy-coupling mechanisms (Hempfling and Vishniac, 1967; Timmer-ten-Hoor, 1976; Kelly et al., 1977;

Eccleston and Kelly, 1978; Justin and Kelly, 1978; Kelly, 1982, 1990; Jones and Kelly, 1983). Overall oxidation reactions exploited by the different groups of known chemolithotrophs are given in Table 1, and our current knowledge of the energy calculated to be available from these reactions and the observed growth or energy yields achieved by some chemolithotrophic bacteria are given in Table 2. Kelly (1990a, 1999) has reviewed this topic in more detail.

Except in the case of hydrogen oxidation, where the electrode potential is more negative than the NAD+/NADH couple, all these oxidations couple electron transport to the cytochrome system of the bacteria, and NAD+ reduction requires energy-dependent electron flow from cytochromes (Kelly, 1978, 1990a). This dependence is a biochemical hindrance to the growth of such chemolithotrophs because their energy metabolism is often largely concerned with the generation of NADH. Less of the energy available from an oxidation can be coupled more directly to biosynthesis than would occur, for example, during growth on hydrogen or pyruvate as an energy substrate. In addition, many of the most-studied chemolithotrophs use the Calvin cycle to fix carbon dioxide as a main source of carbon, and more than 80% of the total

Table 1. Known chemolithotrophic oxidation reactions.

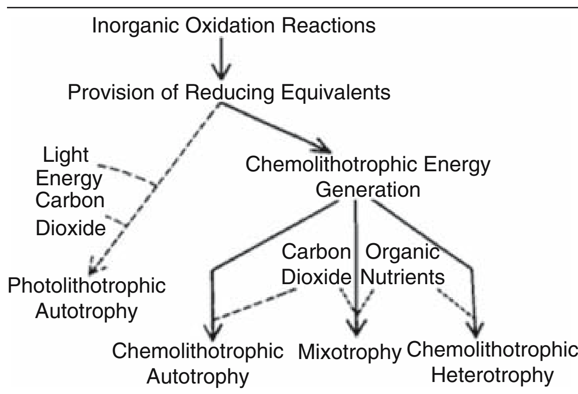
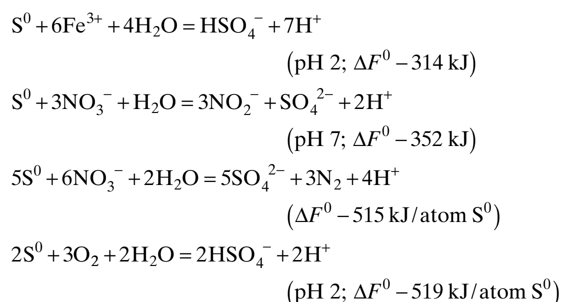


Table 2. Energetics and growth yields from the chemolithotrophic oxidations of Table 1.



$S^0 + 6Fe^{3+} + 4H_2O = HSO_4^- + 7H^+$ $(pH\ 2;\ \Delta F^0 - 314\ kJ)$

$S^0 + 3NO_3^- + H_2O = 3NO_2^- + SO_4^{2-} + 2H^+$ $(pH\ 7;\ \Delta F^0 - 352\ kJ)$

$5S^0 + 6NO_3^- + 2H_2O = 5SO_4^{2-} + 3N_2 + 4H^+$ $(\Delta F^0 - 515\ kJ/atom\ S^0)$

$2S^0 + 3O_2 + 2H_2O = 2HSO_4^- + 2H^+$ $(pH\ 2;\ \Delta F^0 - 519\ kJ/atom\ S^0)$

energy budget of non-hydrogen-oxidizing chemolithotrophs is indicated for use in converting carbon dioxide to an amount of carbohydrate, equivalent to that found in the growth nutrient of most heterotrophs. This combination of energy requirements explains why the growth yields of chemolithotrophs (already limited by the relatively low molar energy yield of their substrates) are generally apparently rather meager. In fact, they convert energy with reasonable efficiency, in spite of the biochemical problems to be surmounted (Justin and Kelly, 1978; Kelly, 1990a, 1999).

Chemolithotrophy and Autotrophy among Heterotrophs

A consequence of the historical development of the concepts of chemolithotrophy and "chemoautotrophy" was that any capacity for heterotrophic growth in an organism isolated originally as an autotroph tended to be regarded as an additional property of an organism that was primarily an "autotroph." Thus, the first isolates of the facultatively autotrophic thiobacilli were classified as *Thiobacillus novellus* rather than as a *Pseudomonas* that could grow chemolithoautotrophically. This procedure of slotting such organisms into "filing boxes" on the basis of their autotrophy, essentially devaluing their heterotrophic potential as a secondary character, was introduced by Winogradsky and used for most of the following hundred years. The practice has had both good and bad consequences for our understanding of the chemolithotrophs and their place in the natural environment. It has been good because it focused attention on the "obligate chemolithotroph" as an accepted physiological phenomenon, exemplified by clearly definable genera (*Thiobacillus*, some strains of *Beggiatoa*, *Hydrogenobacter*, *Hydrogenovibrio*, *Aquifex*, and the nitrifying bacteria being good examples; Kelly and Harrison, 1989; Nishihara et al., 1990, 1991, 1998; Huber et al., 1992; Shima and Suzuki, 1993; Nelson and Hagen, 1996), and it has enabled and motivated numerous investigators to probe the biochemistry of chemolithotrophic processes. It has been a hindrance to the broader understanding of chemolithotrophy in the natural environment because for many years minds were closed to the idea that chemolithotrophy could be a property of heterotrophs.

The period of microbiologists' reluctance to seek "new" chemolithotrophs (Schlegel, 1975), ended with a new understanding that mixed physiology and metabolic flexibility involving chemolithotrophy and autotrophy is the basis of

successful growth and survival in more organisms than was dreamed previously. Even *Beggiatoa*, so central in the formulation of the concept of the Anorgoxydant is proving a bizarre physiological kaleidoscope.

One of the first cracks in the defense of the concept that chemolithotrophy is a unique and essential taxonomic character was the reclassification of the hydrogen bacteria. For years, autotrophic growth with hydrogen oxidation as a source of energy justified the grouping of these bacteria (all known examples of them were also heterotrophic) into an artificial taxon known as "hydrogen bacteria." In 1969, these were scattered by new thinking and reclassified into diverse standard genera of heterotrophs, on the basis of their morphology and heterotrophic physiology, with their hydrogen-based chemolithotrophy and autotrophy being regarded simply as additional physiological properties (Davis et al., 1969).

The other genus of questionable validity is *Thiobacillus*, whose members obtain energy from oxidizing inorganic sulfur. As currently constituted, this genus contains diverse obligate chemolithotrophs, which differ from each other so widely that they should be reclassified into new genera (Kelly, 1989; Kelly and Harrison, 1989; Kelly and Wood, 1999), as well as facultatively heterotrophic species, which should be reassigned to existing genera of chemoorganotrophs or to new genera, created for them using heterotrophic metabolism and molecular biological criteria. For example some species, such as those described in the previous edition (Kelly, 1991) as *T. novellus* and *T. versutus*, are extremely versatile heterotrophs that can exhibit mixotrophy (in which heterotrophic and chemolithoautotrophic mechanisms operate simultaneously) and are taxonomically remote from obligately chemolithotrophic species. Since the previous edition of *The Prokaryotes*, *T. versutus* has been recognized as a species of *Paracoccus* (Katayama et al., 1995), and 16S rRNA gene sequence analysis has placed *T. novellus* in the α-Proteobacteria, whereas the type species, *T. thioparus*, is a member of the β-Proteobacteria (Lane et al., 1992). Other species, including the obligate chemolithotrophs *T. tepidarius*, *T. neapolitanus*, *T. halophilus*, *T. hydrothermalis* and *T. thiooxidans* have been found to be members of the γ-Proteobacteria, and unlikely to be related even at the genus level to the type species of *Thiobacillus* (McDonald et al., 1997; Kelly and Wood, 1999).

Among the early isolates of autotrophic organisms that had become regarded as probable heterotrophs, or at best mixotrophs, were *Gallionella* and *Beggiatoa*, but their ability to grow respectively as iron- or sulfide-oxidizing chemolithotrophs is now well established (Keil, 1912; Hanert, 1981; Nelson and Jannasch, 1983; Nelson et al., 1986a, 1986b, 1989a; Kelly, 1988). There have been unequivocal demonstrations of an obligately autotrophic, sulfide-oxidizing marine strain of *Beggiatoa* that used a biosynthetic citric acid cycle, lacked 2-oxoglutarate dehydrogenase, and showed virtually no regulatory effect by organic substrates on its constitutive ribulose bisphosphate carboxylase (Hagen and Nelson, 1996). An intriguing observation was that growth of *Beggiatoa alba* on acetate was greatly stimulated by the availability of sulfide, which was apparently used as an energy source enabling surplus assimilation of acetate over that obtainable heterotrophically (Güde et al., 1981). Similarly intriguing is the presence of the autotrophic enzyme, ribulose bisphosphate carboxylase in non-autotrophic strains of *Beggiatoa*, the enzyme seemingly being under repression control by acetate (Nelson et al., 1989b). *B. leptomitiformis* grows mixotrophically on succinate medium with thiosulfate or tetrathionate, which are oxidized to generate ATP by oxidative phosphorylation (Grabovich et al., 1998), further extending the range of strains of filamentous sulfur bacteria exhibiting this kind of mixed energy generation. Another filamentous genus believed to be capable only of mixotrophic growth with inorganic sulfur compounds was *Thiothrix*, but then *T. ramosa* was shown to be able to grow autotrophically using thiosulfate oxidation as sole energy source (Odintsova et al., 1993). Interestingly, while *Thiothrix* and *Beggiatoa* are both members of the γ-Proteobacteria, *Thiothrix* was not part of the novel monophyletic lineage comprising *Beggiatoa* and *Thioploca* (Teske et al., 1996).

Among the unicellular sulfur bacteria, recent work has shown just how blurred is the physiological distinction between the classical definition of *Thiobacillus* and heterotrophs capable of chemolithotrophy. Thus, obligately heterotrophic marine pseudomonads showing thiosulfate-stimulated growth (and carbon dioxide fixation) have been isolated and shown to produce sulfate, as would thiobacilli (Ruby et al., 1981). Even *Pseudomonas aeruginosa* shows increased growth yields in chemostat culture on glucose, when oxidizing thiosulfate to tetrathionate (Mason and Kelly, 1988). Chemolithoautotrophy on thiosulfate is also known in new and old genera of heterotrophs, including *Thiosphaera* (Robertson and Kuenen, 1983; now reclassified as *Paracoccus pantotrophus*, Ludwig et al., 1993; Kelly et al., 1999), *Paracoccus denitrificans*, and other hydrogen-oxidizing heterotrophs (Friedrich and Mitrenga, 1981; Kelly, 1988, 1989). An organism, called *Thiobacillus* Q, isolated as the predominant organism from

ditch water, using a chemostat limited by acetate plus thiosulfate, proved to be incapable of autotrophic growth, and thus to be another example of a heterotroph capable also of chemolithoheterotrophy (Gommers and Kuenen, 1988). It thus bears some similarity to *Thiobacillus intermedius* (London, 1963; now reclassified as *Thiomonas* intermedia; Moreira and Amils, 1997) and the original description of *Thiobacillus perometabolis* (now *Thiomonas perometabolis*), which grew best as chemolithotrophic heterotrophs (London and Rittenberg, 1967; Katayama-Fujimura and Kuraishi, 1983). Mixotrophy and chemolithotrophic heterotrophy have been shown also in the extreme thermoacidophile, *Sulfolobus* and in iron-oxidizing mesophiles and thermophiles (Wood and Kelly, 1983; Barros et al., 1984; Wood et al., 1987). Metabolic flexibility exhibited by such bacteria and the facultatively heterotrophic thiobacilli is of great survival significance to such organisms (Whittenbury and Kelly, 1977; Robertson and Kuenen, 1991) during competition in the natural environment. Thus, mixotrophy can enable these bacteria to dominate in mixed populations when both chemolithotrophic and chemoorganotrophic nutrients are present (Gottschal et al., 1979; Kelly and Kuenen, 1984). The observation of such flexibility should stimulate us to ask just how adaptable the physiology of the lithotrophs may be: whether, for example, the photolithotrophs function significantly as sulfur-oxidizing chemolithotrophs in darkness when given access to low concentrations of oxygen. Thus, *Thiocapsa* can grow in darkness using aerobic oxidation of thiosulfate or sulfide to support carbon dioxide fixation (Kondratieva et al., 1976; Kondratieva, 1989). Pigmentation is lost under such conditions, but ribulose bisphosphate carboxylase specific activity is similar under all photo- or chemolithotrophic conditions. The bacteria thus have the adaptive ability to behave physiologically like thiobacilli. Phenomena of this kind could prove to have considerable ecological and biogeochemical significance in the natural environment. Their widespread demonstration would further prove the undesirability of establishing dogmatic definitions of physiological types of organisms without the possibility of overlap areas. Thus, organisms of seemingly very different fundamental physiology could, under appropriate conditions, behave similarly. Indeed the converse question should be addressed: are any missing links of physiological types of obligate or facultative autotrophs possible? What of obligate hydrogen oxidizers and facultatively heterotrophic nitrifying bacteria, neither of which was proved to exist when one of us wrote the equivalent chapter in the first edition of *The Prokaryotes* (Kelly, 1981). There is now also no doubt that numerous organisms exist that have no autotrophic potential but use energy from lithotrophic oxidations to support growth on organic carbon sources. A remarkably versatile example is *Sulfurospirillum deleyianum*, which can use hydrogen as an electron donor, with its oxidation coupled to the reduction of inorganic or organic acceptors (oxygen, nitrate, nitrite or reduced sulfur compounds, or dimethylsulfoxide or C4-organic acids). This organism also uses sulfide as an electron donor (with acetate as carbon source), reducing nitrate and nitrite to ammonia to generate metabolic energy (Eisenmann et al., 1995). Some of these concepts are summarized in Fig. 1.

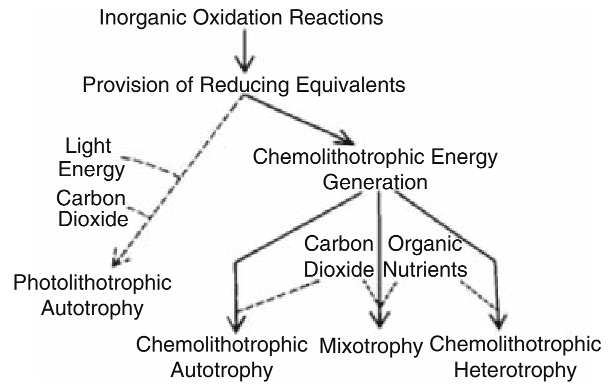


Fig. 1. Scheme illustrating the relation of inorganic oxidations to chemolithotrophic and photolithotrophic metabolism.

For many decades, the species of the nitrifying bacteria were uniformly believed to be obligate chemolithoautotrophs, in accordance with Winogradsky's original description of them and use of their properties to define the Anorgoxydant. Subsequently, facultative heterotrophy was proved in nitrite-oxidizing species, in which acetate or pyruvate was used to support aerobic and anaerobic growth (Smith and Hoare, 1968; Bock, 1976; Freitag et al., 1987; Bock et al., 1988). Also, one of the most abundant of the nitrite-oxidizing bacteria in the natural environment, *Nitrobacter vulgaris*, has been shown to grow faster heterotrophically than autotrophically, and can reduce nitrate (to nitrite, ammonia, NO and N_2O) during anaerobic growth on acetate or pyruvate (Bock et al., 1990). The complexity of habitat niches and complexity of the involvement of nitrite-oxidizing bacteria in soil ecosystems, is thus greater than was long believed. As yet, nitrite- (or ammonia-) dependent chemolithotrophic heterotrophy has not been shown, but mixotrophic growth of *N. vulgaris* can be faster than during heterotrophy (Bock et al., 1990).

The Overlap of Autotrophy, Methylotrophy and Chemolithotrophy

The methane-oxidizing bacteria, such as *Methylococcus*, are dependent on methane oxidation (or in some cases also methanol) for energy and carbon, but even in these the autotrophic enzyme ribulose bisphosphate carboxylase has been found (Taylor, 1977), and could contribute to the synthesis of phosphoglycerate during growth on methane (Stanley and Dalton, 1982). Interestingly, the amount of the carboxylase in *M. capsulatus* in chemostat culture decreased with oxygen-limitation (Khmelenina et al., 1992), possibly suggesting its function as an oxygenase has a greater importance. The carbon monoxide-oxidizing bacteria also fix carbon dioxide by means of the Calvin cycle, as do *Thiobacillus novellus* and *Paracoccus* versutus during growth on methylamine, methanol or formate as substrates (Kelly et al., 1979; Kelly and Wood, 1982, 1984; Meyer, 1989). Thus, autotrophy can occur in organisms growing on methylotrophic or other one-carbon energy substrates and does not have to be linked to chemolithotrophic energy sources. Growth of some thiobacilli and hyphomicrobia has been shown using one-carbon sulfur compounds as sole substrates (for review, see Kelly and Smith, 1990b). These substrates, including dimethyl sulfoxide, dimethyl sulfide, dimethyl disulfide and methanethiol, provide a somewhat remarkable meeting place or common feeding ground for sulfur-dependent chemolithoautotrophs and truly methylotrophic organisms. Thus some strains of *Thiobacillus thioparus* oxidize both the methyl and the sulfur groups and obtain energy from their oxidation. The thiobacilli cannot grow on one-carbon compounds, such as methylamine, which is used by the hyphomicrobia, and the latter cannot grow on sulfide or thiosulfate unless a compound such as methylamine is also supplied. Remarkably, the hyphomicrobia can grow mixotrophically on methylamine and thiosulfate, when they derive energy from oxidizing the latter, and use this to increase the proportion of methylamine-carbon incorporated by the serine pathway. They are thus chemolithotrophic methylotrophs that show no capacity for autotrophy, and serve to illustrate further that chemolithotrophic energy-generating processes can underpin or enhance the metabolism and growth not only of diverse autotrophs, but also of methylotrophs and heterotrophs. It also illustrates that substrates such as inorganic sulfide, methylated sulfides, methylamines, and other intermediates of sulfur or one-carbon metabolism may be competed for in the natural environment by organisms of diverse basic physiology, including specialist thiobacilli and hyphomicrobia, as well as some chemolithotrophic heterotrophs.

Chemoorganotrophic Potential among Obligate Chemolithotrophs

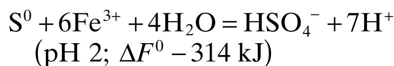
So far we have dwelt on the diversity of organisms, both autotrophs and heterotrophs in which the potential for chemolithotrophic energy conservation from inorganic oxidations resides. In writing such an introduction twenty or fifty years ago, the emphasis would have been placed on the uniqueness of the chemolithotrophic mode of growth and the puzzle of why in some organisms the chemolithoautotrophic mode of growth is obligatory (van Niel, 1943; Umbreit, 1947; Kelly, 1967, 1971). The reason why some bacteria exhibit this obligate chemolithotrophy is still inadequately explained (Kelly, 1971; Zavarzin, 1989), and it is probable that there is no single, simple, and universal explanation. Certainly, impermeability to organic nutrients is not the reason, as many obligate thiobacilli and nitrifying bacteria incorporate organic compounds at the expense of chemolithotrophically-generated energy. Also it has long been realized that the hypothesis that fundamental differences exist between the central metabolic processes of chemolithotrophs and heterotrophs was not tenable. Indeed the problem is made more intriguing by the evidence for storage polymers in obligate strains of thiobacilli and *Nitrobacter* (van Gool et al., 1971). *Thiobacillus neapolitanus* has been shown not only to accumulate polyglucose to levels exceeding 20% of the cellular protein content (Beudeker et al., 1981a), but also to consume this under conditions of (aerobic) carbon dioxide starvation, and to degrade it under anaerobic conditions by a heterolactic fermentation pathway (Beudeker et al., 1981b). A wide range of organic-nitrogen compounds, such as purines and urea, can be used to provide nitrogen for the chemolithoautotrophic growth of *Thiobacillus thiooxidans* (Brierley and Brierley, 1968; Metzdorf and Kaltwasser, 1988), and some obligate strains exhibit requirements for trace compounds such as vitamins. Thus the explanation for obligate chemolithotrophy must lie in the nature of the central regulatory processes that control carbon flow to biosynthesis in such bacteria, and this also must determine their seeming inability to oxidize exogenously supplied organic nutrients as sources of energy. The significant chemolithotrophic-energy-dependent incorporation of some compounds (such as amino acids) indicates that in the natural envi-

ronment the obligate chemolithotroph, while likely to be mainly autotrophic, must exhibit a degree of chemolithotrophic heterotrophy. They are thus at one end of a continuum of physiological behaviour, as discussed by Kelly and Kuenen (1984).

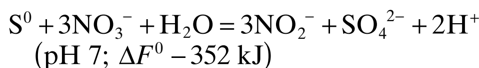
It has become clear that chemolithotrophy is not a restricted property of a few quaint but ecophysiologically-specialized bacteria, but it is a metabolic mode shared among many heterotrophs and may enable them to prevail over less versatile species in the competition for resources in nutrient-restricted natural environments.

Some Novel Chemolithotrophic Reactions and Some "New" Chemolithotrophs

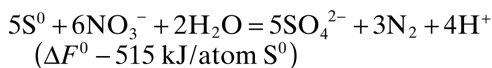
Potentially energy-yielding novel reactions have been found in sulfur-oxidizing thiobacilli and *Sulfolobus*, and in iron-oxidizing thermophiles. *Thiobacillus ferrooxidans* and *T. thiooxidans* can oxidize sulfur at the expense of ferric iron reduction as the respiratory oxidant (Brock and Gustafson, 1976). *T. ferrooxidans* does this anaerobically and thus may be capable of anaerobic growth using this system. It is noteworthy that previous reports of ferric iron reduction by heterotrophs have attributed the process to nitrate reductase (Thauer et al., 1977), but the probable inability of these bacteria to denitrify may suggest the existence of a specialist enzyme system, possibly a relict of a metabolic process that evolved before the appearance of nitrate respiration in early microorganisms. The amount of energy available from sulfur oxidation coupled to iron reduction is comparable with that from nitrate reduction or from aerobic sulfur or iron oxidation:



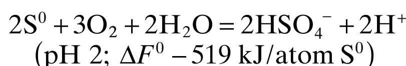
$$S^0 + 6Fe^{3+} + 4H_2O = HSO_4^- + 7H^+ \quad (\text{pH } 2;\ \Delta F^0 - 314 \text{ kJ})$$



$$S^0 + 3NO_3^- + H_2O = 3NO_2^- + SO_4^{2-} + 2H^+ \quad (\text{pH } 7;\ \Delta F^0 - 352 \text{ kJ})$$



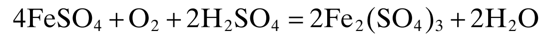
$$5S^0 + 6NO_3^- + 2H_2O = 5SO_4^{2-} + 3N_2 + 4H^+ \quad (\Delta F^0 - 515 \text{ kJ/atom } S^0)$$



$$2S^0 + 3O_2 + 2H_2O = 2HSO_4^- + 2H^+ \quad (\text{pH } 2;\ \Delta F^0 - 519 \text{ kJ/atom } S^0)$$

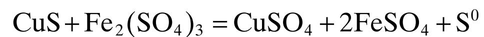
Iron-oxidizing, moderately thermophilic, chemolithotrophic heterotrophs were described by Brierley et al. (1978, 1980; Clark and Norris, 1996), growing on ferrous iron or pyrite as a substrate, but incapable of sustained growth on sulfur. Those isolates did, however, grow on copper sulfide (CuS), but only if small amounts (e.g., 1 mM) of iron (Fe^{2+} or Fe^{3+}) were present. Such organisms might simply be exhibiting an unusually high requirement for assimilable iron to grow on sulfur compounds. It is also possible that their main source of energy could be the oxidation of ferrous iron, with its constant regeneration by either biological oxidation of the CuS with Fe^{3+} or purely chemical reaction of CuS with ferric sulfate, according to the following processes:

1. Biological, energy-yielding aerobic ferrous iron oxidation:



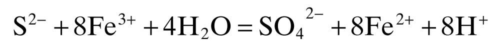
$$4FeSO_4 + O_2 + 2H_2SO_4 = 2Fe_2(SO_4)_3 + 2H_2O$$

2. Sulfide oxidation at the expense of ferric iron reduction:



$$CuS + Fe_2(SO_4)_3 = CuSO_4 + 2FeSO_4 + S^0$$

3. Chemical reduction of ferric iron:



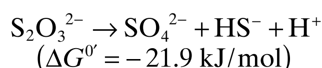
$$S^{2-} + 8Fe^{3+} + 4H_2O = SO_4^{2-} + 8Fe^{2+} + 8H^+$$

Measurement of growth yields or carbon dioxide fixation in such an organism should elucidate the main energy-yielding processes because considerably greater yields should result if both sulfide and iron oxidation were energy coupled, rather than solely the latter.

Earlier, we noted that all then known hydrogen-oxidizing autotrophs could be classified as heterotrophs (Davis et al., 1969). The vacant metabolic niche of the obligately chemolithoautotrophic hydrogen-oxidizer was filled in 1980 with the demonstration that such bacteria exist in some hot springs (Kawasumi et al., 1988). Several species of the thermophilic (and one case of a halophilic) *Hydrogenobacter* genus have been described (Kristjansson et al., 1985; Nishihara et al., 1990) and found growing on hydrogen optimally at 70°C, fixing carbon dioxide by a reductive tricarboxylic acid cycle, but shown to be incapable of growth on a wide range of simple and complex organic materials. Nishihara et al. (1989) isolated another obligately chemolithoautotrophic, halophilic hydrogen bacterium that grew best at 37°C (with no growth at 45°C) and used the Calvin cycle to fix carbon dioxide. This bacterium represented a further metabolic signature on the bewildering palimpsest of microbial physiology. Interestingly, it also grows on inorganic sulfur compounds and if originally it had been isolated from enrichment culture (for example on thiosulfate), it would have been classifiable as "*Thiobacillus*," but only if its ability to use hydrogen had not been tested. It is interesting to note the degree of overlap among the obligate and facultative hydrogen-oxidizers in their ability also to use inorganic sulfur oxidation for energy.

This metabolic overlap, and presence of versatile physiology is perhaps nowhere more remarkably emphasized than in *Acidianus* (Segerer et al., 1986; Zillig et al., 1986; Fuchs et al., 1996). *Acidianus* species are extremely thermophilic (85–90°C) and acidophilic (pH 2.0–2.5) archaebacteria. They are capable of aerobic autotrophic growth on elemental sulfur, which they oxidize to sulfate, while fixing carbon dioxide by a not yet fully resolved mechanism that resembles the 3-hydroxypropionate pathway postulated in *Chloroflexus* (Holo, 1989; Ishii et al., 1996). These bacteria are also capable of strictly anaerobic autotrophic growth during which they reduce elemental sulfur to hydrogen sulfide at the expense of hydrogen oxidation (Kelly, 1985, 1988; Segerer et al., 1985, 1986; Zillig et al., 1985, 1986). Thus, chemolithotrophy can exist in the same organisms using aerobic sulfur oxidation or anaerobic hydrogen oxidation, while simultaneously dissolving the previous distinction of sulfur oxidation and reduction as properties of quite distinct physiological groups of organisms.

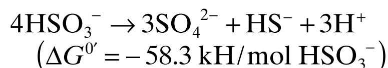
This distinction has been weakened further by the work of Cypionka (e.g., Bak and Cypionka, 1987) on sulfate-reducing bacteria that can grow using "sulfur fermentation" as a means of generating chemolithotrophic energy (Kelly, 1987, 1988, 1990a). Thus, *Desulfovibrio sulfodismutans* and *Desulfobacter curvatus* are among organisms able to conserve energy from the anaerobic disproportionation of thiosulfate or sulfite to produce mixtures of sulfate and sulfide as end products; thus, both sulfur (or sulfite) reduction to sulfide and anaerobic oxidation to sulfate occur in their energy-generating mechanism. The overall process is a fermentation, in that the combined oxidation state of the end products equals that of the substrate (Bak and Cypionka, 1987; Bak and Pfennig, 1987; Kelly, 1987, 1988):



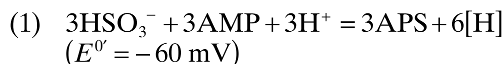
$$S_2O_3^{2-} \rightarrow SO_4^{2-} + HS^- + H^+ \quad (\Delta G^{0\prime} = -21.9\ \text{kJ/mol})$$

Recent studies have shown that there is differential fractionation of the sulfur isotopes (^{32}S and ^{34}S) within thiosulfate, when this is the sulfur compound being disproportionated by *Desulfovibrio desulfuricans*. Disproportionation of thiosulfate yielded sulfate that was isotopically heavier than the inner- sulfur (sulfonate- , $-SO_3-$) of thiosulfate, and sulfide which was isotopically lighter than the outer- (sulfane-, -S-) of thiosulfate (Cypionka et al., 1998; Smock et al., 1998). This means that the disproportionation of thiosulfate does not result in its direct conversion to sulfate and sulfide, but that intermediate reactions occur during which there is fractionation of ^{32}S and ^{34}S (Cypionka et al., 1998).

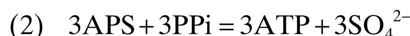
Enzymological studies have partially resolved the probable mechanism of these processes and how the free energy is conserved (Krämer and Cypionka, 1989). The overall reaction, and three component steps leading to sulfate and sulfide formation from sulfite, with ATP formation, may be:



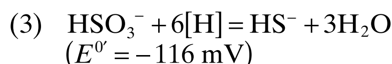
$$4HSO_3^- \rightarrow 3SO_4^{2-} + HS^- + 3H^+ \quad (\Delta G^{0\prime} = -58.3\ \text{kH/mol}\ HSO_3^-)$$



$$(1)\quad 3HSO_3^- + 3AMP + 3H^+ = 3APS + 6[H] \quad (E^{0\prime} = -60\ \text{mV})$$



$$(2)\quad 3APS + 3PPi = 3ATP + 3SO_4^{2-}$$



$$(3)\quad HSO_3^- + 6[H] = HS^- + 3H_2O \quad (E^{0\prime} = -116\ \text{mV})$$

(where APS is adenylyl sulfate and PPi is pyrophosphate).

The problem implicit in this scheme is that the reductant-donating reaction [1] is less electronegative than that producing sulfide (3). This led Krämer and Cypionka (1989) to postulate energy-dependent reversed electron transport as part of the overall process. This would be needed if an alternative means of oxidizing sulfite to sulfate were not found in these anaerobes. Such an oxidation could well involve energized membrane functions that lead to further ATP synthesis (in a coupled sulfite oxidation and sulfite reduction) and possibly to $NAD(P)^+$ reduction. These sulfite or thiosulfate-fermenting chemolithotrophs are in fact chemolithotrophically heterotrophic in that they have been grown on sulfur compounds as sole energy source and on acetate as carbon source. The sulfur fermentation continued also when hydrogen was provided as an oxidizable substrate (Krämer and Cypionka, 1989).

Hydrogen oxidation is of course a source of energy not only to many aerobes and the sulfur-reducing archaebacteria (already discussed), but also to the sulfate-reducing bacteria and the methanogens (Table 3, equation 1). The chemolithotrophy and autotrophy of the sulfate-reducing bacteria have a long and somewhat bizarre history. For some time, *Desulfovibrio* was regarded as a facultative autotroph, oxidizing hydrogen to reduce sulfate and fixing carbon dioxide as a source of carbon. The autotrophic status of *Desulfovibrio* species (Butlin and Adams, 1947) was demoted to that of "not to be true autotrophs" (Mechalas and Rittenberg, 1960; Postgate, 1979), and then the genus was reinstated as having strains truly capable of chemolithotrophy on hydrogen and sulfate (Badziong et al., 1978; Thauer, 1989). Now some sulfate-reducers also are known to be capable of chemolithotrophic autotrophy, such as

Desulfobacterium autotrophicum, which uses the reductive acetyl CoA pathway to fix carbon dioxide, and *Desulfobacter hydrogenophilus*, which uses the reductive tricarboxylic acid cycle (Fuchs, 1989).

Clearly the physiological types of chemolithotrophs, and metabolic combinations involving chemolithotrophy, are many and various, and many more novelties can be expected as *The Prokaryotes* is progressively updated.

Evolutionary Aspects of the Origin of Chemolithotrophy

For perhaps two billion years, since the origin of life on earth, metabolic evolution occurred in a globally anaerobic environment. Before the appearance of the oxygen atmosphere, the widely accepted view of life processes was that of a fermentative metabolism acting at the expense of organic materials (the "prebiotic soup") accumulated during an era of prebiotic chemical evolution (Oparin, 1957; Maden, 1995). Thus, the first living entity was regarded as a completely heterotrophic unit using abiotically preformed organic molecules (Horowitz, 1945). Metabolism during this period might have included the development of some steps of the tricarboxylic acid cycle as an anaerobic process, and of bacterial photosynthesis at the expense of organic compounds, with hydrogen and sulfide as reductants. During this time, the earliest chemolithotrophs were postulated to have appeared: these were proposed to be methanogens, which reduced carbon dioxide with hydrogen, and the sulfate-reducing bacteria, which used hydrogen as well as other reductants.

There is, however, no longer a universal acceptance of the view that the earliest organisms were heterotrophs (Maden, 1995; Edwards, 1998). One view gaining strong support is that the earliest self-sustaining metabolism was rooted in chemolithoautotrophy (Wächterhäuser, 1988, 1990a, 1990b, 1992). All of the most deeply rooted lines of Bacteria and Archaea are hyperthermophiles, and many of these are autotrophs (Woese, 1987; Burggraf et al., 1992; Stetter, 1992). Indeed, Stetter (1992) has proposed that the deepest branches of the phylogenetic tree comprise chemolithotrophic autotrophs, thus indicating autotrophy as a primordial metabolic process. It is also possible that chemolithotrophy predated autotrophy, and that the first chemolithotrophs were chemolithotrophic heterotrophs, using inorganic energy sources as well as prebiotic organic molecules.

There is a widely accepted view that the first autotrophic processes were akin to those now seen in the sulfur- and sulfate-reducing bacteria and Archaea (i.e., cyclic processes involving organic acids). The process once regarded as typifying autotrophy, namely the Calvin reductive pentose phosphate cycle, is regarded as being of much later origin and as having evolved from earlier sugar-metabolizing pathways (Maden, 1995). The Calvin cycle is apparently not functional in carbon dioxide fixation by Archaea and hydrogen- or sulfur-oxidizing hyperthermophiles (Fuchs and Stupperich, 1985; Maden, 1995; Schönheit and Schäfer, 1995), and this is used as evidence that it cannot have been a primordial pathway. It is noteworthy, however, that genes apparently coding for a novel (or even primordial) form of ribulose bisphosphate carboxylase occur in *Pyrococcus* and other Archaea (Ezaki et al., 1998), but their origin and whether they are the result of later lateral gene transfer, is not known. If these genes are relict rather than a result of lateral transfer, the Calvin cycle could be of much more ancient origin, arising in chemolithotrophic heterotrophs in which biosynthesis using simple exogenous sugars had already evolved. An argument for a later origin also has been applied to the carbon monoxide dehydrogenase pathway (acetyl coenzyme A pathway), which requires additional carbon dioxide fixing processes to support biosynthesis beyond the level of two-carbon units (Maden, 1995). Wächterhäuser (1990a, 1990b, 1992) postulates that the first pathway of autotrophic carbon fixation was an archaic precursor of the reductive citric acid cycle, whose descendent is found in examples of modern eubacteria, green sulfur bacteria, hyperthermophilic hydrogen bacteria, and Archaea (Evans et al., 1966; Schauder et al., 1987; Wächterhäuser, 1990a, 1990b; Shima and Suzuki, 1993; Beh et al., 1993; Maden, 1995).

Studies of anciently duplicated genes encoding components of the protein-targeting machinery have been suggested to help identify the root of the universal tree of life (Gribaldo and Cammarano, 1998). The key protein of the signal recognition particle (SRP54 in Eukarya and Fm in Bacteria) and the protein involved in the recognition and binding of the ribosome SRP nascent polypeptide complex (SR alpha in Eukarya and Ftsy in Bacteria) are products of ancient gene duplication that appears to predate the divergence of all extant taxa (Gribaldo and Cammarano, 1998). This leads to the reasoning that the first bifurcation in the tree of life separated the lineage leading to Bacteria from a common ancestor to Archaea and Eukarya (Gogarten and Taiz, 1992; Gogarten, 1995; Gribaldo and Cammarano, 1998). Phylogenetic studies on dissimilatory sulfite reductases and adenylyl sulfate (APS) reductase (Hipp et al., 1997; Wagner et al., 1998), using organisms as diverse as *Chromatium*, *Archaeoglobus* and *Desulfovibrio*, showed gene homology for these

reductase enzymes. The degree of similarity of the DNA sequences for the bacterial and Archaeal genes was such that the most likely explanation was a common origin from ancestral reductase genes. This suggests that the genes for the ancestral reductases were present before the split between the domains of Bacteria, Archaea and Eukarya took place. This kind of evidence lends considerable weight to a view of primordial organisms that were chemolithotrophs capable of the dissimilatory processing of inorganic sulfur compounds, including the earliest sulfur- and sulfate-reducing organisms.

While the dating of early evolutionary processes is exceedingly conjectural, the consensus view at present is that chemolithotrophic and autotrophic processes existed early in the development of living systems. Currently, however, there is debate about (1) the validity of the concept of the divergence of the Bacteria and the Archaea (Gupta, 1998a, 1998b), and (2) the nature and dating of the progenitor organisms that led to modern life forms (Gogarten-Boeckels et al., 1995; Gogarten et al., 1996). The first argument does not greatly affect our view of a very ancient origin of chemolithoautotrophy, but the theory advanced by the latter authors may push back the origin of these processes to a time in Earth's history even prior to the cessation of heavy meteoritic bombardment (Chyba, 1992; Gogarten-Boeckels et al., 1995). Their hypothesis is that prokaryotic forms evolved and could have reached a sophisticated state of development by about 3.7 billion years (3.7 Gyr) ago. These primordial prokaryotes would have colonized extremely hot environments but did not arise necessarily in such environments. The deep phylogenetic separation of modern Archaea and Bacteria, and their fundamental differences in cell biology, could be evidence of their descent from distinct primordial ancestors, each extant at 3.7 Gyr, rather than a common cenancestor. If a mass-extinction-scale extra-terrestrial impact event occurred at 3.7 Gyr, resulting in near-boiling of oceans, then only isolated examples of ancient primordial, hyperthermophilic prokaryotic types might have survived, and two of these might have led by subsequent separate evolution to the Bacteria and Archaea (and presumably the Eukarya). This scenario would make the common ancestor of all current life forms a chemolithotrophic autotroph, which originated little more than 0.5 Gyr after the formation of the Earth.

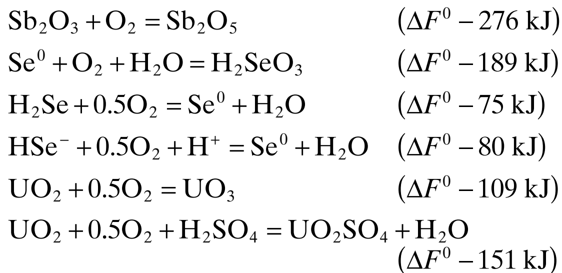
If one accepts the view that elemental oxygen and nitrate are both relatively very recent additions to the natural environment (Broda, 1977a; Gautier, 1992), the latter only appearing when the biosphere became less reducing, then the modern chemolithotrophs, dependent on oxygen or nitrate respiration, must be regarded as relatively recent evolutionary products. Their origins, however, are clearly traced from the types of inorganic oxidative metabolism (e.g., of hydrogen by sulfate-reducers, and of both hydrogen and sulfide by photolithotrophs) that evolved in the anaerobic phase of evolution. The basic patterns for chemolithotrophic and autotrophic biochemistry could well have been laid in this period; thus, the principle for sulfur-compound oxidation now seen in thiobacilli could have developed in ancient phototrophs. Interestingly, in relation to the idea of primordial organisms being anaerobic hyperthermophiles, there are several examples of modern thermophiles or hyperthermophiles capable of hydrogen oxidation either aerobically or by nitrate reduction to dinitrogen. Examples are *Hydrogenobacter acidophilus* (optimum temperature 65°C and pH 3–4; Shima and Suzuki, 1993) and the Archeon *Pyrobaculum aerophilum* (optimum growth at 100°C; Volkl et al., 1993). Better known are the examples of *Sulfolobus* and *Acidianus* (some with temperature optima approaching 100°C), capable of aerobic sulfur oxidation or anaerobic hydrogen oxidation coupled to sulfur reduction (Fuchs et al., 1996); however, such organisms and *Pyrobaculum* are clearly unlike any primordial Archeon in being facultative aerobes, but their unusual combination of chemolithotrophic potentials could be relict indicators of the earliest chemolithotrophic biochemical processes.

The mechanisms for ammonia (and possibly nitrite) oxidation could stem from more ancient methane-oxidizing organisms: methane oxidation by ammonia-oxidizers supports this view, and the similarities of the DNA sequences for genes encoding ammonia and methane monooxygenases (Holmes et al., 1995) are consistent with evolution from common ancestral enzyme forms. The major changes were, of course, the development of enzyme systems enabling the reaction with oxygen and in some cases the coupling of energy conservation to the oxidation of inorganic substances.

The questions remain: are other lithotrophs to be discovered in nature, or are such theoretically possible chemolithotrophs missing from nature? If so, did they once exist?

As already stated, any exergonic oxidation reaction with a reasonably electronegative potential might support metabolism and growth. The oxidation of ferrous iron is a good example of a process of rather low energy yield and unfavorable electrode potential (relative to NAD^+ reduction) that has been very successfully exploited in the evolution of organisms like *Thiobacillus ferrooxidans*. Among some

possible reactions would be the oxidation of metals that show several valence states. Thus, manganese oxidation (possibly a source of energy for ill-defined organisms like *Metallogenium*) could well prove to be the basis of a chemolithotrophic process, and the demonstration of ribulose 1,5-bisphosphate carboxylase genes in a marine manganese-oxidizing bacterium suggests that autotrophy driven by manganese oxidation may be possible (Caspi et al., 1996). There is also reasonable evidence that cytochrome-mediated oxidation of cuprous copper and stannous tin occurs in *T. ferrooxidans* (Lewis and Miller, 1977), which appears anyway to obtain energy from the former. Other metal oxidations mentioned in the literature are of antimony (for which there is evidence of an organism; Lyalikova, 1972), selenium and uranium, for which possible reactions are:



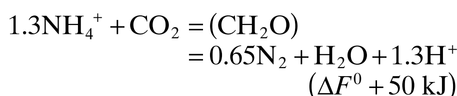
$$Sb_2O_3 + O_2 = Sb_2O_5 \quad (\Delta F^0 - 276\ \text{kJ})$$
$$Se^0 + O_2 + H_2O = H_2SeO_3 \quad (\Delta F^0 - 189\ \text{kJ})$$
$$H_2Se + 0.5O_2 = Se^0 + H_2O \quad (\Delta F^0 - 75\ \text{kJ})$$
$$HSe^- + 0.5O_2 + H^+ = Se^0 + H_2O \quad (\Delta F^0 - 80\ \text{kJ})$$
$$UO_2 + 0.5O_2 = UO_3 \quad (\Delta F^0 - 109\ \text{kJ})$$
$$UO_2 + 0.5O_2 + H_2SO_4 = UO_2SO_4 + H_2O \quad (\Delta F^0 - 151\ \text{kJ})$$

All of these oxidations yield at least as much energy as iron oxidation, presuming the ΔF0 values to reflect free energy changes that could be trapped metabolically.

Oxidation of anions also could be energy yielding: the organisms using sulfur oxyanions and nitrite are well known. Other energy substrates could be compounds of tellurium, molybdenum, vanadium, or any salt of a multivalent element capable of further oxidation. Another possible substrate might be phosphine (PH_3), which could be oxidized through several intermediates to orthophosphate, with an overall large change in free energy:

$$PH_3 + 2O_2 = H_3PO_4 \quad (\Delta F^0 - 1166\ \text{kJ})$$

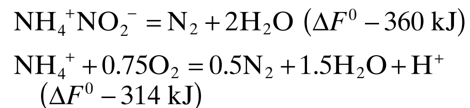
Broda (1977b) discussed the apparently unfulfilled expectation that two further types of ammonia-oxidizing bacteria might exist. The first of these was anaerobic, photosynthetic ammonia bacteria, which use NH_4^+ in a way analogous to sulfide, use light-dependent CO_2-fixation, and generate dinitrogen from ammonia:



$$1.3NH_4^+ + CO_2 = (CH_2O) = 0.65N_2 + H_2O + 1.3H^+ \quad (\Delta F^0 + 50\ \text{kJ})$$

Such phototrophic organisms, if they ever existed, could have been the precursors of the modern aerobic nitrifying bacteria.

Anaerobic ammonia oxidation had not been shown in known species of modern nitrifying bacteria, and this was attributed to the presumed need for an oxygenation of ammonia to hydroxylamine which was obligatorily dependent on elemental oxygen. Broda (1977b) speculated that anaerobic ammonia oxidation using nitrite as oxidant is feasible, with dinitrogen as the end product. Similarly, aerobic ammonia oxidation to N_2 is theoretically possible as a chemolithotrophic process (Broda, 1977b):



$$NH_4^+NO_2^- = N_2 + 2H_2O \quad (\Delta F^0 - 360\ \text{kJ})$$
$$NH_4^+ + 0.75O_2 = 0.5N_2 + 1.5H_2O + H^+ \quad (\Delta F^0 - 314\ \text{kJ})$$

Both these reactions are more exergonic overall than aerobic ammonia oxidation to nitrite. That comparable processes may indeed be catalysed by anaerobic ammonia-oxidizing bacteria is indicated by the observation in a commercial denitrification plant that the overall nitrogen balance of the system could be explained only if ammonia oxidation with nitrate as oxidant was occurring (Mulder, 1989). Novel anaerobic, ammonium-oxidizing (“Anammox”), bacteria were shown to be highly enriched in this plant, apparently growing autotrophically by obtaining energy from the conversion of ammonia and nitrite (via nitrite, hydroxylamine and hydrazine) to nitrogen gas (van der Graaf et al., 1996, 1997). The well-known aerobic nitrifier, *Nitrosomonas*, has been shown to couple anaerobic ammonia oxidation to the reduction of nitrogen dioxide, with the formation of NO and nitrite (Schmidt and Bock, 1997).

The sulfur-oxidation-dependent reduction of ferric iron referred to earlier also could be a relict of a primordial, energy-yielding, proto-chemolithotrophic process. In the pre-oxic biosphere, most iron was probably in the ferrous state, but because of the absence of autooxidation, ferrous iron may have been a substrate for a form of photosynthesis. In any case, it was oxidized to ferric iron over possibly 10^9 years following the appearance of O_2-photosynthesis, but before free oxygen began to accumulate in the atmosphere. Abundant sulfide and iron could thus have made a selective “niche” for such chemolithotrophy.

Probably many of the theoretically feasible reactions for energy generation will not be found in any organisms, past or present, because the substrates of the reactions were never sufficiently abundant on earth for any chance evolution of an enzyme system that

used the reaction to have survived. The modern chemolithotrophs seem to be so successful by one of two physiological specializations. Either they are specialist organisms, like the obligately chemolithotrophic sulfur oxidizers, which do not compete with heterotrophs because they use an inorganic substrate not available to most of the latter and are not dependent on organic carbon; or they are versatile facultative organisms, capable in organic, nutrient-rich environments of "switching off" their chemolithotrophic autotrophic metabolism and competing as successful heterotrophs. The place of mixotrophy and chemolithotrophic heterotrophy in the natural environment is less certain but probably confers advantage on such organisms, as they can compete simultaneously for distinct and unrelated sources of energy and carbon. In rather selective environments (such as acid, hot, or metal-rich habitats) where the chemolithotroph is best adapted to survive (e.g., the sulfur-oxidizing thermophiles of hot springs or the metal-tolerant, acidophilic, iron-oxidizing organisms of mineral leaching systems), mixotrophy could be a great selective advantage, where there is perhaps minimal competition with pure heterotrophs. Mixotrophy, in particular, potentially enables an organism to exploit as many nutritional facets of its environment as possible.

Our growing understanding of the complexity of the metabolic possibilities among chemolithotrophs and heterotrophs with chemolithotrophic potential, as well as the realization of the extreme antiquity of chemolithoautotrophic processes, not only helps explain why so many organisms can live together in seeming contradiction of the competitive exclusion principle, but also means that even greater metabolic variety can be expected to be discovered.

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Prokaryotes (2006) 2:457–495
DOI: 10.1007/0-387-30742-7_16

CHAPTER 1.16

Oxidation of Inorganic Nitrogen Compounds as an Energy Source

EBERHARD BOCK AND MICHAEL WAGNER

Introduction

Life depends on the element nitrogen. In nature, nitrogen exists mainly in the oxidation states -III (NH_3), O (N_2), +I (N_2O), +II (NO), +III (NO_2^-), +IV (NO_2), and +V (NO_3^-). Owing to nitrogen transformations by the activity of living organisms and to chemical instability, any form of oxidation state has only a transient existence. Dinitrogen (N_2) is the most inert and frequent constituent of the atmosphere.

Taking into account also abiotic transformations, three cycles of nitrogen can be distinguished:

1. The cycle of the atmosphere
2. The interaction between the atmosphere and the biosphere
3. The cycle of the biosphere

The nitrogen cycle mediated by the biosphere (Fig. 1) can also be characterized by mobilization and immobilization of nitrogen compounds. Most of the reactions are catalyzed exclusively by prokaryotes. By microbial nitrogen fixation, dinitrogen is reduced to ammonia and subsequently transferred to amino acids and assimilated into cell material. On the other hand, ammonia is released from organic nitrogen compounds by microbial activity called "ammonification" or "mineralization." Ammonia (NH_3)/ammonium (NH_4^+) is the most frequently found form of nitrogen in the biosphere and is transferred efficiently over long distances via volatilization. In contrast, nitrite is usually found in trace amounts in aerobic habitats and only accumulates at low oxygen partial pressure, e.g., in soil with high water potential. Because of the toxicity of nitrite for living organisms, the maintenance of low nitrite concentration in aerobic habitats is essential. Under oxic conditions, ammonia and nitrite are not stable and are converted to nitrate by nitrifying bacteria. Nitrification, the biological oxidation of reduced forms of inorganic nitrogen to nitrite and nitrate, is catalyzed by two physiological groups of bacteria. Ammonia-oxidizing bacteria, which use ammonia and not ammonium as substrate (Suzuki et al., 1974), gain energy from oxidation of ammonia to nitrite, and nitrite-oxidizing bacteria thrive by oxidizing nitrite to nitrate. In sea- and freshwater as well as in soil, nitrite produced by the ammonia oxidizers is immediately consumed by nitrite oxidizers and thus the nitrite concentration is extremely low in these environments (El-Demerdash and Ottow, 1983; Schmidt, 1982). Nitrate can be assimilated by plants and microorganisms. Under anoxic or oxygen-limited conditions, nitrate is used as electron acceptor for anaerobic respiration (if organic matter is available) and thereby converted to ammonia (respiratory ammonification) or dinitrogen (denitrification).

This chapter focuses on nitrifying bacteria, which use the oxidation of inorganic nitrogen compounds as their major energy source. Lithotrophic nitrifiers are Gram-negative bacteria and conventionally have been placed in the family Nitrobacter iaceae (Buchanan, 1917; Watson, 1971a; Watson et al., 1989). However, phylogenetically the lithoautotrophic ammonia oxidizer s, characterized by the prefix *Nitroso*-, and nitrite oxidizer s, characterized by the prefix *Nitro*-, are not closely related (Teske et al., 1996; Purkhold et al., 2000). Comparative 16S rRNA sequence analysis demonstrated that all recognized ammonia oxidizer s are either members of the β- or γ-subclass of Proteobacteria (Fig. 2). The genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a closely related monophyletic assemblage within the β-subclass of Proteobacteria (Head et al., 1993; Woese et al., 1984; Teske et al., 1994; Utåker et al., 1995; Pommerening-Röser et al., 1996; Purkhold et al., 2000), whereas the genus *Nitrosococcus* constitutes a separate branch within the γ-subclass of Proteobacteria (Woese et al. 1985; Purkhold et al., 2000). Among the nitrite oxidizer s, the genera *Nitrobacter*, *Nitrococcus* and *Nitrospina* were assigned to the α-, γ-, and γ-subclass of Proteobacteria, respectively (Orso et al., 1994; Teske et al., 1994). Nitrite oxidizer s of the genus *Nitrospira* are affiliated with the

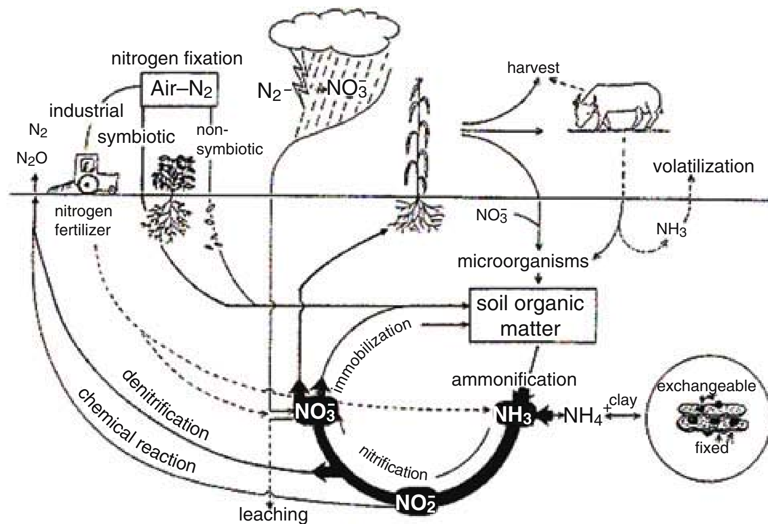
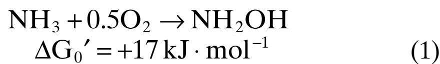


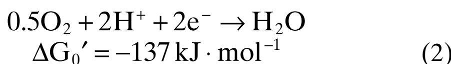
Fig. 1. Nitrogen cycle mediated by the biosphere.

recently described *Nitrospira*-phylum, which represents an independent line of descent within the domain Bacteria (Ehrich et al., 1995).

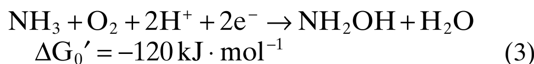
The most important character of lithotrophic nitrifying bacteria is energy generation via ammonia oxidation to nitrite (3. Biochemistry of ammonia oxidizing bacteria) and nitrite oxidation to nitrate (5. Biochemistry of nitrite oxidizing bacteria), respectively, according to the following equations:



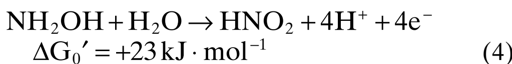
$$NH_3 + 0.5O_2 \rightarrow NH_2OH \quad \Delta G_0' = +17\,kJ \cdot mol^{-1} \qquad (1)$$



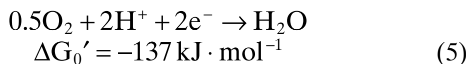
$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad \Delta G_0' = -137\,kJ \cdot mol^{-1} \qquad (2)$$



$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad \Delta G_0' = -120\,kJ \cdot mol^{-1} \qquad (3)$$

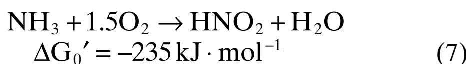


$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad \Delta G_0' = +23\,kJ \cdot mol^{-1} \qquad (4)$$

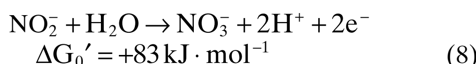


$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad \Delta G_0' = -137\,kJ \cdot mol^{-1} \qquad (5)$$

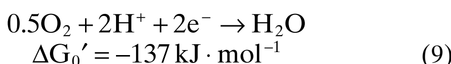
$$NH_2OH + 1.5O_2 \rightarrow HNO_2 + 2e^- + 2H^+ \quad \Delta G_0' = -114\,kJ \cdot mol^{-1} \qquad (6)$$



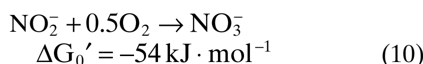
$$NH_3 + 1.5O_2 \rightarrow HNO_2 + H_2O \quad \Delta G_0' = -235\,kJ \cdot mol^{-1} \qquad (7)$$



$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad \Delta G_0' = +83\,kJ \cdot mol^{-1} \qquad (8)$$



$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad \Delta G_0' = -137\,kJ \cdot mol^{-1} \qquad (9)$$



$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad \Delta G_0' = -54\,kJ \cdot mol^{-1} \qquad (10)$$

Equations 1 and 2 describe the two half-reactions of ammonia oxidation to the intermediate hydroxylamine (NH_2OH). The total reaction is given in equation 3. For hydroxylamine oxidation, no oxygen is consumed (equation 4). Subsequently two electrons are transferred back to reaction 2, and the remaining two electrons pass to the respiratory chain (equation 5). The second step of ammonia oxidation, the hydroxylamine oxidation, is depicted in equation 6. The overall reaction (equation 7) shows that biogenic ammonia oxidation causes nitric acid production. The $\delta G_0'$ value of reaction 7 is significantly higher than that of nitrite oxidation (equation 10). Nitrite oxidation starts with equation 8. Electrons are released and penetrate the respiratory chain at the cytochrome *c* level (equation 9). There is no acid production when nitrite is oxidized to nitrate (equation 10).

Ammonia oxidation is initiated by the enzyme ammonia monooxygenase (AMO; 3.2 Enzymes involved in ammonia oxidation; Ammonia monooxygenase), which oxidizes ammonia to hydroxylamine. Substrates for AMO are ammonia (Wood, 1986), dioxygen, and two electrons. One atom of molecular oxygen is reduced to water, while the second oxygen atom is incorporated to form hydroxylamine. The intermediate hydroxylamine is further oxidized to nitrite by hydroxylamine oxidoreductase (HAO; 3.2 Enzymes involved in ammonia oxidation; hydroxylamine oxidoreductase). Two of the four electrons derived are required for AMO activity, and the other two are used for energy generation (3.3 Rlectron Flow and Energy Transduction). The AmoA protein is assumed to contain the active site of AMO (Hyman and Arp, 1992). A second AMO subunit named "AmoB" has been identified (Bergmann and Hooper, 1994a). The

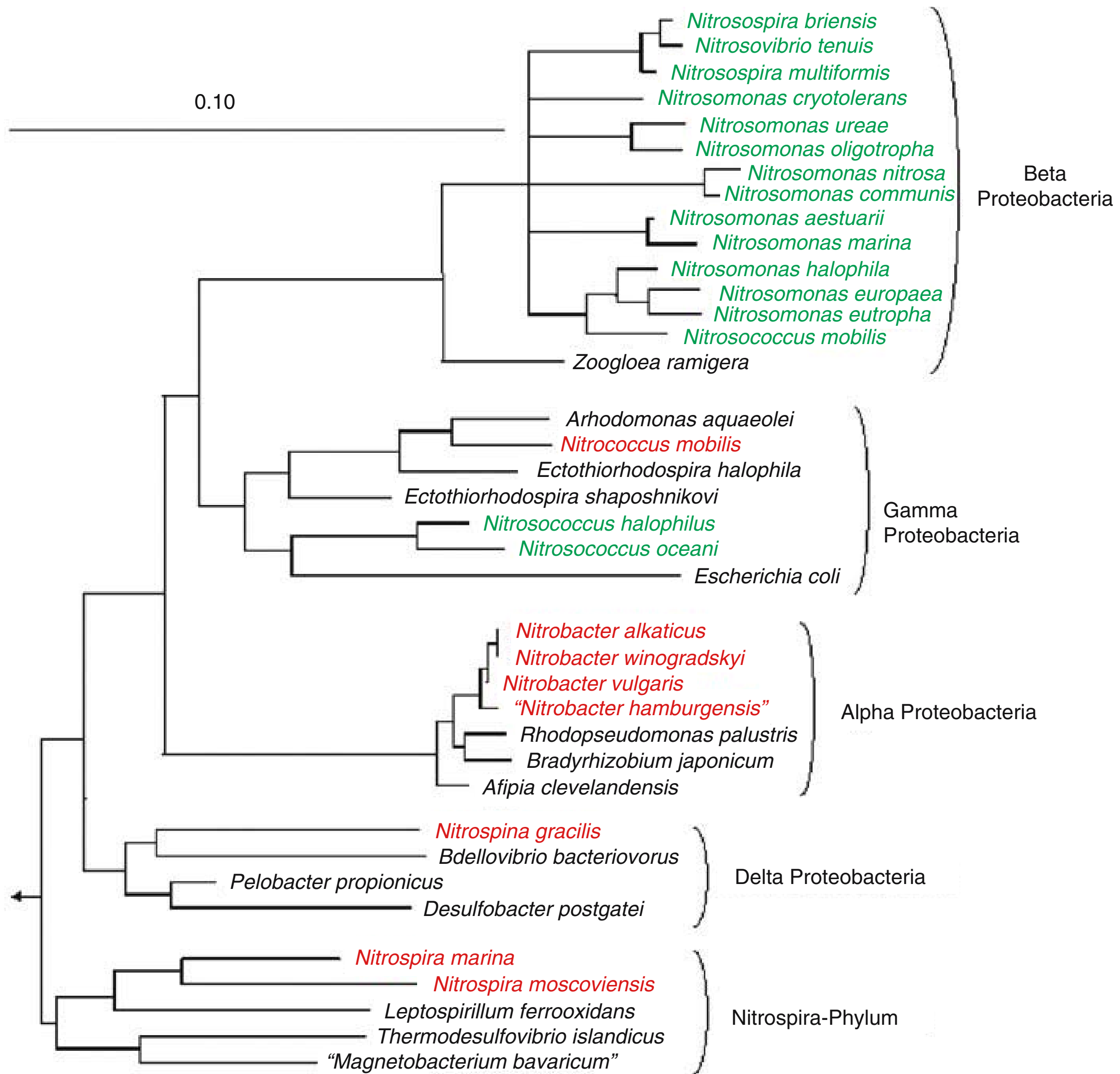


Fig. 2. 16S rRNA-based tree reflecting the phylogenetic relationship of ammonia- and nitrite-oxidizing bacteria. The consensus tree is based on the results of a maximum likelihood analysis of the 16S rRNA primary structure data from the nitrifying bacteria shown in the tree and a selection of reference sequences. Only homologous positions that share identical residues in at least 50% of all available almost complete bacterial 16S rRNA sequences were included for tree reconstruction. In the tree, ammonia oxidizer s are labeled green, and nitrite oxidizer s are depicted in red. It should be noted that the assignment of the genus *Nitrospina* to the δ-Proteobacteria is tentative and might change if additional reference sequences become available. Multifurcations connect lineages for which no unambiguous branching order could be retrieved using different treeing methods. Bar represents 10% estimated sequence divergence.

gene cluster encoding AMO contains a third open reading frame termed "*amoC*," which is located upstream of the genes *amoA* and *amoB* (Klotz et al., 1997; 4. Genetics of ammonia oxidizer s). Neither AmoA nor AmoB have been purified in the active state as yet. The enzyme HAO is a trimer of 63-kDa subunits, including seven c-type hemes and a novel heme (P-460) per monomer (Arciero and Hooper, 1993; Hoppert et al., 1995; Igarashi et al., 1997). The enzyme is located in the periplasmic space, but anchored in the cytoplasmic membrane.

Nitrite oxidation is initiated by the enzyme nitrite-oxidoreductase (NO_2-OR; 5.2. Enzymes involved in nitrite oxidation) which occurs as characteristic membrane-associated two-dimensional crystals in all nitrite oxidizer s. These regularly arranged particles are located on the surface of the cytoplasmic—and if present—intracytoplasmic membranes of nitrite-oxidizing bacteria. In all *Nitrobacter* species and in *Nitrococcus*, particles are arranged in rows, whereas in Nitrospina and both Nitrospira species, hexagonal patterns were observed. The NO_2-OR con-

sists of two subunits (Meincke et al., 1992). For *Nitrobacter hamburgensis*, the molecular weight of one particle was found to be 186 kDa representing an αβ-heterodimer (Spieck et al., 1996). The full sequence of the β-subunit as well as a partial sequence of the α-subunit of the NO_2-OR of *Nitrobacter hamburgensis* shows similarities to nitrate reductases of several chemoorganotrophic bacteria (6. Genetics of nitrite oxidizer s). During oxidation of nitrite to nitrate, the additional oxygen atom of nitrate is derived from water (Aleem et al., 1965b) and two electrons are released for energy generation.

In addition to lithotrophic nitrifiers, various heterotrophic bacteria, fungi and algae (Focht and Verstraete, 1977; Killham, 1986; Papen et al., 1989) are capable of oxidizing ammonia to nitrate. However, in contrast to lithotrophic nitrification, heterotrophic nitrification is not coupled to energy generation (7. Heterotrophic nitrification). Consequently, heterotrophic nitrifiers are dependent on the oxidation of organic substrates (Focht and Verstraete, 1977; Kuenen and Robertson, 1987). During heterotrophic nitrification, ammonia or reduced nitrogen from organic compounds (e.g., the amino group of amino acids) is co-oxidized to hydroxylamine, gaseous nitrogen oxides, nitrite or nitrate. For example, methane-oxidizing bacteria were shown to co-oxidize ammonia to nitrite by a biochemically well-characterized particulate (membrane-bound) methane monooxygenase and a unique hydroxylaminoxidoreductase (Anthony, 1982; Yoshinari, 1985; O'Neil and Wilkinson, 1977; Zahn et al., 1994; Bergmann et al., 2000). The methane monooxygenase is assumed to be biochemically related to the AMO of ammonia-oxidizing bacteria, and methane-oxidizing bacteria are potential contributors to nitrification in the rhizosphere of rice plants (Bodelier and Frenzel, 1999). Conversely, ammonia oxidizer s are able to oxidize methane to methanol (Hyman and Wood, 1983; Ward, 1987; Jones and Morita, 1983; Steudler et al., 1996), but up to now, there is no evidence that ammonia oxidizer s significantly contribute to the oxidation of atmospheric methane (CH_4) in natural systems (Jlang and Bakken, 1999; Bodelier and Frenzel, 1999). In general, heterotrophic nitrification is considered to contribute only marginally to the global nitrogen cycle (Brady, 1984; Brown, 1988) but nevertheless might be of local importance especially in heath and conifer forest soils (for example, see van de Dijk et al., 1980; Schimel et al., 1984).

Lithotrophic nitrifiers are autotroph ic bacteria that fix carbon dioxide (CO_2) via the Calvin-Benson cycle (Harms et al., 1981), and to a lesser extent, via phosphoenolpyruvate carboxylase (Takahashi et al., 1993). In the past, they were thus described as obligate lithoautotrophs and were thought to find organic compounds toxic. However, this assumption is not correct for several nitrifier species. Clark and Schmidt (1967) demonstrated that ammonia oxidizer s of the genus *Nitrosomonas* and nitrite oxidizer s of the genus *Nitrobacter* are capable of growing mixotrophically with ammonia or nitrite as electron donors and with a combination of carbon dioxide and organic compounds as carbon source. Compared to purely autotroph ic growth, the addition of organic compounds stimulated cell growth and increased cell yield (Steinmüller and Bock, 1976; Matin, 1978; Krümmel and Harms, 1982; Watson et al., 1986). Furthermore, the nitrite oxidizer s *Nitrobacter winogradskyi*, *N. hamburgensis* and *N. vulgaris* can grow chemoorganotrophically with acetate or pyruvate as electron donor and dioxygen or nitrate (in absence of dioxygen) as electron acceptor (Bock, 1976; Freitag et al., 1987). However, for these organisms, heterotrophic growth was always slower than lithotrophic growth. Recently, Daims and coworkers (Daims et al., 2000; H. Daims et al., 2001) showed that in nitrifying activated sludge, not yet cultured *Nitrospira*-related nitrite oxidizer s fix CO_2 and simultaneously take up pyruvate but not acetate, butyrate and propionate. In addition, some strains of *Nitrosomonas* can utilize organic substances like urea or glutamine as source of their substrate ammonia for lithotrophic growth (Koops et al., 1991).

The transformation of ammonia to nitrate via nitrite by the nitrifying bacteria has various direct and indirect implications for natural and man-made systems. For example, nitrifying bacteria contribute directly or indirectly to loss of nitrogen compounds from various environments due to:

1. Leaching of mobile nitrogen compounds produced by nitrifiers. Leaching is the mobilization and transfer of nitrate to rivers, lakes, seawater and groundwater. Nitrification is not desirable in agricultural soil because it induces loss of soil nitrogen. Fertilizer ammonium, which is required for plant growth, adsorbs well to clay particles of soil owing to its positive charge (Fig. 1). When converted to nitrate, the inorganic soil nitrogen becomes mobile and thus susceptible to denitrification and leaching. In some countries, nitrification inhibitors like nitrapyrin (N-Serve) are used in agriculture to minimize nitrogen loss (Huber et al., 1977; Keeny, 1986; Slangen and Kerkhoff, 1984; Lipschultz et al., 1981; Poth and Focht, 1985).
2. Denitrification. Denitrification is the microbial reduction of nitrate via nitrite, nitric oxide (NO) and nitrous oxide (N_2O) to dinitrogen (N_2). This type of anaerobic respiration can be per-

formed by a variety of phylogenetically different heterotrophic microorganisms. By aerobic oxidation of ammonia to nitrate, nitrifying bacteria produce the electron acceptor for subsequent denitrification in many natural and engineered systems. During the last years, nitrifiers also have been shown to be able to denitrify (3.5 denitrification catalyzed by ammonia oxidizer s).

3. Chemodenitrification of nitrite (produced by the ammonia oxidizer s) in acidic environments. Chemodenitrification is defined as nonenzymatically catalyzed loss of nitrogen due to dismutation of nitric acid at pH values <4.5 leading to the formation of nitrate and gaseous nitric oxide. In the atmosphere, nitric oxide is unstable and reacts with oxygen or ozone to form gaseous nitrogen dioxide, which chemically dismutates to nitrous and nitric acid in the presence of water.

In addition to the well-recognized metabolic activity, nitrifiers react to and produce gaseous nitrogen oxides like N_2O, NO and nitrogen dioxide (NO_2). For example, the presence of NO is required for ammonia oxidation of *Nitrosomonas eutropha* (Zart et al., 2000). Furthermore, cell growth and the ammonia-oxidizing activity of this species is enhanced by NO_2 (Zart and Bock, 1998). On the other hand, NO is always produced during ammonia oxidation (Stüven and Bock, 2001; Schmidt et al., 2001b). In the absence of oxygen, *Nitrosomonas eutropha* (and to a certain extent *Nitrobacter* sp.) are able to denitrify concomitant with the production of nitric oxide, nitrous oxide and dinitrogen (in the case of *N. eutropha*; Poth and Focht, 1985; Freitag et al., 1987; Zart and Bock, 1998; Bock et al., 1995). Furthermore, NO_2 has recently been reported to be produced by *Nitrosomonas europaea* if grown in coculture with *Paracoccus denitrificans* (Stüven and Bock, 2001). Owing to the above-mentioned activities, ammonia-oxidizing bacteria are considered to contribute to the increasing nitrous oxide level in the atmosphere (Bouwman, 1993). While flux mechanisms for nitric oxide exchange are frequently studied (e.g., Conrad, 1996), the processes of nitrogen dioxide production and release from soil have rarely been investigated (Williams et al., 1992; Baumgärtner et al., 1991). Nitric oxide, nitrogen dioxide and nitrous oxide circles between soil and atmosphere are of great importance because N_2O is a greenhouse gas and NO and NO_2 act as effectors for metabolic activity of microorganisms. The latter effect is of particular importance considering the increasing amounts of the nitrogen oxides NO and NO_2 in the anthropogenically polluted atmosphere originating from methane-, oil- and coal-combustion. Compared to these sources, the contribution of microbiologically produced NO and NO_2 however is marginal.

The production of nitric and nitrous acid by nitrifiers also contributes to biodeterioration and can cause harmful effects for plants. With an outdoor exposure experiment lasting for 7 years, Mansch and Bock (1998) could demonstrate that the ammonia concentration of the atmosphere in the city of Duisburg (Germany) was high enough to support cell growth of lithotrophic ammonia oxidizer s in natural sandstone. The formation of nitrous and nitric acid by such endolithic nitrifiers causes biodeterioration of carbonaceous masonry (Bock and Sand, 1993). Furthermore, in unbuffered environments (e.g., forest soils), the oxidation of ammonia to nitric and nitrous acid leads to acidification followed by the formation of aluminum ions (Al^{3+}) from insoluble aluminates, which are toxic to the roots of trees (Mulder et al., 1989; Stams et al., 1991). However, it should be kept in mind that plants can also benefit from nitrifying activity. Especially, many tree species prefer nitrate instead of ammonia as nitrogen source.

Nitrification is also important in biotechnology for efficient removal of ammonium from sewage (Painter, 1988; Eighmy and Bishop, 1989). Nitrifiers oxidize ammonium, which together with urea is the most frequently found nitrogen compound in sewage, to nitrate which can subsequently be removed from the sewage by denitrifying bacteria via anaerobic respiration. This treatment, which is an integral part of modern nutrient removal at wastewater treatment plants, prevents environments from increasing amounts of ammonia (causing eutrophication and oxygen depletion) and reduces the toxic effects of ammonium to aquatic life. However, the slow growth rate of nitrifiers and their susceptibility to changes in pH and temperature as well as to toxic sewage compounds causes frequent failure of nitrification in municipal and industrial wastewater treatment plants. In the future, wastewater treatment plants also might exploit the unique physiology of recently identified but not yet cultured novel planctomycetes that can catalyze anaerobic oxidation of ammonium to dinitrogen with nitrite as electron acceptor (Strous et al., 1999; Schmid et al., 2000; see Anaerobic Ammonium Oxidation Catalyzed By Deep Branching Planctomycetes in this Chapter).

Nitrifying bacteria are slow-growing organisms because their cell growth is inefficient. For example, nitrite oxidizer s oxidize 85–115 mol of nitrate to generate the energy required for assimilation of 1 mol of carbon dioxide (Bömeke, 1954). Thus, it is not surprising that the shortest generation times measured in laboratory experiments did not exceed 7 h for *Nitrosomonas* and 10 h for *Nitrobacter* (Bock et al., 1990). For cell division in natural environ-

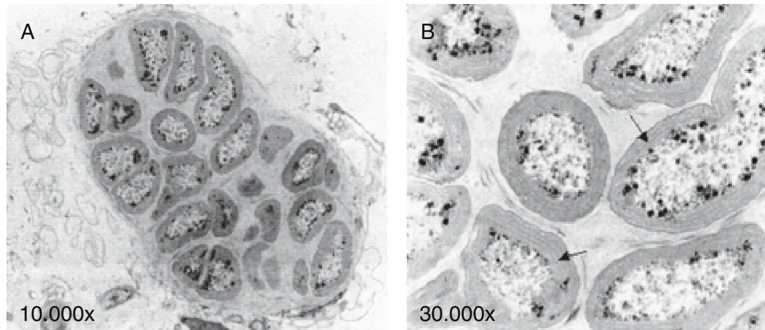


Fig. 3. Transmission electron micrographs of ultrathin sections of an ammonia oxidizer microcolony in activated sludge (A). Arrows indicate intracytoplasmic membranes (B). Modified from Wagner et al. (1995).

ments, most nitrifier species even need several days to weeks depending on substrate, oxygen availability, the temperature and pH values. The slow growth rates of nitrifiers have severely hampered cultivation-dependent approaches to investigate the number, community composition and dynamics of nitrifiers in different environments. The number of nitrifiers in complex systems has been traditionally determined by the most-probable-number (MPN) technique (Matulewich et al., 1975). However, this method is time-consuming and the nitrifier cell counts determined usually do not correlate well with nitrifying potential estimated for the same environmental sample under optimized laboratory conditions (Belser and Mays, 1982; Belser, 1979; Groffmann, 1987; Mansch and Bock, 1998). These discrepancies illustrate that not all nitrifiers can be cultivated using standard methods (Stephen et al., 1998; Juretschko et al., 1998; Purkhold et al., 2000). Furthermore, in many environments nitrifiers form dense microcolonies of ten to several thousand cells embedded in extracellular polymeric substances (EPS; Fig. 3). Since these microcolonies are resistant to the dispersal techniques implemented in standard cultivation protocols, the use of these protocols dramatically underestimates the number of nitrifiers occurring in microcolonies (Watson et al., 1989; Stehr et al., 1995; Wagner et al., 1995).

For direct microscopic enumeration of nitrifiers in complex samples, the fluorescent antibody (FA) technique can be applied (Belser, 1979; Fliermanns et al., 1974), but for antibody production, the target cells have to be isolated first as pure culture and the produced antibodies often recognize only a few strains of a species (Belser and Schmidt, 1978). Recently monoclonal antibodies targeting the nitrite oxidoreductase were developed that allow group-specific detection of nitrite-oxidizing bacteria (Bartosch et al., 1999). In addition, polyclonal antibodies specifically recognizing the AmoB protein of β-subclass ammonia oxidizer s are available (Pinck et al., 2001). Alternatively, nitrifiers can be detected in environmental samples independent from their

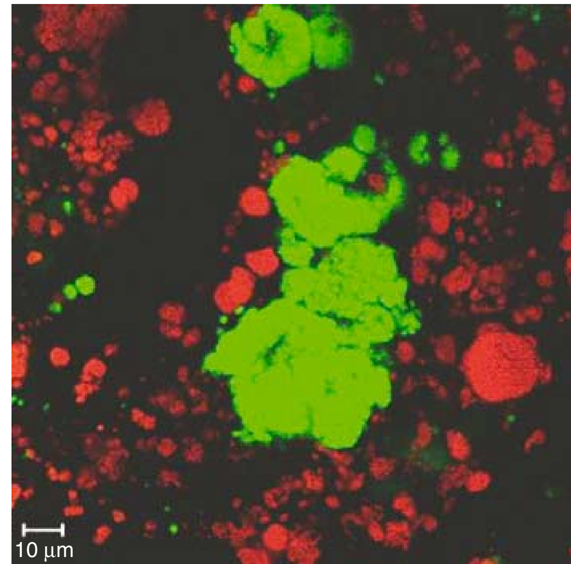


Fig. 4. In situ detection (with fluorescently labeled 16S rRNA-targeted oligonucleotide probes) of ammonia-oxidizing and nitrite-oxidizing bacteria in a nitrifying biofilm from a municipal wastewater treatment plant. Ammonia oxidizer s are stained red, whereas nitrite-oxidizing bacteria of the genus *Nitrospira* appear green. Bar = 10 μm.

culturability by using a variety of different polymerase chain reaction (PCR)-techniques for specific amplification of 16S rRNA gene fragments (e.g., Degrange and Bardin, 1995; Hiorns et al., 1995; Voytek and Ward, 1995; McCaig et al., 1994; Kowalchuk et al., 1997; Utåker and Nes, 1998) or a fragment of the *amoA* gene (e.g., Rotthauwe et al., 1997; Purkhold et al., 2000). Quantitative population structure analysis of nitrifying bacteria within their natural habitat can most precisely be obtained by applying the recently developed set of rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization (FISH; Wagner et al., 1995; Wagner et al., 1996; Mobarry et al., 1996; Juretschko et al., 1998; Daims et al., 2000; Fig. 4).

Nitrifying bacteria are present in oxic and even anoxic environments. They are widely distributed in fresh water, seawater, soils, on/in

rocks, in masonry, and in wastewater treatment systems. Nitrifiers also could be enriched or isolated from extreme habitats like heating systems with temperatures of up to 47°C (Ehrich et al., 1995; E. Lebedeva, personal communication) and permafrost soils up to a depth of 60 m at a temperature of down to –12°C. Although the pH optimum for cell growth is 7.6–7.8, nitrifiers were frequently detected in environments with suboptimal pH (e.g., acid tea soils and forest soils at pH values below 4) but also in highly alkaliphilic soda lakes at a pH of 9.7–10.5 (Sorokin et al., 2001). Growth under suboptimal acidic conditions might be possible by ureolytic activity, by aggregate formation (De Boer et al., 1991), or as biofilms (e.g., on clay particles; Allison and Prosser, 1993). In many environments, nitrifier sensitivity to sunlight is of ecological importance. The light sensitivity of ammonia- and nitrite oxidizer s increases from blue light to long wave UV (Hooper and Terry, 1974; Hyman and Wood, 1984a; Shears and Wood, 1985). Based on spectroscopic similarities, Shears and Wood (1985) postulated a model of the ammonia monooxygenase light inhibition similar to the three-stage catalytic cycle of the tyrosinase reaction. In *Nitrobacter*, which is more sensitive to visible light than *Nitrosomonas* (Bock, 1965), the photooxidation of *c*-type cytochrome s is assumed to cause light-induced cell death (Bock, 1970).

Although *Nitrosomonas euopaea* and *Nitrobacter* sp. are the most commonly investigated ammonia- and nitrite oxidizer s in laboratory studies, molecular analysis revealed that other nitrifiers are of higher importance in many natural and engineered systems. For example, stone material of historical buildings and many soil systems seem to be dominated by members of the genera *Nitrosovibrio* and *Nitrosospira*, respectively (Spieck et al., 1992; Hiorns et al., 1995; Stephen et al., 1996; Meincke et al. 1989), whereas different *Nitrosomonas* species and *Nitrosococcus mobilis* are the most abundant ammonia oxidizer s in wastewater treatment plants (Juretschko et al., 1998; Purkhold et al., 2000). Interestingly, not yet cultured members of the genus *Nitrospira* and not *Nitrobacter* are the most abundant nitrite oxidizer s in sewage treatment plants and aquaria filters (Burrell et al., 1998; Juretschko et al., 1998; Wagner et al., 1996; Daims et al., 2000).

Phylogeny of Lithotrophic Nitrifying Bacteria

Traditionally, nitrifying bacteria have been lumped together into one coherent group, the family Nitrobacter iaceae (Watson, 1971a; Watson et al., 1989). Based on their ability to lithotrophically oxidize either ammonia to nitrite or nitrite to nitrate, nitrifying bacteria were separated into two groups, the ammonia- and the nitrite oxidizer s. The assignment of ammonia- and nitrite-oxidizing bacteria into genera was dependent primarily upon their morphological features like cell size, shape, and the arrangement of the intracytoplasmic membranes (Watson et al., 1989). The physiological and morphological grouping of the nitrifying bacteria is in contradiction to data obtained from molecular phylogenetic studies which show at least subdivision level diversity within and between the ammonia- and nitrite oxidizer s (Head et al., 1993; Orso et al., 1994; Teske et al., 1994; Purkhold et al. 2000; Ehrich et al., 1995). Significant differences between ammonia- and nitrite-oxidizing bacteria are also indicated by the fact that both physiological groups possess very different key enzyme systems for the energy-gaining oxidation of ammonia and nitrite, respectively (Enzymes involved in ammonia oxidation; Enzymes involved in nitrite oxidation). With the exception of the nitrite oxidizer s of the genera *Nitrospina* and *Nitrospira*, all known nitrifiers are closely related to phototrophs and thus presumably originated in several independent events by conversion of photosynthetic ancestors to chemolithotroph s (Teske et al., 1994). Consistent with this conversion hypothesis, all nitrifying bacteria related to phototrophs retain the general structural features of the putative ancestor's photosynthetic membrane complex, while nitrite oxidizer s of the genera *Nitrospina* and *Nitrospira* lack intracytoplasmic membranes (ICMs). However, it should be noted that the ammonia oxidizer s of the genera *Nitrosospira* and *Nitrosovibrio* lack an extensive intracytoplasmic membrane system (Koops and Möller, 1992).

Phylogeny of Ammonia Oxidizer s

Chemolithotroph ic ammonia oxidizer s were isolated for the first time at the end of the nineteenth century (Winogradsky, 1892). Since then, 16 species of ammonia oxidizer s have been described (Jones et al., 1988; Koops et al., 1976; Koops et al., 1990; Koops et al., 1991; Watson, 1965), and according to DNA-DNA hybridization experiments, at least 15 additional genospecies are "hidden" in existing culture collections (Koops et al., 1991; Koops and Harms, 1985; Stehr et al., 1995). Our current perception of evolutionary relationships of ammonia-oxidizing bacteria is mainly based on comparative sequence analysis of their genes encoding the 16S rRNA and the active site polypeptide of the ammonia monooxygenase (AmoA). During

the last decade, the genes for both biopolymers were sequenced for all recognized ammonia oxidizer species (Alzerreca et al., 1999; Head et al., 1993; Pommerening-Röser et al., 1996; Teske et al., 1994; Purkhold et al., 2000; Rotthauwe et al., 1995; Rotthauwe et al., 1997; McTavish et al., 1993; Horz et al., 2000) and the deduced phylogeny now provides an encompassing and relatively robust framework for assignment of 16S rDNA and *amoA* sequences of 1) ammonia oxidizer isolates (Stehr et al., 1995; Suwa et al., 1997; Utåker et al., 1995; Juretschko et al., 1998) and 2) cloned sequence fragments directly retrieved from the environment (e.g., Stephen et al., 1996; Rotthauwe et al., 1995; Purkhold et al., 2000).

According to comparative 16S rRNA sequence analysis, all recognized ammonia oxidizer s are members of two monophyletic lineages within the β- and γ-subclass of Proteobacteria (Fig. 5). The marine species *Nitrosococcus halophilus* and *Nitrosococcus oceani*, which are distantly related to methane-oxidizing bacteria, cluster together in the γ-subclass of Proteobacteria. All other ammonia oxidizer s form a monophyletic assemblage within the β-subclass of Proteobacteria, most closely related to the iron-oxidizer *Gallionella ferruginea*. This lineage encompasses the genera *Nitrosomonas* (including *Nitrosococcus mobilis*, which is actually a member of the genus *Nitrosomonas*), *Nitrosovibrio*, *Nitrosolobus* and *Nitrosospira*. It has been suggested (Head et al., 1993) and subsequently questioned (Teske et al., 1994) that the latter three genera should be reclassified into the single genus *Nitrosospira*. The nitrosomonads can be further subdivided into the *N. europaea/Nc. mobilis* cluster, the *N. marina* cluster, the *N. oligotropha* cluster, and the *N. communis* cluster (Purkhold et al., 2000). *Nitrosomonas cryotolerans* forms a separate lineage within the β-Proteobacteria. The genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* are closely related and form a cluster to the exclusion of the nitrosomonads. Similar but not identical evolutionary relationships were obtained if comparative analysis of AmoA sequences were performed (Purkhold et al., 2000). In the AmoA tree, the *N. europaea/Nc. mobilis* cluster, the *N. marina* cluster, and the *Nitrosospira*-cluster are retained, whereas the members of the *N. oligotropha* cluster and the *N. communis* cluster form no monophyletic assemblages.

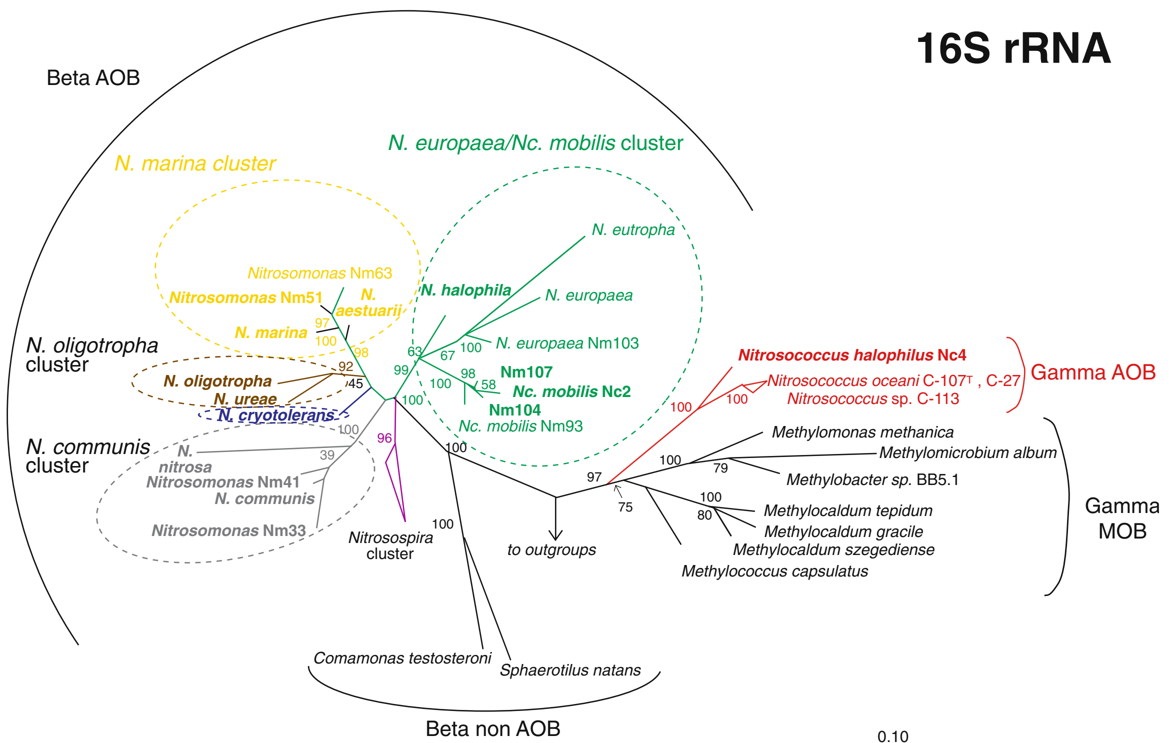


Fig. 5. Phylogenetic neighbor-joining 16S rRNA tree reflecting the relationships of ammonia-oxidizing bacteria and several reference organisms. The multifurcation connects branches for which a relative order could not be unambiguously determined by applying different treeing methods. Parsimony bootstrap values for branches are reported. Missing bootstrap values indicate that the branch in question was not recovered in the majority of bootstrap replicates by the parsimony method. AOB = ammonia-oxidizing bacteria; MOB = methane-oxidizing bacteria. The bar indicates 10% estimated sequence divergence. Modified from Purkhold et al. (2000).

Phylogeny of Nitrite Oxidizer s

Four different genera, *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*, of lithotrophic nitrite-oxidizing bacteria have been described. From 16S rRNA sequence analysis, the first three genera were assigned to different subclasses of the Proteobacteria, whereas *Nitrospira* is the name-giving genus of an independent bacterial phylum (Fig. 5). The genus *Nitrobacter* contains the four closely related species (*N. hamburgensis*, *N. vulgaris*, *N. winogradskii* and *N. alkalicus*) within the α-subclass of Proteobacteria. Nitrite oxidizer s of the genus *Nitrobacter* are phylogenetically related to *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, *Afipia clevelandensis* and the phototroph *Rhodobacter palustris* (Seewaldt et al., 1982; Orso et al., 1994; Teske et al., 1994) with which *Nitobacter* shares a nearly identical arrangement of ICMs.

The genus *Nitrococcus* represented by the single marine species *Nitrococcus mobilis* is, like the marine ammonia oxidizer s of the genus *Nitrosococcus*, a member of the ectothiorhodospira branch of the γ-subclass of Proteobacteria, consistent with an assumed photosynthetic ancestry of these nitrifiers. *Nitrococcus* and *Nitrosococcus* are the only nitrite- and ammonia oxidizer s that are relatively closely related, but the closest relatives of *Nitrococcus mobilis* are the phototrophic bacteria *Arhodomonas aquaeoli*, *Ectthiorhodospira halochloris* and *Ectthiorhodospira halophila* (Teske et al., 1994).

The genus *Nitrospina* with the marine *Nitrospina gracilis* as the only species (represented by two isolates, one from the Atlantic and the other from the Pacific) has been provisionally assigned to the δ-subclass of Proteobacteria and is the only member of a deep branch within this subclass (Teske et al., 1994). *Nitrospina gracilis* shows no ICMs.

The genus *Nitrospira* encompasses the marine species *Nitrospira marina* and *Nitrospira moscoviensis*, isolated from a municipal water heating system. The genus *Nitrospira* forms a monophyletic grouping with the genera *Thermodesulfovibrio*, *Leptospirillum* and with "*Magnetobacterium bavaricum*." This phylogenetic assemblage has recently been identified as a novel phylum within the domain Bacteria and was named "*Nitrospira phylum*" (Ehrich et al., 1995). There is accumulating molecular evidence that *Nitrospira*-related nitrite oxidizer s are of major importance for nitrite-oxidation in wastewater treatment plants and aquarium filters (Burrell et al., 1998; Juretschko et al., 1998; Hovanec et al., 1998; Daims et al., 2000), and also occur in many natural environments including the rhizosphere (Fig. 6). Like *Nitrospina gracilis*, members of the genus *Nitrospira* do not possess ICMs and are apparently not closely related to phototrophic bacteria.

Biochemistry of Ammonia-Oxidizing Bacteria

Ammonia oxidizer s are lithoautotrophic organisms using carbon dioxide as the main carbon source (Bock et al., 1991). Their only way to gain energy is the two-step oxidation of ammonia to nitrite (Hooper, 1969). Investigations of the K_m values and pH optima indicate that ammonia (NH_3) rather than ammonium (NH_4^+) is the substrate of ammonia oxidizer s (Suzuki et al., 1974; Drozd, 1976). This is in accordance with results showing that the ammonia-oxidizing enzyme might be located in the cytoplasmic membrane (Suzuki and Kwok, 1981a; Tsang and Suzuki, 1982), since membranes are highly permeable to ammonia but not to ammonium (Kleiner, 1985). First, ammonia is oxidized to hydroxylamine (Kluyver and Donker, 1926) by the ammonia monooxygenase (AMO; Hollocher et al., 1981). This enzyme does not possess high substrate specificity and also oxidizes several apolar compounds such as methane, carbon monoxide or some aliphatic and aromatic hydrocarbons (Hooper et al., 1997). These compounds can act as competitive inhibitors of ammonia oxidation (Hyman et al., 1988; Keener and Arp, 1993). The second step is performed by the hydroxylamine oxidoreductase (HAO). This enzyme oxidizes hydroxylamine to nitrite (Wood, 1986). Two of the four electrons released (Andersson and Hooper, 1983) are required for the AMO-reaction (Tsang and Suzuki, 1982), whereas the remaining ones are used for the generation of proton motive force (Hollocher et al., 1982) to regenerate ATP and NADH (Wheelis, 1984; Wood, 1986). Most of the investigations on energy metabolism of ammonia-oxidizing bacteria have been carried out with *Nitrosomonas europaea*. Keeping in mind that the ammonia oxidizer s encompass five different genera affiliated to two proteobacterial subclasses (Phylogeny of ammonia oxidizer s), additional species should be investigated to obtain a more encompassing picture of the biochemistry of the ammonia-oxidizing system (Giannakis et al., 1985).

Ammonia and Hydroxylamine as Substrates

The overall process of ammonia oxidation to nitrite may be characterized as a two-stage process:

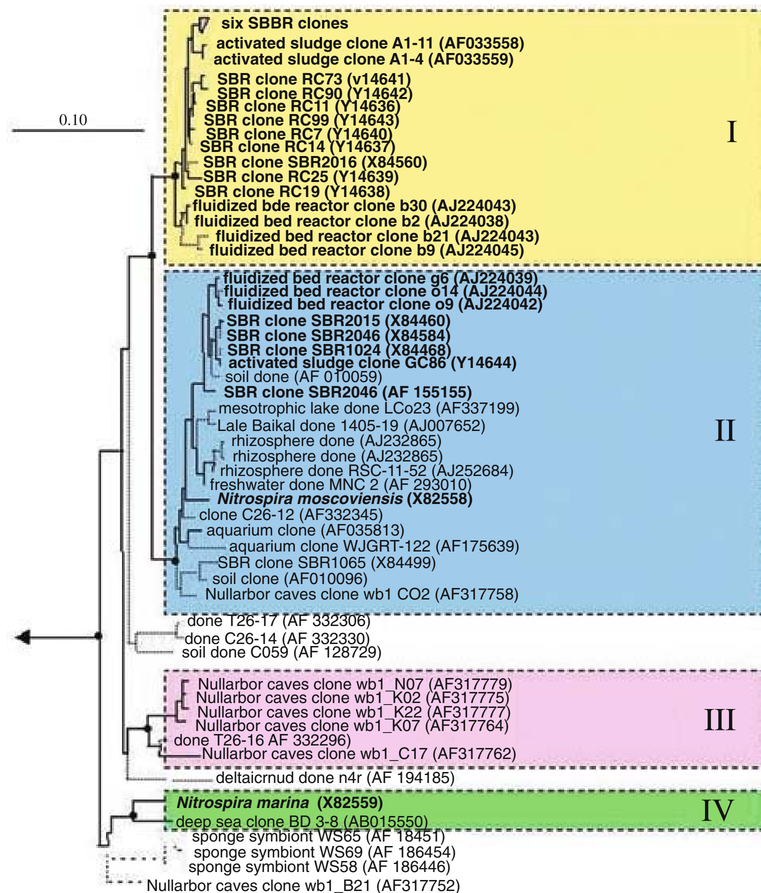


Fig. 6. Phylogenetic tree of the genus *Nitrospira* based on comparative analysis of 16S rRNA sequences. The basic tree topology was determined by maximum likelihood analysis of all sequences longer than 1,300 nucleotides. Shorter sequences were successively added without changing the overall tree topology. Branches leading to sequences shorter than 1,315 nucleotides are dotted to point out that the exact affiliation of these sequences cannot be determined. Black spots on tree nodes symbolize high parsimony bootstrap support above 90% based on 100 iterations. The scale bar indicates 0.1 estimated changes per nucleotide. The four sublineages of the genus *Nitrospira* are delimited by horizontal dashed lines and marked by the numbers I to IV. Two of the four sublineages entirely consist of 16S rDNA sequences amplified from environmental samples. Modified from H. Daims et al., 2001.

$$NH_3 + 0.5O_2 \rightarrow NH_2OH \qquad (11)$$

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \qquad (12)$$

However, this two-stage scenario is a simplification. For lithotrophic ammonia oxidizer s, ammonia is essential as the primary substrate. The intermediate hydroxylamine (NH_2OH) is the real energy source. The coupling between ammonia- and hydroxylamine oxidation, a complex mechanism not yet established in detail, is suggested by several observations. The addition of hydroxylamine to ammonia-oxidizing cells shortened the lag-phase of ammonia oxidation (Hooper, 1969), probably by providing reductants to the monooxygenase. It is generally assumed that partial reduction of *c*-type cytochrome s is necessary to start ammonia oxidation. Cytochrome reduction was attained by addition of hydroxylamine to cell-free preparations of *Nitrosomonas europaea* (Suzuki et al., 1981b). If both ammonia and hydroxylamine are used, the molar growth yield of hydroxylamine was found to be twice the amount of ammonia (Böttcher and Koops, 1994; De Bruijn et al., 1995). On the other hand, increasing amounts of hydroxylamine are inhibitory to ammonia oxidation (Hyman and Wood, 1984b; Poth and Focht, 1985; Abeliovich and Vonshak, 1993), probably due to imbalancing the redox state of AMO and HAO (Wood, 1986). Another result is more difficult to understand. All attempts to grow ammonia oxidizer s on hydroxylamine as the only substrate have failed, although hydroxylamine is oxidized to nitrite (Lees, 1952; Hoffman and Lees, 1953; Engel and Alexander, 1958; Nicholas and Jones, 1960). This failure is most likely not caused by the toxicity of hydroxylamine, because addition of hydroxylamine in the presence of ammonia promotes substrate oxidation and cell growth. As demonstrated recently, *Nitrosomonas eutropha* cells are capable of growing on hydroxylamine as the only substrate when AMO is simultaneously inhibited by acetylene (S. Oesterreicher, personal communication; Fig. 7). Without addition of acetylene, *N. eutropha* cells lyse within 3 days when hydroxylamine is oxidized to nitrite, although within the first day NADH and ATP are still formed (C. Look, personal communication). It is important to note that during these experiments ammonia was present as nitrogen source because hydroxylamine could not be assimilated. The observation that reduc-

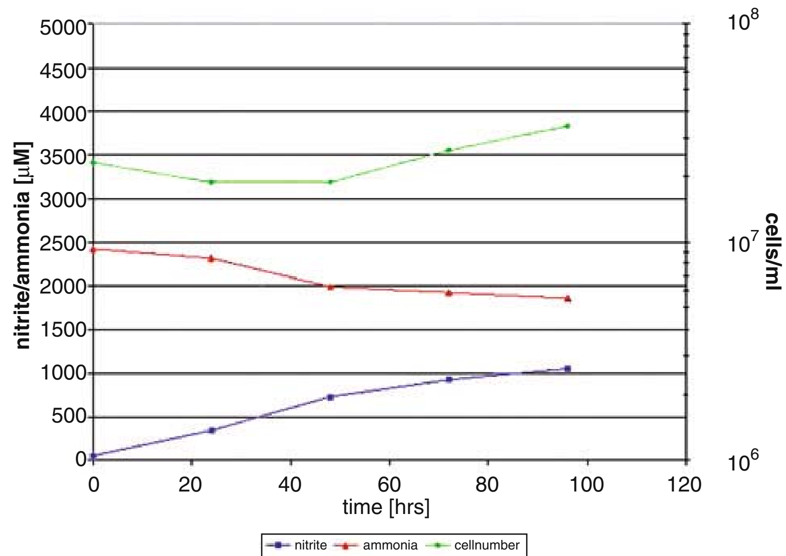


Fig. 7. Growth of *Nitrosomonas eutropha* in the presence of ammonia, 2,315 parts per million (ppm) acetylene, and hydroxylamine (4 mmol) as substrate (48–96h). The AMO was inhibited by acetylene, while cell growth was detectable after a lag phase of 2 days. Most of the hydroxylamine undergoes deterioration in contact with atmospheric oxygen. As calculated from additional nitrite formation, 400 µmol of hydroxylamine were oxidized to nitrite resulting in an increase of cell number.

tion of a functionally active AMO in the absence of ammonia leads to cell death could be explained by the formation of toxic oxygen radicals by this enzyme under these conditions. This suicidal activity of ammonia oxidizer s also might cause nitrification breakdown in wastewater treatment plants, if 1) plenty of organic substrate is available as additional alternative electron donor and 2) ammonia is present in very low concentrations.

Enzymes Involved in Ammonia Oxidation

Ammonia Monooxygenase The first intermediate of ammonia oxidation is assumed to be hydroxylamine (Genes encoding AMO, HAO, and related Enzymes). In the presence of hydrazine (an irreversible inhibitor of hydroxylamine oxidation; Nicholas and Jones, 1960; Hynes and Knowles, 1978), the production of small quantities of hydroxylamine from ammonia was observed (Hoffman and Lees, 1953; Yoshida and Alexander, 1964). Using $^{18}O_2$, it could be demonstrated that more than 92% of the oxygen in hydroxylamine originates from dioxygen (Dua et al., 1979). The enzyme AMO, catalyzing the conversion of ammonia to hydroxylamine, has not yet been purified as active protein, but Hyman and Wood (1985a) were able to identify a membrane-associated ^{14}C-labeled protein, putatively representing a component of AMO, when whole cells of *Nitrosomonas europaea* were incubated with [^{14}C]acetylene. The N-terminal amino acid sequence of the [^{14}C]acetylene-labeled protein (AmoA) was determined. Based on this sequence, an oligonucleotide was derived and was used to identify and clone the gene *amoA*. The AmoA protein is a 31.8 kDa (McTavish et al., 1993), probably containing the active site of AMO (Hyman and Arp, 1992), and consists of five transmembrane sequences and one periplasmic loop. In the same operon, a second gene *amoB* is located adjacent to *amoA*. From the deduced amino acid sequence, the protein has a molecular weight of 43 kDa (Bergmann and Hooper, 1994a), and is characterized by two transmembrane domains and two periplasmic loops (Vanelli et al., 1996). Upstream of the genes *amoA* and *amoB*, a third open reading frame *amoC* is located which might encode a chaperone helping the AmoA and AmoB protein subunits to integrate into the membrane properly (Klotz et al., 1997).

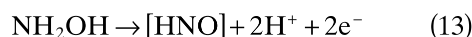
Indirect evidence indicates that AMO is a copper-containing monooxygenase (Rees and Nason, 1966; Tomlinson et al., 1966; Loveless and Painter, 1968; Dua et al., 1979; Hollocher et al., 1981; Wood, 1988a; Hooper and Terry, 1973). Quantitative immunoblot analysis using polyclonal antibodies revealed that total cell protein of *Nitrosomonas eutropha* consisted of approximately 6% AmoA and AmoB, when cells were grown using standard conditions (Pinck et al., 2001). The specific cellular amount of AMO in cells of *Nitrosomonas eutropha* was regulated by ammonium concentration. At high ammonium concentrations, less AMO was found than under ammonium-limiting conditions. Furthermore, AMO seems to be strongly protected from degradation. Cells starving one year for ammonia still contained high amounts of AMO, although they showed far less ammonia oxidation activity than growing cells. Hence, the amount of AMO does not directly correlate with the activity of ammonia oxidation.

Most information about the reactions catalyzed by AMO originates from studies with intact cells. In addition to oxidizing ammonia, AMO can hydroxylate non-growth-supporting substrates such as hydrocarbons and alcohols (Hooper and Terry, 1973; Suzuki et al., 1976; Tsang and Suzuki, 1982; Hyman and Wood, 1983; Hyman and Wood, 1984a; Hyman and Wood, 1984b; Hyman et al., 1985b; Voysey and Wood, 1987). This is not only of theoretical interest but also could be of importance for microbial ecology (Hall, 1986). For example, pure cultures of ammonia oxidizer s are able to oxidize methane, but could not grow on this alternative electron donor (O'Neil and Wilkinson, 1977; Hyman and Wood, 1983; Jones and Morita, 1983). Recent data, however, suggest that at least in the rice rhizosphere, ammonia oxidizer s do not significantly contribute to the methane oxidation (Bodelier and Frenzel, 1999; Co-oxidation and Inhibition of AMO). This capability reflects structural and functional homologies between the ammonia- and the methane monooxygenase of ammonia oxidizer s and methanotrophs, respectively (Bedard and Knowles, 1989). Since substrates or competetive inhibitors of AMO are apolar, it seems reasonable to assume that its active site is hydrophobic. As suggested by Hooper et al. (1997), the reaction is started by the activation of oxygen rather than the substrate. Oxygen might be activated by reduction with a reduced metal-containing center of the enzyme followed by the release of water to form a reactive oxygen species. This compound may extract an electron from the substrate (hydroxylation of the substrate) or interact with nitric oxide to form the real oxidant nitrogen dioxide /dinitrogen tetroxide (see also Fig. 17).

HYDROXYLAMINE OXIDOREDUCTASE The key enzyme of hydroxylamine oxidation, HAO, is a multiheme enzyme, located in the periplasmic space (Olson and Hooper, 1983; Hooper et al., 1984b; Hooper and DiSpirito, 1985; Genes encoding AMO, HAO, and related Enzymes) The enzyme complex has a relative molecular weight of 180,315–190,315 and consists of an α3 oligomer closely associated with three heme centers including seven *c*-type hemes and a novel heme, P-460, per monomer (Arciero and Hooper, 1993; Hoppert et al., 1995; Igarashi et al., 1997; Bergmann and Hooper, 1994b). The P-460 was found to be a CO-binding heme (Hooper et al., 1978; Lipscomb et al., 1982b). According to spectroscopic and chemical investigations, the P-460 iron resides in a heme-like macrocycle, but the presumed porphyrin must have some unusual features (Andersson et al., 1984). In total, HAO constitutes about 40% of the *c*-type heme of *Nitrosomonas europaea* (Hooper et al., 1978). The *c*-type hemes of HAO can be placed into two classes with different oxidation-reduction midpoint potentials and protein environments, respectively (Lipscomb and Hooper, 1982a; Prince et al., 1983; Hooper, 1984a; Collins et al., 1993; Arciero et al., 1991). A detailed discussion of possible interactions of the described redox centers of the HAO can be found in Hooper (1989).

Hydroxylamine is supposed to bind at the HAO near the P-460 center. Electrons are released and transferred to *c*-hemes (Hooper and Terry, 1977; Hooper and Balny, 1982; Olson and Hooper, 1983). Initially, Hooper and Balny (1982) postulated that HAO catalyzes a two-electron dehydrogenation of hydroxylamine and a subsequent net addition of one oxygen atom from dioxygen. Later, they favored a mechanism in which water was the source of the second oxygen atom of the metabolic final product nitrite (Andersson and Hooper, 1983; Hooper, 1984a).

The oxidation of hydroxylamine to nitrite was postulated to be a two-step reaction with enzyme-bound nitroxyl (HNO) as an intermediate (Andersson and Hooper, 1983):



$$NH_2OH \rightarrow [HNO] + 2H^+ + 2e^- \quad (13)$$

$$[HNO] + H_2O \rightarrow HNO_2 + 2H^+ + 2e^- \quad (14)$$

However, in cell-free extracts of *Nitrosomonas europaea*, nitric oxide was suggested as another possible intermediate of hydroxylamine oxidation (Hooper and Terry, 1979). Experiments with ^{15}N-label showed that nitric oxide was produced by hydroxylamine oxidation and not by nitrite reduction. The authors discussed a mixed-function hydroxylation of nitric oxide to be involved in the oxidation from (HNO) to nitrite, with all intermediates being enzyme-bound. Miller and Wood (1983) analyzed CO-binding cytochrome s of the *b* type in *Nitrosomonas europaea* and discussed their possible function in binding nitric oxide resulting from hydroxylamine oxidation.

Electron Flow and Energy Transduction

ELECTRON FLOW The first step of ammonia oxidation to nitrite, the conversion to hydroxylamine, is endergonic. Thus, hydroxylamine is the real energy-generating substrate. If all subsequent steps of the hydroxylamine oxidation to nitrite are coupled to electron transport chains, a maximum yield of four electrons can result. The number of electrons passing to the terminal oxidase(s), however, is uncertain because four systems (ammonia monooxygenase, nitrite reductase, cytochrome oxidase and NADH production) are fed with electrons from the oxidation of hydroxylamine to nitrite (Wood, 1986).

Electrons from HAO reduce cytochrome c_{554}, a 25-kDa tetraheme protein (Andersson et al., 1986b). Because both ammonia and hydroxylamine oxidation seem to be balanced at a steady state, cytochrome c_{554} is thought to be the first electron transfer branch point. Two of the four electrons released from the hydroxylamine oxidation must pass to the monooxygenase reaction, the latter two flow to a second branch point, e.g., cytochrome c_{552} and then to one of the terminal oxidases cytochrome aa_3 (DiSpirito et al., 1986) or nitrite reductase. Once per 5.7 cycles, two electrons are assumed to enter a reverse electron transfer pathway for NADH production (Wood, 1986). Cytochrome c_{554} is a probable candidate for the proposed central role because it is a two-electron carrier (Arciero et al., 1991; Bergmann et al., 1994c). The electron carriers downstream have not been investigated in detail. However, the production of nitric and nitrous oxide by *Nitrosomonas europaea* and *N. eutropha* suggested that nitric oxide reductase as well as nitrous oxide reductase might be present (Hooper et al., 1997). Yamanaka and Shinra (1974) postulated the path of electrons from HAO to the terminal oxidase to be:

HAO → cytochrome c_{554} → cytochrome c_{552} → terminal oxidase. However, several other membrane-bound redox carriers have been identified in Nitrosomonas europaea. The function of a tetraheme *c*-type cytochrome (Cyt *c* B) is unknown (Bergmann et al., 1994c). The periplasmic diheme cytochrome *c* peroxidase (Arciero and Hooper, 1994) of Nitrosomonas europaea might protect enzymes like HAO, which are easily inactivated by hydrogen peroxide (H_2O_2; Hooper and Terry, 1977). Wood (1986) suggested a more conventional construction of the electron transport chain, involving ubiquinone and membrane-bound *b*- and *c*-type cytochrome s. Wood (1986) discussed a possible proton motive Q-cycle as described by Mitchell (1975). Ubiquinone (Q8 species) and membrane-bound cytochrome s of types *b* and *c* were identified in Nitrosomonas europaea (Hooper et al., 1972; Tronson et al., 1973; Miller and Wood, 1983). In Fig. 8, a model of the electron flow in ammonia oxidizer s is depicted.

Fig. 8. Model of the electron flow in ammonia-oxidizing bacteria. The part of the figure dealing with nitric oxide, nitrogen dioxide and dinitrogen tetroxide is hypothetical (Novel aspects). CM = cytoplasmic membrane, i = inside the cell/cytoplasmic space, and o = outside of the cell/periplasmic space, and TCA = the tricarboxylic acid cycle. Figure was kindly provided by I. Schmidt.

Energy Transduction ATP synthesis driven by proton motive force occurs in *Nitrosomonas* (Drozd, 1976; Drozd, 1980; Hollocher et al., 1982; Kumar and Nicholas, 1982). This energy transduction is assumed to proceed at the level of hydroxylamine oxidation, but the process is not as yet well understood.

A simplified scheme would be a two-proton release per electron pair translocated from hydroxylamine to the electron transport chain via the periplasmic located HAO outside the membrane. In addition, the consumption of two protons in the cytochrome oxidase reaction is probably located on the cytoplasmic site of the membrane. However, the exact amount of ATP gained by oxidation of hydroxylamine is not known, since the total number of electrons fed into the energy-generating respiration chain per mol hydroxylamine oxidized varies, depending upon growth stage and environmental conditions. This variation reflects the fact that the production of NADH by reverse electron flow is not constant and coupled to hydroxylamine oxidation by an unknown mechanism.

According to Hollocher et al. (1982), the H^+/O ratio depends on the substrate concentration. From their measurements, they extrapolated the maximum values to be 3.4 and 4.4 for ammonia and hydroxylamine oxidation, respectively. In addition, Drozd (Drozd, 1976; Drozd, 1980) stated the maximum P/O ratio from hydroxylamine oxidation to be only one. Measurements of respiration-driven proton translocation indicated the association of only one proton translocation loop, with the transport of

two electrons channeled from hydroxylamine to the terminal oxidase.

NADH Production Aleem (1966) showed that cell-free extracts of *Nitrosomonas europaea* catalyzed an ATP-dependent $NAD(P)^+$ reduction with hydroxylamine as substrate. The reaction was interpreted as ATP-driven reverse electron flow. This hypothesis is in accordance with the postulate that the transmembrane oxidation-reduction loops of respiration chains are reversible, with the exception of the cytochrome -*c* oxidase loop. However, in vivo, the proton motive force resulting from the hydroxylamine oxidation might perhaps drive the reverse direction of the electron flow directly, without support of ATP as previously demonstrated for the nitrite oxidizer s *Nitrobacter winogradskyi* and *Nitrobacter vulgaris* (Freitag and Bock, 1990).

Co-Oxidation and Inhibition of AMO

The ammonia monooxygenase (AMO) is a nonspecific enzyme. Ammonia oxidizer s are capable of co-oxidizing a range of hydrocarbons (including methane and even xenobiotics), which raised interest in exploiting these microorganisms for bioremediation (Vanelli et al., 1990). The broad substrate range of AMO also is responsible for inhibition of ammonia oxidizer s by a variety of substances (Table 1). During oxidation of acetylene via AMO, reactive intermediates that bind irreversibly to AMO are formed in the presence of oxygen. The same mechanism causes the inhibition of AMO by trichlorethylene. The acetylene inhibition can be ameliorated by high ammonia concentrations via an unknown mechanism (Hyman and Wood, 1985a). Competitive inhibitors of AMO are methylfluorides, dimethylether (Voysey and Wood, 1987; Miller et al., 1993; Hyman et al., 1994) alkanes, alkenes (Hyman et al., 1988), and aromatic compounds (e.g., aniline; Keener and Arp, 1994; Voysey and Wood, 1987; Hyman and Wood, 1983; Jones and Morita, 1983). Carbon monoxide (CO) not only binds irreversibly to cytochrome s but also competitively inhibits AMO, the enzyme that oxidizes it to carbon dioxide (Tsang and Suzuki, 1982; Erickson et al., 1972). Since copper is a cofactor of AMO (Loveless and Painter, 1968; Hooper and Terry, 1973), metal chelators such as allylthiourea and diethyldithiocarbamate are noncompetitive, reversible inhibitors (Lees, 1952).

In addition to some of the above-mentioned inhibitors, Table 1 lists other inhibitors of ammonia oxidation that do not directly interact with AMO. Ammonia oxidation is much more strongly inhibited by all listed physical parameters and chemical compounds than is hydroxylamine oxidation.

Denitrification Catalyzed by Ammonia Oxidizers

Ammonia-oxidizing bacteria not only catalyze aerobic ammonia oxidation but also show denitrifying activity with nitrite as electron acceptor. For example, small amounts of nitric oxide and nitrous oxide are produced during denitrification with ammonia as electron donor at reduced oxygen concentrations (Hooper, 1968; Goreau et al., 1980; Remde and Conrad, 1990; Stüven et al., 1992). When using $^{14}NH_4^+$ and $^{15}NO_2^-$, Poth and Focht (1985) demonstrated that nitrous oxide

Table 1. Inhibitors of ammonia oxidation.

Inhibitor	Optimum	Substrate: NH_3[a]	Substrate: NH_2OH[a]
Allylthiourea	10^{-6}[b]	18	100
KCN	5×10^{-6}[b]	22	83
Na_2S	10^{-4}[b]	0	9
NH_2NH_2	2×10^{-3}[b]	16	86
CO (95% O_2, 5% CO)	0.05[b]	8	100
mCCP[c]	10^{-5}[b]	17	128
Dinitrophenol (DNP)	2×10^{-4}[b]	27	100
Methylene blue	10^{-4}[b]	0	100
Methanol	5×10^{-3}[b]	0	100
Ethanol	0.09[b]	0	100
Acetate	0.1[b]	91	100
Light	420lux	0	100
Temperature	15°C	23	50

[a]Nitrite producing rates (%) of whole cells using ammonia as substrate are listed. For comparison, the respective rates for hydroxylamine oxidation are shown. Nitrite-producing rate of the untreated control equals 100%.
[b]Concentration in mol per liter.
[c]*m*-Chlorcarbonyl cyanide phenylhydrazone.
Modified from Hooper and Terry (1973).

was produced at low oxygen tension by nitrite reduction and not by hydroxylamine oxidation. The reaction is thought to be catalyzed by a periplasmic soluble cytochrome oxidase/nitrite reductase induced at low oxygen partial pressure (Miller and Wood, 1982; Miller and Nicholas, 1985; DiSpirito et al., 1985). Additionally, the formation of dinitrogen was observed (Poth, 1986; Bock et al., 1995), indicating that at least some strains of *Nitrosomonas* possess a nitrous oxide reductase. However, this enzyme has not been isolated as yet from denitrifying ammonia oxidizer s.

Ammonia oxidizer s show relatively high denitrification activities when they are cultivated under oxygen-limited conditions in the presence of organic matter (mixotrophic growth conditions; Bock et al., 1995). However, under these conditions, ammonia oxidation rates are low (Zart et al., 1996). For this reason, the denitrifying potentials of ammonia oxidizer s cannot be efficiently exploited for one-step nitrogen removal in wastewater treatment plants.

In the absence of dissolved oxygen, *Nitrosomonas eutropha* and *Nitrosomonas europaea* are capable of anoxic denitrification using molecular hydrogen, or simple organic compounds such as acetate, pyruvate, or formate as electron donors and nitrite as electron acceptor (Bock et al., 1995; Abeliovich and Vonshak, 1992; Stüven et al., 1992).

Genetics of Ammonia Oxidizer s

Relatively little information regarding the genetic makeup of ammonia oxidizer s is available. Most studies focussed on *Nitrosomonas europaea* (genome of ca. 2.2 Mb) whose genomic sequence is currently being determined (spider.jgi-psf.org). For the other ammonia oxidizer s of the β- and λ-subclasses of Proteobacteria (Phylogeny of ammonia oxidizer s), sequence information is restricted to the genes coding for the 16S rRNA (for a review, see Purkhold et al., 2000), the 16S-23S rDNA intergenic spacer region (Aakra et al., 1999), as well as the ammonia monooxygenase operon (Rotthauwe et al., 1995; Purkhold et al., 2000; Alzerreca et al., 1999). Recently, a gene for a copper-containing dissimilatory nitrite reductase (*nirK*) has been detected by PCR and was sequenced for several β-subclass ammonia-oxidizing bacteria is underway (Casciotti and Ward, 2001). Ammonia oxidizer s can also harbor plasmids, as demonstrated by the isolation and characterization of two cryptic plasmids in a *Nitrosomonas* strain retrieved from activated sludge (Yamagata et al., 1999).

Genes Encoding AMO, HAO and Related Enzymes

Genes coding for enzymes involved in the oxidation of ammonia, particularly the ammonia monooxygenase (AMO), the hydroxylamine oxidoreductase (HAO), and the accompanying cytochrome s have been most intensively studied in *N. europaea*, which has multiple copies of these primary nitrification genes (Enzymes involved in ammonia oxidation). *Nitrosomonas europaea* has a duplicated *amo* operon containing a continuous arrangement of the genes *amoC*, *amoA* and *amoB*, which are cotranscribed as a 3.5-kb mRNA and encode the three subunits of AMO, AmoC, AmoB and AmoA (McTavish et al., 1993; Klotz et al., 1997; Sayavedra-Soto et al., 1998). A third copy of *amoC*, which is not associated with the genes for the other subunits of this enzyme, has recently been identified (Sayavedra-Soto et al., 1998). Multiple *amo* operons also have been found in several other ammonia oxidizer s (Table 2). Furthermore, *N. europaea* has at least three copies of each of the genes coding for the hydroxylamine oxidoreductase (*hao*) and cytochrome c_{554} (*cycA* or *hcy*; McTavish et al., 1993; Hommes et al., 1994). Each copy of the *hao* gene is located 950 bp upstream of a copy of the *hcy* gene, but both genes are always found to be within different operons (Bergmann et al., 1994c; Sayavedra-Soto et al., 1994). Downstream of two of the *hcy* genes, an ORF (*cycB*) predicted to encode another tetraheme cytochrome *c* was detected (Bergmann et al., 1994c). The nucleic acid sequences of the multiple copies of all abovementioned genes (except for the unlinked *amoC* genes) are either identical or highly similar within a single ammonia oxidizer species, whereas much lower similarities occur between the respective genes of different species. Thus, it is likely that the multiple gene copies originated from relatively recent gene duplication events and were not caused by lateral gene transfer (Klotz and Norton, 1998). It has been speculated that the presence of multiple genes might 1) allow more-rapid generation of the respective mRNA during ammonia flushes within the local environment of the ammonia oxidizer s (Hommes et al., 1998) or 2) be responsible for maintaining a certain ratio of the gene products (Bergmann et al., 1994c).

In addition to those genes with products directly involved in ammonia oxidation, genes of *N. europaea* encoding the enolase (*eno*) and CTP synthase (*pyrG*) were sequenced (Mahony and Miller, 1998). The enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, and its gene was found to be linked on the chromosome with the *pyrG* gene, albeit both

Table 2. Number of *amo* operons and *amoC* copy numbers in different ammonia oxidizer s.

Organism	*amo* operon number	*amoC* copy number[a]
Nitrosomonas europaea ATCC 19178	2	3
Nitrosomonas eutropha C-91	2	3
Nitrosospira briensis C-128	3	4
Nitrosospira sp. NpAV	3	4
Nitrosolobus multiformis ATCC25196	3	4
Nitrosospira sp. 39-19	3	4
Nitrosovibrio tenuis NV-12	2	3
Nitrosococcus oceani	1	1
Nitrosococcus sp. C-113	1	1

[a]Several ammonia oxidizers contain in their genomes an additional *amoC* copy not linked to other *amo* genes. In *N. europaea*, the additional *amoC* copy has 60% nucleic acid sequence similarity to each of the other two *amoC* copies.
From Norton et al. (1996); Alzerreca et al. (1999), and data from GenBank.

genes are not cotranscribed. A similar arrangement of both genes is present in the *Escherichia coli* genome (where they are cotranscribed), though these genes are not linked in other investigated bacterial genomes.

Unfortunately, no sequence information regarding the genes involved in CO_2 fixation/carboxysome formation of the autotroph ic ammonia oxidizer s is currently available.

Regulation of AMO and HAO

One unusual feature of *N. europaea* is that it possesses multiple copies of those genes directly involved in ammonia oxidation. This is remarkable, since, with the exception of rRNA and tRNA genes, only relatively few cases of gene duplications have been described for bacteria (e.g., Hass et al., 1992; Sela et al., 1989; Tubulekas and Hughes, 1993; Kusian et al., 1995). The significance of the *N. europaea* ammonia oxidation genes being present in multiple copies has been investigated using techniques for transformation and insertional mutagenesis (Hommes et al., 1996; Hommes et al., 1998). Disruption of each of the two *amoA* copies showed that each copy was functional in *N. europaea* and that neither copy is essential in the cell. However, knockout of one of the *amoA* copies, but not of the other has a significant influence on the growth rates of the cells (Hommes et al., 1998), suggesting different regulation of each copy. Surprisingly, however, the putative σ^{70}-type promotors of both *amoA* genes were found to be identical (Hommes et al., 2001), indicating that the differential transcription of both genes (Hommes et al., 1998) involves regions upstream of the promotor where the DNA sequences of both copies diverge (Hommes et al., 2001). Similar results were obtained with cells carrying single mutations in each of the *amoB* genes (Stein et al., 2000). Insertional mutagenesis of each of the three *hao* gene copies, all of which possess σ^{70}-type promotors (Hommes et al., 2001), showed that none of them was essential and that their inactivation could be compensated fully by the two remaining *hao* genes (Hommes et al., 1996). However, owing to the presence of three *hao* gene copies, differences in their regulation might only become apparent after simultaneous inactivation of two of the copies.

Ammonia-oxidizing bacteria thrive in environments where ammonia is often present in very low concentrations. In these habitats, the capability to efficiently make use of temporal flushes of ammonia might represent an important selective advantage for an ammonia oxidizer. Therefore, the genetic and physiological responses of ammonia oxidizer s under conditions of ammonium limitation (ammonium present in amounts that can be metabolized to completion), of starvation (absence of ammonium), and in the presence of excess ammonium were intensively investigated. *Nitrosomonas cryotolerans* and *N. eutropha* survive ammonia starvation for at least 25 weeks (Jones and Morita, 1985) and one year, respectively (Pinck et al., 2001). In contrast to energy-starved heterotrophic bacteria, *N. cryotolerans* cells after 10 weeks of starvation 1) do not miniaturize, 2) maintain stable levels of intracellular ATP, and 3) show no changes in the total protein, DNA or RNA levels (Johnstone and Jones, 1988). Furthermore, quantitative FISH demonstrated that ammonia oxidizer s in activated sludge maintain relatively stable cellular rRNA concentrations during starvation for one month or inhibition with allylthiourea for several days (Wagner et al., 1995; Morgenroth et al., 2000). During prolonged starvation for several months or years ammonia oxidizer s lose ammonia-oxidizing activity but still contain significant amounts of AMO inasmuch as this enzyme is degraded more slowly in comparison to the mean cellular protein (Pinck et al., 2001). Under conditions of ammonia starvation, the mRNA of the *amo* gene disappears within 8 hours, though the ammonia and hydroxylamine oxidation activities do not change over a period of 24 h (Stein and Arp, 1998a). Limiting ammonium concentrations results in a large loss of ammonia-oxidizing activity (85%) after 24 h, but it neither affects the steady-state levels of *amoA* mRNA nor the result in degradation of the AmoA subunit (Stein and Arp, 1998a). Interestingly, short-chain alkanes and other substrates having a high binding affinity for AMO ameliorate the inactivating effects of ammonia limitation by protecting the

energy-generating activity of *N. europaea* from potentially toxic by-products of its metabolism (Stein and Arp, 1998a, b; Ammonia and hydroxylamine as Substrates). Interestingly, *N. europaea* cells grown in biofilms recover much faster after ammonium starvation than their planktonic counterparts. Preliminary data suggest that this phenomenon might be caused by cell-to-cell communication via *N*-(3-oxohexanoyl)-L-homoserine lactone (Batchelor et al., 1997). As expected, ammonium/ammonia induces the transcription of the ammonia monooxygenase and hydroxylamine oxidoreductase genes as well as the transcription of several additional genes that were not further characterized in *Nitrosomonas europaea* (Sayavedra-Soto et al., 1996). Furthermore, the activity of AMO is regulated by the presence of ammonia at translational (Hyman and Arp, 1995; Stein et al., 1997) and posttranslational (Stein et al., 1997) levels.

Biochemistry of Nitrite-Oxidizing Bacteria

The second step of nitrification, the oxidation of nitrite to nitrate, is performed by nitrite-oxidizing bacteria. Although at least four different genera of nitrite oxidizer s exist in nature (Phylogeny of nitrite oxidizer s), most of our knowledge on the physiology and biochemistry of these organisms stems from research on *Nitrobacter* species and thus cannot be generalized for all nitrite oxidizer s.

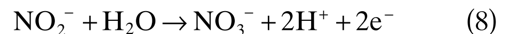
The key enzyme of nitrite-oxidizing bacteria is the membrane-bound nitrite oxidoreductase (Tanaka et al., 1983), which oxidizes nitrite with water as the source of oxygen to form nitrate (Aleem et al., 1965b). The electrons released from this reaction are transferred via *a*- and *c*-type cytochrome s to a cytochrome oxidase of the aa_3-type. However, the mechanism of energy conservation in nitrite oxidizer s is still unclear. Neither Hollocher et al. (1982) nor Sone et al. (1983) were able to find an electron transport chain linked to proton translocation in nitrite-oxidizing cells of *Nitrobacter winogradskyi*. The first product of energy conservation was shown to be NADH and not ATP (Sundermeyer and Bock, 1981).

Except for *Nitrobacter*, all other isolated nitrite oxidizer s are obligate lithotrophs with nitrite serving as the only energy source. Although many strains of Nitrobacter are able to grow heterotrophically, growth is very inefficient and slow (Smith and Hoare, 1968; Bock, 1976). Additionally, inorganic substrates other than nitrite, namely nitric oxide, can be used for lithotrophic growth, indicating metabolic diversity among *Nitrobacter* species (Freitag et al., 1987).

In anoxic environments, *Nitrobacter* cells are able to grow by denitrification (Freitag et al., 1987; Bock et al., 1988). Nitrate can be used as acceptor for electrons derived from organic compounds to promote anaerobic growth. Since the oxidation of nitrite is a reversible process, the nitrite oxidoreductase can reduce nitrate to nitrite in the absence of oxygen (Sundermeyer-Klinger et al., 1984). Furthermore, the nitrite oxidoreductase copurifies with a nitrite reductase that reduces nitrite to nitric oxide (Ahlers et al., 1990).

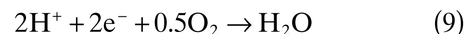
Nitrite as a Substrate

The utilization of nitrite as an energy source has been the subject of several reviews (Wood, 1986; Hooper, 1989; Bock et al., 1991; Yamanaka et al., 1981; Tanaka et al., 1983; Sundermeyer-Klinger et al., 1984; Fukuoka et al., 1987). Nitrite is oxidized to nitrate and the oxygen atom in the nitrate molecule is derived from water (Aleem, 1965a; Kumar et al., 1983; Hollocher, 1984) according to equation (8).



$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (8)$$

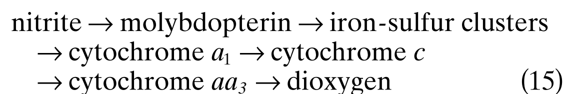
The two electrons released are transported to oxygen, as described in equation (9).



$$2H^+ + 2e^- + 0.5O_2 \rightarrow H_2O \quad (9)$$

The produced nitrate is inhibitory for *Nitrobacter* species at concentrations between 30 and 65 mM, probably owing to feedback inhibition.

The electron flux from nitrite to oxygen could pass the following electron carriers (Bock et al., 1992):



$$\text{nitrite} \rightarrow \text{molybdopterin} \rightarrow \text{iron-sulfur clusters} \rightarrow \text{cytochrome } a_1 \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } aa_3 \rightarrow \text{dioxygen} \quad (15)$$

Enzymes Involved in Nitrite Oxidation

NITRITE OXIDOREDUCTASE Nitrite oxidation is a reversible process. The enzyme nitrite oxidoreductase (NO_2-OR) catalyzes the oxidation of nitrite to nitrate and the reduction of nitrate to nitrite (Genetics of nitrite oxidizer s). The NO_2-OR is an inducible membrane protein present in Nitrobacter cells, which are either grown lithotrophically with nitrite or heterotrophically in the presence of nitrate. Depending upon the enzyme isolation technique, the molecular features of NO_2-OR vary considerably. Cytochrome s of the *a*- and *c*-type were present when the enzyme of *Nitrobacter wino-*

gradskyi was solubilized with Triton X-100 and purified by ion exchange and size exclusion chromatography (Tanaka et al., 1983). The purified protein was composed of three subunits of 55, 29 and 19 kDa. Cytochrome a_1 and c were also found when *n*-octylglycoside was chosen as detergent. However, using sodium deoxycholate and subsequent isolation by sucrose gradient centrifugation, only cytochrome c could be detected (Sundermeyer-Klinger et al., 1984). In this preparation, the holoenzyme of *Nitrobacter hamburgensis* consisted of three subunits with relative weights of 116–130, 65 and 32 kDa. No cytochrome s were found when the NO_2-OR was isolated from membranes by heat treatment. In this case, only two subunits of 115–130 and 65 kDa were present for *Nitrobacter winogradskyi*, *Nitrobacter vulgaris* and for *Nitrobacter hamburgensis* (Bock et al., 1990).

All preparations of the NO_2-OR contain molybdenum (Mo) and iron-sulfur clusters. In membranes of *Nitrobacter winogradskyi*, signals attributed to molybdenum were detected by electron proton resonance spectroscopy (Ingledew and Halling, 1976). In isolated NO_2-OR, molybdenum occurred in the form of molybdopterin (Krüger et al., 1987). The molybdenum content varied between 0.13 (Sundermeyer-Klinger et al., 1984) and 1.4 γ-atoms per molecule (Fukuoka et al., 1987). This difference can probably be explained by the fact that molybdenum often is lost during the enzyme isolation procedure. Molybdenum is essential for nitrite oxidation, and when it is replaced by tungsten, lithoautotrophically growing cells of *Nitrobacter hamburgensis* are inhibited, whereas heterotrophically growing cells are not. Flavoproteins are absent in NO_2-OR preparations. When isolated with Triton X-100, manganese was found to be associated with the NO_2-OR (Tanaka et al., 1983). The pH optimum of the NO_2-OR for nitrite oxidation differs from that for nitrate reduction. Optimal pH for nitrite oxidation with ferricytochrome c_{550}, ferricyanide, or chlorate as oxidants is about 8.0. With reduced methyl or benzyl viologen as reductants, the optimal pH for nitrate reduction ranges from 6.0 to 7.0. The apparent K_m value for nitrite oxidized by the NO_2-OR with the aid of different electron acceptor s varied with the test conditions between 0.5 and 2.6 mM (Tanaka et al., 1983) or 0.5 and 3.6 mM (Sundermeyer-Klinger et al., 1984), whereas the K_m value for nitrate amounted to about 0.9 mM.

It is important to note that the specific activities of NO_2-OR are influenced by the purification steps of the isolation procedure. As shown in Table 3, the nitrite oxidation activity and the nitrate reduction activity are highest in the membrane fraction. Both activities decrease to about 80% when NO_2-OR is isolated from membranes without detergent. If Triton X-100 or sodium deoxycholate is used for isolation, this effect is even more pronounced (Yamanaka and Fukumori, 1988; Sundermeyer-Klinger et al., 1984).

Table 3. Activity variations of the nitrite oxidoreductase isolated from mixotrophically grown cells of *Nitrobacter hamburgensis* depending on the isolation procedure.

	Specific activity (units)	
Fraction	NO_2^--oxidizing activity[a]	NO_3^--reducing activity[b]
Crude extract, 8,000 × g	1.728	2.101
Supernatant	2.338	1.839
Membranes	6.047	3.270
Membranes after heat treatment	2.582	4.882
Purified enzyme	2.506	1.740

[a]The unit of activity for NO_2^- oxidizing was determined with ClO_3^- as electron acceptor. One unit is defined as the oxidation of 1 μM nitrite per minute and per milligram of protein.
[b]The nitrate reductase activity was measured with reduced methyl viologen (MVH) as electron donor. One unit is defined as the reduction of 1 μM nitrate and per minute and per milligram of protein.

CYTOCHROME *c* OXIDASE In *Nitrobacter* species, absorption peaks at 605 nm in difference spectra indicate a cytochrome c oxidase of the aa_3-type. This membrane-bound enzyme was purified to an electrophoretically homogeneous state (Yamanaka et al., 1981; Sewell et al., 1972), and the function of cytochrome aa_3 was determined as a terminal oxidase by photoactivation of CO-inhibited oxygen consumption. In contrast to mitochondrial terminal oxidases, cytochrome aa_3 of *Nitrobacter winogradskyi* is composed of two subunits with 40 and 27 kDa in a molar ratio of l:l (Yamanaka et al., 1979). One molecule of the enzyme contains two molecules of heme a, two atoms of copper, one atom of magnesium but no zinc (Yamanaka and Fukumori, 1988). The K_m values were estimated to be 110 and 24 μM for horse heart cytochrome c and ferricytochrome c (both of which can serve as electron donors) of *Nitrobacter winogradskyi*, respectively. Phospholipids isolated from *Nitrobacter winogradskyi* did not stimulate the oxidation rate of native ferrocytochrome c or horse heart cytochrome c (Yamanaka and Fukumori, 1988). If cytochrome aa_3 was incorporated in phospholipid vesicles or membrane vesicles, respiratory control was observed, but proton-pumping activity was not (Sone et al., 1983; Sone, 1986).

NITRITE REDUCTASE In *Nitrobacter vulgaris*, a membrane-bound nitrate reductase (NiR) was copurified with the nitrite oxidoreductase (Ahlers et al., 1990). The NiR reduces nitrite to

nitric oxide, which is released under reduced oxygen partial pressure from the cells to the environment. Therefore, this enzyme seems to be a dissimilatory nitrite reductase of the denitrification type.

In the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of NO_2-OR and NiR, three bands are visible. In addition to the two proteins with M_r 115,000 and 65,000, which are constituents of the NO_2-OR, a third protein with M_r 63,000, possibly representing the NiR, is detectable. The pH optimum of the NiR was shown to be 6.1 and the K_m value for nitrite was 0.263 mM. The isoelectric point (IEP) was calculated to be at pH 5.5–6.0. Reduced horse heart cytochrome *c* can serve as an electron donor for nitrite reduction in *Nitrobacter winogradskyi* and *Nitrobacter vulgaris*. The biological function of NiR is difficult to understand, since ATP generation has not been detected during nitrite reduction (Freitag and Bock, 1990).

Electron Flow and Energy Transduction

As shown in Fig. 9, the first step, the electron transfer from nitrite to cytochrome a1 is catalyzed by the enzyme nitrite oxidoreductase. Cytochrome a_1 was shown to be necessary to channel electrons from nitrite to cytochrome *c* (Yamanaka and Fukumori, 1988). Cytochrome a_1 of *Nitrobacter winogradskyi* is not autoxidizable (Tanaka et al., 1983) and shows a typical absorption maximum at 589 nm. It is always found in nitrite-oxidizing and nitrate-reducing cells of all *Nitrobacter* species. On the other hand, *Nitrospira marina* does not possess any cytochrome of the *a*-type (Watson et al., 1986).

The electrons enter the underlying respiratory chain at the level of cytochrome *c* (Aleem, 1968; Cobley, 1976b; Aleem and Sewell, 1981; Sundermeyer and Bock, 1981; Tanaka et al., 1983). The reduction of cytochrome *c* is a thermodynamically unfavorable step, which is slow in cell-free extracts. Electrons derived from the nitrite/nitrate couple have a redox potential of $E_{m,7}$ = +420 mV, whereas those of ferrocytochrome *c*/ferricytochrome *c* have a potential of $E_{m,7}$ = +260 mV. A relatively high nitrite concentration would cause a lowering of the redox potential, but in natural habitats, high nitrite concentrations are rarely found (Schmidt, 1982). Actually, a highly active cytochrome aa_3 pushes nitrite oxidation by the removal of electrons from cytochrome *c*. In addition, the concentration of cytochrome aa_3 also varies dependent upon the oxygen concentration. SDS-PAGE experiments demonstrated that cells of Nitrobacter vulgaris grown under high oxygen partial pressure possess high nitrite-oxidizing activity and a high cytochrome aa_3 content, whereas those cells

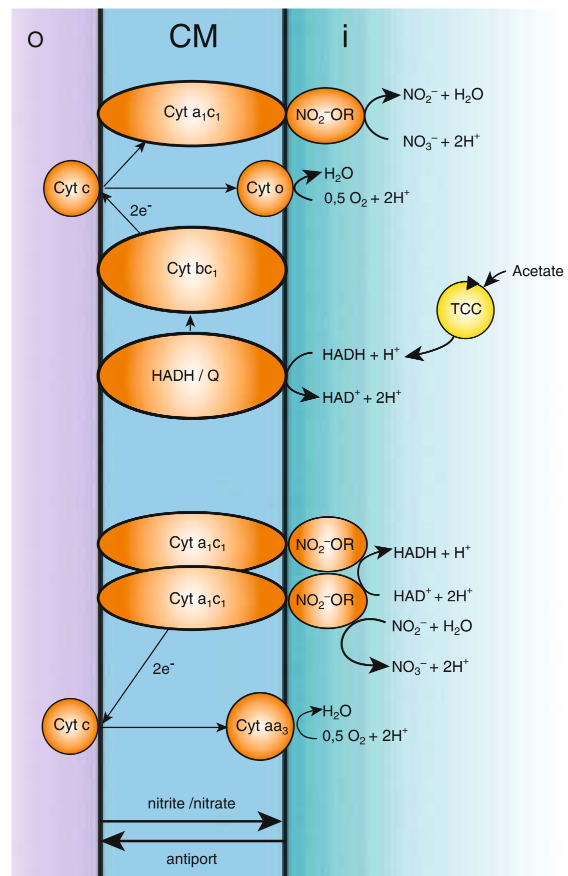


Fig. 9. Model of the electron flow in *Nitrobacter*. Depicted are the pathways of denitrification and heterotrophic growth (upper part) and the nitrification pathway (lower part). CM = cytoplasmic membrane, i = inside the cell/cytoplasmic space, o = outside of the cell/periplasmic space, NO_2-OR = nitrite oxidoreductase, and TCA = the tricarboxylic acid cycle. Figure was kindly provided by I. Schmidt.

grown under low oxygen tension have a low activity and a low cytochrome aa_3 content (E. Bock, unpublished observation).

The nitrite-oxidizing system of *Nitrobacter vulgaris* can be remodeled by reassociation of n-octylglycoside-isolated NO_2-OR with cytochrome aa_3. The activity of the nitrite-oxidizing system increased with increasing amounts of cytochrome *c* oxidase (Fig. 10). Present alone, NO_2-OR or cytochrome aa_3 was unable to oxidize nitrite to nitrate. The in vitro modeling of the nitrite-oxidizing system of *Nitrobacter vulgaris* shows clearly that both enzymes are essential for the oxidation of nitrite to nitrate, with oxygen as the terminal electron acceptor. At a fixed NO_2-OR content, the enzyme activity is regulated by the concentration of cytochrome aa_3.

In addition to oxygen, CO_2 can serve as electron sink in *Nitrobacter*. Cytochrome *c* oxidation

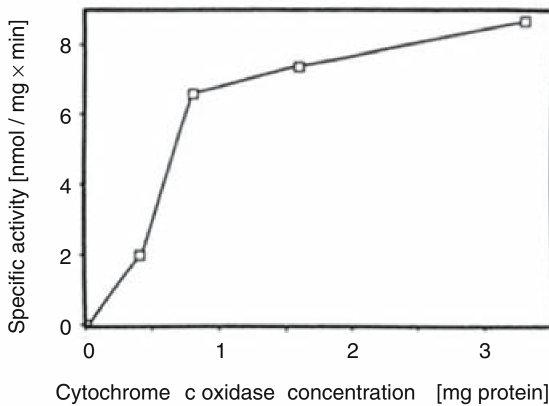


Fig. 10. Increase of the specific nitrite-oxidizing activity in cell-free enzyme preparations of *Nitrobacter vulgaris*. Isolated nitrite oxidoreductase was complemented with increasing amounts of cytochrome oxidase (aa_3) for 20 h at 28°C. The specific activity was measured as nitrite oxidized to nitrate with oxygen as the electron acceptor.

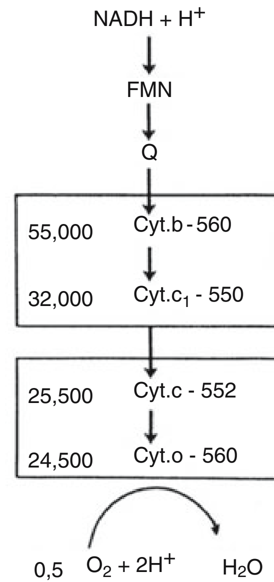


Fig. 11. Components of the conventional respiratory chain of *Nitrobacter*. The blocks symbolize proteins and their subunits in M_r.

generates energy that is necessary for autotroph ic carbon dioxide fixation. Since lithoautotrophic growth is inefficient, 85–115 mol of nitrite have to be oxidized to assimilate 1 mol of carbon dioxide (Bömecke, 1954).

Electron Flow of the Conventional Respiratory Chain The electrons from nitrite meet the underlying respiratory chain at the level of cytochrome *c* (not shown in Fig. 9). This chain functions in lithotrophically, mixotrophically, and heterotrophically growing cells as well as in endogenous respiring cells of *Nitrobacter* in the absence or presence of oxygen (Fig. 11). Electrons from NADH + H^+ (NADH) pass via flavin mononucleotide (FMN) and ubiquinone to a cytochrome bc_1-complex and finally to the terminal oxidase.

The responsible NADH oxidase has not yet been isolated. However, FMN was found to be present in heterotrophically grown *Nitrobacter* cells (Kirstein et al., 1986). Ubiquinone Q_{10} was the isoprenoid in the respiratory chain (Aleem and Sewell, 1984).

The cytochrome bc_1 is supposed to consist of a cytochrome b_{560} (55 kDa) and cytochrome *c*1-550 (32 kDa; M. Rudert, unpublished observations). The terminal oxidase (presented in Fig. 11) is a protein complex isolated from *Nitrobacter vulgaris* cells (M. Rudert, unpublished observations) consisting of a 25-kDa cytochrome c_{552} and a 24.5-kDa cytochrome *o*. This oxidase is assumed to be active at high oxygen partial pressure and is also present in lithotrophically grown cells of *Nitrobacter winogradskyi* (Aleem and Sewell, 1984) as well as in heterotrophically grown cells of *Nitrobacter hamburgensis* (Kirstein et al., 1986). At low oxygen tension, cytochrome aa_3 might perform this function (Fig. 9).

Additional electron carriers have been described by different authors. Kurokawa et al. (1987) isolated a flavin adenine dinucleotide-containing flavoenzyme from lithotrophically grown cells of *Nitrobacter winogradskyi* with NAD(P)H cytochrome *c* reductase and transhydrogenase activities. Two cytochrome s of the *b*-type, b_{560} and b_{564}, were found in *Nitrobacter hamburgensis*. Cytochrome b_{560} is typical for heterotrophically grown cells and might belong to the bc_1 complex (Kirstein et al., 1986). The function of cytochrome b_{564} is unknown. Several additional membrane-bound and soluble cytochrome s of the *c*-type have been described (Chaudhry et al., 1981; Miller and Wood, 1982). As reported by Tanaka et al. (1982) and Yamanaka et al. (1982), the amino acid composition of a soluble cytochrome *c* of *Nitrobacter winogradskyi* is similar to the mitochondrial cytochrome *c*.

ATP Production A generally accepted concept for the mechanism of energy generation derived from the described electron flow system is not available. Cobley (1976a) reported proton release into the cytoplasm, whereas Wetzstein and Ferguson (1985) detected proton extrusion into the periplasmic space coupled to oxidation of nitrite with artificial electron donors. However, proton pumping activity able to drive a membrane-bound ATPase could neither be mea-

sured for nitrite-oxidizing cells nor for nitrite-oxidizing vesicles (Hollocher et al., 1982).

Apart from the oxidation of exogenous organic substrates, *Nitrobacter* cells can oxidize endogenous material, e.g., poly-β-hydroxybutyrate; this metabolic activity is called "endogenous respiration." It has been shown that both the oxidation of exogenic and endogenic matter cause electron flow via a "normal" respiratory chain. Thus, nitrite-oxidizing *Nitrobacter* can be considered as a regulatory specialist because nitrite oxidation interferes with normal respiration, e.g., nitrite oxidation might inhibit endogenous respiration (Eigener and Bock, 1975). Changing from endogenous respiration to nitrite oxidation, active cells increased their ATP pool to a maximum of 1 mol of ATP by the oxidation of 1 of mol nitrite (Aleem, 1968). All attempts to reproduce this result have failed, but in whole cells and in membrane vesicles, ATP was formed at the expense of NADH oxidation with nitrate (Kiesow, 1964; Freitag and Bock, 1990) and/or oxygen as electron acceptor (Sewell and Aleem, 1979).

In *Nitrobacter*, phosphorylation of ADP is carried out by a membrane-bound ATP synthase. Isolated *Nitrobacter* ATPase is similar to the F1-ATPase from a thermophilic bacterium (Yamanaka and Fukumori, 1988). With respect to ATP production, *Nitrobacter* might be best described as a "normal" respiring organism, but this does not explain why heterotrophic growth is so slow.

NADH PRODUCTION AND CELL GROWTH Lithotrophically grown cells of *Nitrobacter winogradskyi* and *Nitrobacter vulgaris* possess an average poly-β-hydroxybutyrate (PHB) content of 10–30% of the cell dry weight (E. Bock, unpublished observations). This relatively high content indirectly indicates overproduction of NADH. Kiesow (1964) demonstrated in vivo NADH synthesis in nitrite-oxidizing cells by measuring the increase in extinction at 340 nm. Repeating these experiments, we also found NADH formation but only at low oxygen partial pressure. The reaction was sensitive to the uncoupler 2,4-dinitrophenol and insensitive to the ATPase inhibitor *N,N′*-dicyclohexyl carbodiimide (DCCD; Freitag and Bock, 1990).

In Fig. 12, the classical scheme of reverse electron flow for generation of NADH is shown. The functional models proposed by Wood (1986) and Hooper (1989) leave many questions unanswered. For example, the authors cannot explain why nitrite-oxidizing cells or spheroplasts of *Nitrobacter winogradskyi* do not produce a proton gradient, which is necessary to understand reverse electron flow (Hollocher et al., 1982). As stated above, nitrite-oxidizing bacteria are able

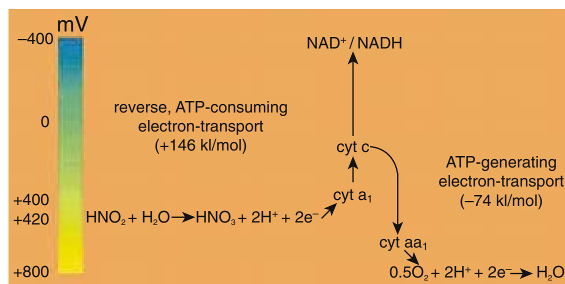


Fig. 12. Classical scheme of ATP-dependent NADH synthesis in nitrite-oxidizing cells of *Nitrobacter*.

to grow with nitrite, although the electron transfer from nitrite to cytochrome *c* is more electronegative than the nitrite/nitrate couple (Aleem, 1968; Ferguson, 1982). In spite of the existing unfavorable redox potential of cytochrome *c*, the electrons released from nitrite are promptly removed by cytochrome aa_3, so that nitrite oxidation can proceed without energy consumption (O'Kelley et al., 1970). As shown by Cobley (Cobley, 1976a; Cobley, 1976b), the membrane potential has a stimulatory effect on the rate of nitrite oxidation. In experiments with whole cells and membrane vesicles, the nitrite-oxidizing activity decreased in the presence of uncouplers, which collapsed the membrane potential. Thus, even the loss of activity of the isolated NO_2-OR might be caused by the loss of the transmembrane electric field, which mediates a conformation transition between an "inactive" and an "active" form of the enzyme (Tsong and Astumian, 1987).

Sundermeyer and Bock (1981) were the first to present evidence that NADH synthesis is the primary energy-conserving process in nitrite-oxidizing cells. In addition to nitrite, nitric oxide was shown to be a suitable electron donor for NADH synthesis (Freitag and Bock, 1990). Figures 13 and 14 show the formation of NADH (increase in absorption at 340 nm) in whole cells of *Nitrobacter winogradskyi*. When nitrite was added to aerobic cell suspensions, the dissolved oxygen tension dropped within 5 min to less than 4% of saturation. As shown in Fig. 13, the NADH pool of the cells first decreased for 5 min and then increased at a constant rate. When nitrite was added to anaerobic cell suspensions, the NADH content increased without any lag phase (Fig. 14). As shown in the figures, the rates of NADH formation with nitric oxide as substrate were faster than those with nitrite. Compared to $E_{m,7}$ = +420 mV for the nitrite/nitrate couple, the redox potential of the nitric oxide/nitrite couple is $E_{m,7}$ = +374 mV (Wood, 1978), if water is the reactant. Thermodynamically, there is no great difference between the two reactions;

nevertheless, nitric oxide was the better substrate than nitrite when *Nitrobacter winogradskyi* was grown lithoautotrophically.

It is generally accepted that NADH generation in *Nitrobacter* cells is an ATP-independent reaction as shown for *Thiobacillus ferrooxidans* (Lu and Kelly, 1988). But it is still unclear how NADH is synthesized.

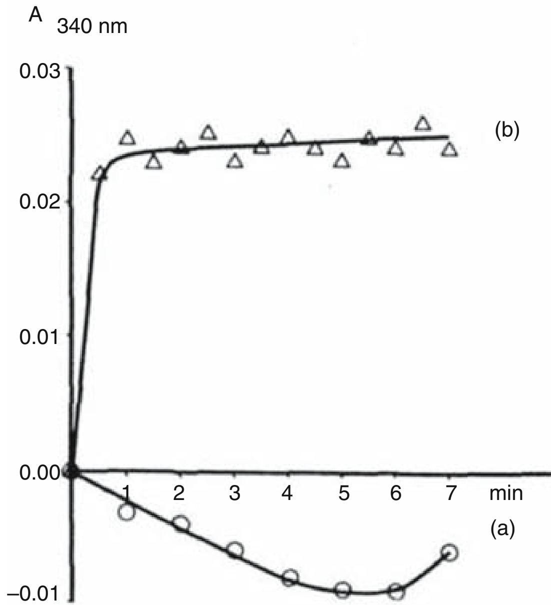


Fig. 13. NADH formation in whole cells of *Nitrobacter winogradskyi* in the presence of nitrite (a) and nitric oxide (b) under oxic conditions. NADH production was measured as the increase in absorption at 340 nm.

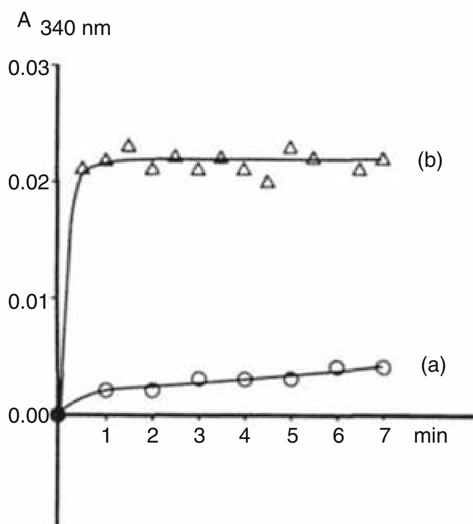


Fig. 14. NADH formation in whole cells of *Nitrobacter winogradskyi* in the presence of nitrite (a) and nitric oxide (b) under anoxic conditions. NADH production was measured as the increase in absorption at 340 nm.

Genetics of Nitrite Oxidizer s

With exception of the 16S rRNA genes (Sorokin et al., 1998; Orso et al., 1994; Teske et al., 1994; Ehrich et al., 1995), no genetic data are available for nitrite-oxidizing bacteria other than *Nitrobacter* (Phylogeny of nitrite oxidizers). For *Nitrobacter* species, sequence of the 16S-23S rRNA intergenic spacer and partial sequences of the 23S rRNA genes have been determined (Grundmann et al., 2000). Furthermore, the genes encoding the two catalytic core subunits of cytochrome *c* oxidase of *Nitrobacter winogradskyi* occur in the same operon. Similar to many ammonia-oxidizing bacteria, *N. winogradskyi* possess at least two copies of these genes in its genome (Berben, 1996). In addition, the sequences of the Calvin cycle genes were determined for *Nitrobacter vulgaris* (Strecker et al., 1994) and *Nitrobacter winogradskyi* (GenBank accession numbers {AF109915} and {AF10 9914}). In *Nitrobacter vulgaris*, the genes for the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase, the glyceraldehyde-3-phosphate dehydrogenase and a regulatory protein of the LysR family are located together within one cluster. Another cluster contains the genes for fructose-1,6- and sedoheptulose-1,7-biphosphatase, phosphoribulokinase, and fructose-1,6- and sedoheptulose-1,7-bisphosphatealdolase. Furthermore, the genes for both subunits (*norA* and *norB*) of the membrane-bound nitrite oxidoreductase (NO_2-OR) from *Nitrobacter hamburgensis* were sequenced. These genes cluster together with an additional ORF (*norX*) in the order *norA*, *norX* and *norB*. The deduced amino acid sequence of protein NorB contains four cysteine clusters with striking homology to those of iron-sulfur centers of bacterial ferredoxins. The β-subunit and the sequenced part of the α-subunit of NO_2-OR exhibit significant sequence similarities with the β- and α-subunits of the two dissimilatory nitrate reductases of several chemo-organotrophic bacteria including *E. coli* (Kirstein and Bock, 1993). This is consistent with biochemical data that suggest a close functional similarity between both enzyme complexes (Sundermeyer-Klinger et al., 1984; Hochstein and Tomlinson, 1988).

Heterotrophic Nitrification

The oxidation of ammonia (van Niel et al., 1993), hydroxylamine (Ralt et al., 1981) or organic nitrogen compounds, e.g., oximes (Castignetti and Hollocher, 1984), to nitrite and nitrate by various chemo-organotrophic microorganisms is called "heterotrophic nitrification." Heterotrophic nitrification is a cometabolism that is

not coupled to energy conservation (Wood, 1988b). Thus, growth of all heterotrophic nitrifiers is completely dependent on the oxidation of organic substrates (Focht and Verstraete, 1977; Kuenen and Robertson, 1987). The final product of heterotrophic nitrification often is nitrite (Castignetti and Gunner, 1980), so that heterotrophic nitrification may supply the substrate for lithotrophic nitrite oxidizer s and heterotrophic denitrifiers. This additional nitrite production (together with the ability of nitrite oxidizer s to grow chemo-organotrophically) might explain why in many environments the number of lithoautotrophic nitrite oxidizer s is much higher than that of lithoautotrophic ammonia oxidizer s (Kuenen and Robertson, 1987).

Recently, attention has been driven to heterotrophic nitrifiers because many of them are capable of aerobic denitrification in the presence of organic matter, leading to the complete elimination of dissolved nitrogen compounds with the formation of gaseous nitrogen oxides and/or dinitrogen gas (Castignetti and Hollocher, 1984; Robertson et al., 1989; Andersson and Levine, 1986a; van Niel et al., 1987). Owing to the simultaneous nitrifying and denitrifying activity, nitrification rates of heterotrophic nitrifiers are often underestimated (Castignetti and Hollocher, 1984; Kuenen and Robertson, 1987). For example, *Paracoccus denitrificans* (formerly called *Thiosphaera pantotropha*) produces nitrite from urea, ammonia and hydroxylamine and is also able to reduce nitrite even under aerobic conditions (Robertson and Kuenen, 1983; Robertson and Kuenen, 1984; Robertson and Kuenen, 1988). Therefore, in cultures of this organism, nitrite only accumulates when the nitrite reductase activity is repressed.

Biochemically, the ammonia-oxidizing enzyme of *Paracoccus denitrificans* shows some similarities to the AMO of lithotrophic ammonia oxidizer s, e.g., the ability to oxidize alkanes, the apparent requirement for copper, and inhibition by light, diethyldithiocarbamate and allylthiourea (Moir et al., 1996a; Crossmann et al., 1997). The purified ammonia-oxidizing enzyme of *P. denitrificans* contains two polypeptides of 38 and 46 kDa, respectively (Moir et al., 1996a). However, the genes encoding for these polypeptides are not closely related to the *amo*-genes of lithotrophic ammonia oxidizer s (Crossmann et al., 1997). The hydroxylamine oxidoreductase from *P. denitrificans* is a monomeric protein of approximately 18.5 kDa containing nonheme-iron (Wehrfritz et al., 1993; Moir et al., 1996b).

The environmental importance of heterotrophic nitrifiers is controversial in the literature. Generally it is assumed that in most environments, the biological conversion of reduced forms of nitrogen to nitrite and nitrate is catalyzed mainly by the lithoautotrophic ammonia- and nitrite-oxidizing bacteria and not by heterotrophic nitrifiers. This reflects that the nitrification rates of heterotrophic nitrifiers are small compared to those of autotroph ic nitrifiers (Robertson and Kuenen, 1988). Therefore, heterotrophic nitrification was thought to occur preferentially under conditions unfavorable for autotroph ic nitrification, e.g., in acidic environments (Killham, 1986). In such environments, heterotrophic bacteria, fungi and even some algae might contribute considerably to nitrification (Schimel et al., 1984; Killham, 1986; Killham, 1987; Robertson and Kuenen, 1990; Spiller et al., 1976). But according to recent reports, even in acidic soils, heterotrophic nitrification contributes to overall nitrate production only to a minor extent (Stams et al., 1990; Barraclough and Puri, 1995).

Novel Aspects

Nitrogenous Oxides Are Essential for Aerobic Ammonia Oxidation

Nitrifying as well as denitrifying bacteria contribute to the net production of nitrogenous oxides from soil (Kester et al., 1996; Kester et al., 1997a, b) and from aquatic environments (Xu et al., 1995). This is noteworthy since the gaseous compounds nitric oxide (NO), nitrogen dioxide (NO_2) and nitrous oxide (N_2O) are of significance for the chemistry of the atmosphere (Johnston, 1972; Crutzen, 1979; Galbally and Roy, 1983). Additionally, nitrous oxide acts as a greenhouse gas (Wang et al., 1976; Andersson and Levine, 1986a). Furthermore, nitric oxide and to a more moderate extent nitrogen dioxide (Mancinelli and McKay, 1983) have strong inhibitory effects on bacteria (Mancinelli and McKay, 1983; Shank et al., 1962). Toxicity of nitric oxide is based on its capability to form metal nitrosyl complexes (mainly with heme proteins, iron-sulfur proteins, and copper-containing proteins; Henry et al., 1991), resulting, for example, in the inhibition of cytochrome oxidases (Carr and Ferguson, 1990). Furthermore, nitric oxide was shown to form S-nitrosothiols from sulfhydryl-groups (Stammler et al., 1992; Hausladen et al., 1996) and to cause $C \rightarrow T$ transitions in the DNA (Wink et al., 1991).

To protect themselves from toxic effects of nitric oxide many bacteria possess detoxifying enzymes. Denitrifying organisms produce nitric oxide by the activity of the nitrite reductase but are able to keep the internal nitric oxide concentration low by reducing it to nitrous oxide via the NO-reductase. Thus, these organisms are

strongly dependent on a close functional coupling between both enzymes (Zumft, 1993). Consistent with this finding, loss of NO-reductase is lethal for denitrifying cells of *Pseudomonas stutzeri* (Braun and Zumft, 1991). Other organisms, for example *Pseudomonas* strain PS88, which do not possess the ability to denitrify, are able to detoxify nitric oxide by means of oxidative processes that convert it to nitrate (Baumgärtner et al., 1996; Hausladen et al., 1996; Koschorreck et al., 1996).

For ammonia-oxidizing bacteria under oxic conditions, NO is also toxic but only in the absence of ammonia (E. Bock, unpublished observation). If ammonia is present, NO and/or NO_2 are even essential for aerobic ammonia oxidation. Therefore, the removal of nitric oxide (NO) from cultures of *Nitrosomonas eutropha* by intensive aeration leads to inhibition of ammonia oxidation. This phenomenon has implications on batch-cultivation of ammonia oxidizer s in the laboratory (Zart and Bock, 1998). Usually, it is necessary to avoid intensive aeration or stirring during the first days of incubation, otherwise, the cells will grow very slowly or even not at all. It was possible to achieve recovery of ammonia oxidation by adding nitric oxide to the air supply. The grade of recovery was dependent on the concentration of nitric oxide supplied (Zart et al., 2000). Inhibition of ammonia oxidation was also observed, when nitric oxide was removed from nitrifying cells of *N. eutropha* by means of DMPS (2,3-dimercapto-1-propane-sulfonic acid) in the presence of Fe^{3+} ions. The addition of nitric oxide lowered inhibition by DMPS. In another assay, ammonia oxidation of *Nitrosomonas eutropha* was inhibited by activity of the NO-detoxifying bacterium *Pseudomonas* PS88 (Baumgärtner et al., 1996; Koschorreck et al., 1996) and could be recovered by addition of NO or lowering the activity of the pseudomonad (Zart et al., 2000). The enhancing effect of nitric oxide and nitrogen dioxide on aerobic nitrification and cell growth of *Nitrosomonas eutropha* could also be demonstrated using fermenter cultures (Zart and Bock, 1998). As shown in Fig. 15, the specific activity of ammonia oxidation increased drastically in the presence of nitric oxide and even more if nitrogen dioxide was added instead of nitric oxide. *Nitrosomonas eutropha* was able to tolerate long exposure (up to 30 days) to nitrogen dioxide or nitric oxide at a concentration as high as 50 ppm when ammonia was oxidized. This is unusual since already 1 ppm nitric oxide is inhibitory for various chemo-organotrophic bacteria (Mancinelli and McKay, 1983). This experiment also shows that nitrite is not a potent inhibitor for *N. eutropha* because nitrite accumulated in this experiment to 100 mM. Thus, ammonia oxidation in

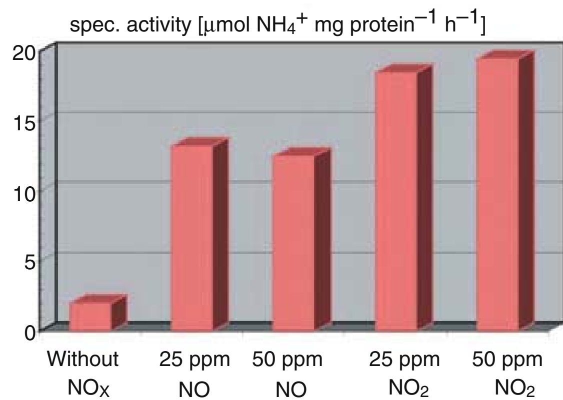


Fig. 15. Increase in specific activity of ammonia oxidation in fermenter cultures of *Nitrosomonas eutropha* upon addition of nitric oxide or nitrogen dioxide to the air supply.

N. eutropha was obviously not inhibited by nitrite accumulation as described for *N. europaea* (Anthonisen et al., 1976; Wullenweber et al., 1978; Drozd, 1980).

The increase of nitrification rates upon nitrogen dioxide or nitric oxide addition was partly due to a significant increase of cell density. In the presence of 50 ppm of nitrogen dioxide, it was possible to obtain up to 2×10^{10} cells ml^{-1} of *N. eutropha* (Zart and Bock, 1998), a cell density of ammonia oxidizer s, which to our knowledge has never been reported before. Generally, in batch cultures of different Nitrosomonas strains, a cell density of about 2×10^8 cells ml^{-1} is rarely exceeded (Engel and Alexander, 1958; Prosser, 1989). In continuous cultures of *N. europaea* with complete biomass retention, Tappe et al. (1996) obtained about 5×10^9 cells ml^{-1}. But it is not clear so far, how nitric oxide and nitrogen dioxide affect the maximum cell density of these organisms.

Beyond this, nitric oxide and nitrogen dioxide had an enhancing effect on the increase of cell number of *Nitrosomonas eutropha* (Zart and Bock, 1998). The specific growth rate (measured as increase in protein) slightly increased upon addition of nitrogen dioxide, but the fission rate increased in a much stronger way. Thus, it is obvious that cell growth was uncoupled from protein increase. Consequently, the cells were depleted of protein when growing in the presence of nitrogen dioxide. This is shown in Fig. 16 where the alteration of cell morphology of *Nitrosomonas eutropha* grown in presence of 50 ppm of nitrogen dioxide is depicted for a period of four weeks. Most striking is the reduction of cell material and the increase of electron dense inclusion bodies. These inclusion bodies were storage material and resembled glycogen-like particles of *Nitrospina gracilis* (Watson and Waterbury,

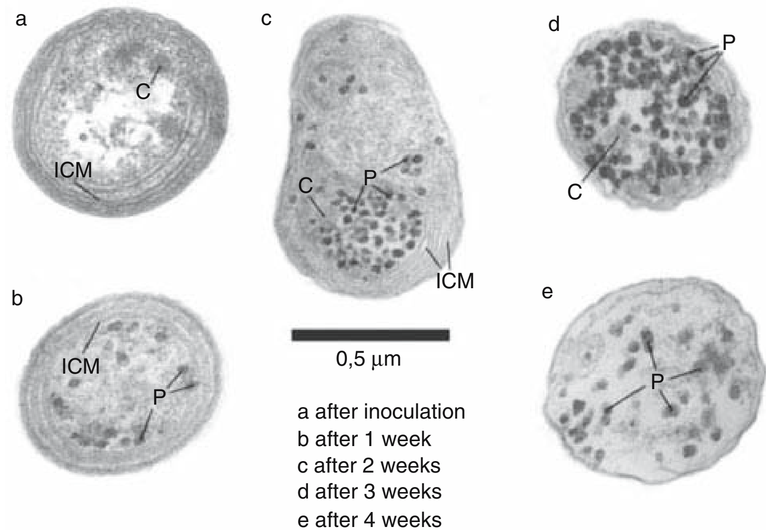


Fig. 16. Electron micrograph of ultrathin sections of cells of *Nitrosomonas eutropha* showing the morphological alteration of cells grown in a fermenter aerated with 50 ppm NO_2. The cells were harvested from the reactor after inoculation (a) and for one (b), two (c), three (d), and four weeks (e) of incubation. ICM = intracytoplasmic membranes, C = carboxysome, and P = unknown particles/ electron dense inclusion bodies.

1971b) and *Nitrosolobus multiformis* (Watson et al., 1971c). Surprisingly, reduction of the protein content per cell was accompanied by increased specific activity of ammonia oxidation.

Although nitrification and cell growth of ammonia oxidizer s were enhanced by adding nitric oxide or nitrogen dioxide to the air supply of the cultures, the cell yield (cell protein produced per mol of ammonia oxidized) and the energy efficiency slightly decreased. This finding might be due to the aerobic production of N_2 induced by the addition of NO_2 (Zart and Bock, 1998). More than 50% of the ammonia was oxidized to dinitrogen (N_2) and traces of nitrous oxide (N_2O) by *Nitrosomonas eutropha*. Increasing amounts of supplementary nitrogen dioxide resulted in increasing nitrogen losses (Zart and Bock, 1998). Previously, significant nitrogen losses were only obtained with extremely oxygen-limited cultures of ammonia-oxidizing bacteria (Bock et al., 1995; Zart et al., 1996).

Recently, it could be demonstrated that the addition of NO_2 to cells of *Nitrosomonas eutropha* grown first under anoxic conditions with hydrogen as electron donor and nitrite as electron acceptor (Schmidt and Bock, 1997) and then shifted to oxic conditions significantly reduced the lag for the initiation of ammonia oxidation (Schmidt et al., 2001b).

Formation of nitric oxide was up to now interpreted as formation of a by-product of ammonia oxidation without significance for the metabolism of ammonia oxidizer s. Inhibition of ammonia oxidation of *Nitrosomonas eutropha* upon withdrawal of nitric oxide from the culture medium indicates that the production of nitric oxide by ammonia oxidizer s seems to be not the formation of a “waste compound” but rather the provision of an important agent for the oxidation of ammonia (Zart et al., 1999). However, nitrogen dioxide rather than nitric oxide could represent the decisive agent, where the latter is acting as precursor for nitrogen dioxide. This hypothesis is based on reports of Schmidt and Bock (Schmidt and Bock, 1997; Schmidt and Bock, 1998) who described anaerobic oxidation of ammonia by *Nitrosomonas eutropha* using nitrogen dioxide as an oxygen donor in the AMO reaction. It seems not unlikely that nitrogen dioxide might be involved in the conversion of ammonia to hydroxylamine under oxic conditions as well. In such a case, nitrogen dioxide might act as cosubstrate for the AMO reaction. But since atmospheric nitrogen dioxide concentration hardly exceeds 800 ppb (Galbally and Roy, 1983; Baumgärtner, 1991), the cells would not be able to cover their requirements by consuming it just from the atmosphere. They might rather produce nitric oxide, which can be oxidized chemically to nitrogen dioxide with dioxygen (Bodenstein, 1918). Although the latter reaction proceeds predominantly in the gas phase (Ford et al., 1993; Wink et al., 1993), nitrogen dioxide-consuming reactions (Lewis and Deen, 1994) and low concentrations of nitric oxide in the liquid (Pires et al., 1994) can alter the development of the aqueous reaction, so that nitrogen dioxide might be produced in biological systems by oxidation of nitric oxide as it is in the gas phase (Huie, 1994).

Considering the finding of Dua et al. (1979) that up to 97% of the oxygen of hydroxylamine originates from molecular oxygen, it is important to note that oxidation of ammonia with nitrogen

dioxide leads to the formation of hydroxylamine and nitric oxide (Schmidt and Bock, 1997). Under oxic conditions, the latter might be reoxidized with dioxygen to form nitrogen dioxide, which would again be available for the oxidation of ammonia by the AMO. Thus, molecular oxygen would not react directly with ammonia but is hypothetically mediated by nitric oxide / nitrogen dioxide. Consequently, it is not necessary to provide nitric oxide or nitrogen dioxide and ammonia in the same ratio since the nitrogenous oxides are permanently recycled. In Fig. 17, a hypothetical model of this NO_x-cycle is depicted which refers to the three-stage catalytic cycle of the tyrosinase reaction (Shears and Wood, 1985). The AMO can have three oxidation states (Shears and Wood, 1985; Bedard and Knowles, 1989; Keener and Arp, 1993). As already mentioned, copper is a constituent of the enzyme and NO/N_2O_4 is involved in ammonia oxidation. Therefore it can be speculated that NO is a cofactor of the AMO. In accordance with Zart and Bock (1998), enzyme-bound N_2O_4 is the final cosubstrate for oxidizing ammonia to hydroxylamine. Dinitrogen tetroxide (N_2O_4) is formed from NO by oxidation with molecular oxygen. The AMO consists of 3 stages: 1) the deoxy-form (reduced), 2) the oxy-form (oxidized), and 3) the metoxy-form (oxidized). The first stage is oxygen-sensitive, the second and the third one are oxygen-stable. The oxygen sensitivity of the deoxy-form is caused by an excess of electrons that might lead to the formation of oxygen radicals, which directly or indirectly react with NO, forming peroxy nitrite (OONO). This compound is toxic and destroys the AMO. This working

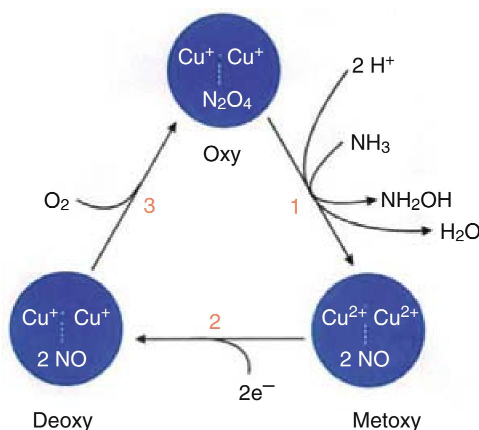


Fig. 17. Hypothetical model of an NO_x-mediated three-stage cycle driving the oxidation of ammonia to hydroxylamine in ammonia-oxidizing bacteria. This cycle does not contribute to the release of NO during aerobic ammonia oxidation (Stüven and Bock, 2001), which is probably caused by chemodenitrification of nitrite at the hydroxylamine oxidoreductase (HAO).

model is in accordance with the observation that purified AMO can oxidize ammonia to hydroxylamine with NADH (H^+) as electron donor under anoxic conditions with N_2O_4 as oxidant. Oxygen was shown to be inhibitory (E. Bock and C. Pinck, unpublished data). Copper in the active center might be responsible for noncovalently binding the nitrogen oxides in form of nitrosyl-complexes. As described, oxygen is reacting with NO of the deoxy-form (Fig. 17/3), transforming NO to N_2O_4 (oxy-form). The copper ions are oxidized from Cu^+ to Cu^{2+}. The N_2O_4-molecule of the oxy-form is the final oxygen-donor for ammonia oxidation to hydroxylamine. By this reaction, the enzyme is transferred to the metoxy-form (Fig. 17/1), which is subsequently converted by reduction to the deoxy-form, thereby completing the cycle (Fig. 17/2). This model also provides an explanation for the inhibition of ammonia oxidation after withdrawal of nitric oxide. As demonstrated recently acetylen inhibits the aerobic and not the anaerobic ammonia oxidation. Therefore acetylene is assumed to bind to the deoxyform of the AMO (Schmidt et al., 2001a). Additionally, the oxidation of ammonia with nitrogen dioxide /dinitrogen tetroxide ($DG0' = -140$ kJ $\cdot$ mol $^{-1}$) is thermodynamically more favorable than the "classical" oxidation of ammonia with dioxygen ($DG0' = -120$ kJ $\cdot$mol $^{-1}$).

Anaerobic Ammonia Oxidation Catalyzed by *Nitrosomonas eutropha*

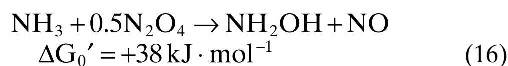
Anaerobic Ammonia Oxidation with Nitrogen dioxide In the absence of dissolved oxygen, ammonia oxidation and cell growth has been observed recently in cultures of *Nitrosomonas eutropha* (Schmidt and Bock, 1997). Those cells were able to replace molecular oxygen by nitrogen dioxide or dinitrogen tetroxide, respectively. Hydroxylamine and nitric oxide were formed in this reaction. While nitric oxide was not further metabolized, hydroxylamine was oxidized to nitrite. However, anoxic ammonia oxidation with nitrogen dioxide was more than tenfold slower than ammonia oxidation with oxygen as electron acceptor.

The activity of ammonia oxidizer s decreases when the oxygen concentration in the medium decreases too. This can be put down to the fact that the oxidant for ammonia oxidation was limited. Therefore, the organisms need another oxidant for anaerobic ammonia oxidation. For a long time, nitrite was discussed as a suitable oxidant (Broda, 1977), but as shown in numerous experiments, it could not serve as electron acceptor for anaerobic ammonia oxidation and cell growth in *Nitrosomonas* (Bock et al., 1995; Zart et al., 1996).

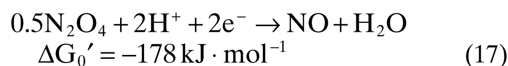
ANAEROBIC AMMONIA OXIDATION IN CELL-FREE EXTRACTS To reveal the stoichiometry of anaerobic ammonia oxidation, consumption and production of ammonia, nitrogen dioxide, nitric oxide, nitrite, nitrous oxide, and dinitrogen were analyzed in cell-free extracts of *N. eutropha*. Many attempts were performed to prepare active cell-free extracts of *Nitrosomonas europaea* under oxic conditions (Suzuki et al., 1970; Suzuki et al., 1981b). One of the most serious problems associated with the characterization of the AMO in extracts has been the instability of the enzyme activity (Suzuki et al., 1974; Suzuki et al., 1981b; Ensign et al., 1993) caused by the sensitivity of reduced AMO to oxygen (C. Pinck and E. Bock, unpublished observation). In contrast, the ammonia-oxidizing enzyme system is stable and active in cell-free extracts under anoxic conditions and thus allowed to characterize the anaerobic ammonia oxidation (Schmidt and Bock, 1998). In a helium atmosphere supplied with 25 ppm of nitrogen dioxide, ammonia and nitrogen dioxide were consumed in a ratio of approximately 1:2 by cell-free extracts of Nitrosomonas eutropha. The production of nitric oxide was closely related to the consumption of nitrogen dioxide. Nitric oxide was released in amounts nearly equimolar to the consumption of nitrogen dioxide. The production rate of nitrite was significantly lower than the oxidation rate of ammonia. It is assumed that nitrogen dioxide and nitrite served as acceptors for electrons derived from ammonia oxidation. Approximately 22% of the nitrite was converted into gaseous nitrogen compounds (nitrogen-loss). The main products of denitrification were dinitrogen and traces of nitrous oxide. During the anaerobic ammonia oxidation, hydroxylamine concentrations between 30 and 40 μM were measured. In control experiments with cell-free extracts of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), *Enterobacter aerogenes* and *Pseudonocardia nitrificans*, neither ammonia oxidation nor nitrogen dioxide consumption could be detected. In sterile control experiments, there was again no ammonia consumption. In addition, no formation of nitric oxide, dinitrogen or nitrous oxide was measurable.

At 25°C and atmospheric pressure, the ratio of nitrogen dioxide (NO_2) to its dimer dinitrogen tetroxide (N_2O_4) is 30:70. To decide whether nitrogen dioxide or dinitrogen tetroxide is the electron acceptor for anaerobic ammonia oxidation, experiments were performed at a temperature of 4°C, conditions under which the dinitrogen tetroxide concentration is almost 100%. Experiments were performed with crude cell-free extracts in the presence of hydrazine as specific inhibitor for HAO. Although the specific activity of the cell-free extracts decreased significantly at 4°C, anaerobic ammonia oxidation could be detected with a stoichiometry of the converted nitrogen-compounds comparable to those observed at 25°C. These results indicate that dinitrogen tetroxide can be used as electron acceptor for anaerobic ammonia oxidation.

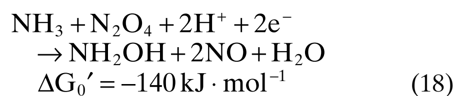
Based on the observed correlations between ammonia and nitrogen dioxide/dinitrogen tetroxide consumption and nitric oxide and hydroxylamine production, the following equations are proposed. Equations 16 and 17 describe the two half-reactions of the anaerobic ammonia oxidation. The total AMO reaction is presented in equation 18:



$$NH_3 + 0.5N_2O_4 \rightarrow NH_2OH + NO \quad \Delta G_0{}' = +38\,kJ \cdot mol^{-1} \tag{16}$$

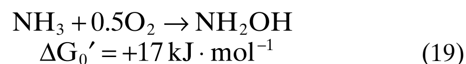


$$0.5N_2O_4 + 2H^+ + 2e^- \rightarrow NO + H_2O \quad \Delta G_0{}' = -178\,kJ \cdot mol^{-1} \tag{17}$$

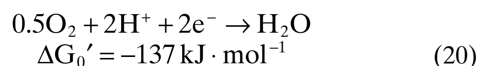


$$NH_3 + N_2O_4 + 2H^+ + 2e^- \rightarrow NH_2OH + 2NO + H_2O \quad \Delta G_0{}' = -140\,kJ \cdot mol^{-1} \tag{18}$$

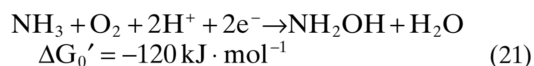
For comparison, the aerobic ammonia oxidation to hydroxylamine is given in equations 19 to 21.



$$NH_3 + 0.5O_2 \rightarrow NH_2OH \quad \Delta G_0{}' = +17\,kJ \cdot mol^{-1} \tag{19}$$



$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad \Delta G_0{}' = -137\,kJ \cdot mol^{-1} \tag{20}$$



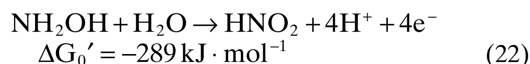
$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad \Delta G_0{}' = -120\,kJ \cdot mol^{-1} \tag{21}$$

The $\delta G_0{}'$ values of the reactions (17 and 20) were calculated under the assumption that the reducing equivalents for the AMO are energetically near the ubiquinone level (+110 mV). Equations (16) to (21) indicate that there are only a few differences between the anaerobic and the aerobic ammonia oxidation of *Nitrosomonas eutropha*. Instead of molecular oxygen in the course of aerobic ammonia oxidation, dinitrogen tetroxide was used as electron acceptor and nitric oxide, an additional product, was released.

It appears likely that the same enzyme is responsible for both the aerobic and the anaerobic ammonia oxidation, since 1) hydroxylamine is an intermediate of both reactions, 2) acetylene inhibits the aerobic as well as the anaerobic ammonia oxidation, and 3) anaerobic ammonia oxidation starts immediately after transferring cell-free extracts to anoxic conditions.

For the oxidation of hydroxylamine under oxic conditions, no oxygen is needed. Therefore, it can be assumed that under anoxic conditions,

hydroxylamine was oxidized according to reaction 22 as well.



$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad \Delta G_0{}' = -289\,kJ \cdot mol^{-1} \qquad (22)$$

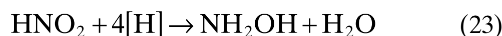
Increasing pool sizes of intracellular ATP and NADH indicated energy conservation in the absence of oxygen but presence of nitrogen dioxide (Schmidt and Bock, 1998). Under these conditions, reducing equivalents were also used for the reduction of carbon dioxide, resulting in cell growth and excretion of extracellular organic compounds like glycerol into the medium.

The relevance of the anaerobic ammonia oxidation with NO_2/N_2O_4 as oxidant for microbial ecology cannot be assessed at present. The nitrogen dioxide concentration of maximal 400–800 ppb in the atmosphere (Crutzen, 1979) should be too low to support anaerobic ammonia oxidation. However, this metabolism might occur in oxygen-limited zones, where locally restricted higher nitrogen dioxide concentrations might exist because of the reaction of molecular oxygen and nitric oxide (Nielsen, 1992).

Anaerobic Ammonium Oxidation Catalyzed by Deep Branching Planctomycetes

Recently, a novel organism was discovered which is capable of catalyzing the anaerobic oxidation of ammonium, with nitrite as electron acceptor (Strous et al., 1999). This organism, which up to now cannot be obtained in pure culture, was enriched from a denitrifying plant reactor where the anaerobic ammonium oxidation process was observed for the first time (Mulder et al., 1995; van de Graaf et al., 1995). By comparative 16S rDNA sequence analysis, the organism was identified as a novel, deep-branching member of the order Planctomycetales and the name "*Candidatus* Brocadia annamoxidans" was proposed (Jetten et al., 2001). The 16S rDNA-based molecular diversity surveys and subsequent fluorescence in situ hybridization analyses of several reactors and wastewater treatment plants with anaerobic ammonia-oxidizing activity demonstrated that at least two different genera that form a monophyletic lineage within the Planctomycetales (Fig. 18) can catalyze this process (Schmid et al., 2000; Schmid et al., 2001). In most plants analyzed so far, "*Candidatus* Kuenenia stuttgartiensis" and not "*Candidatus* Brocadia annamoxidans" is the most abundant anaerobic ammonium oxidizer (Schmid et al., 2000; Jetten et al., 2001).

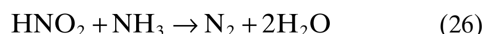
"*Candidatus* Brocadia annamoxidans" is a chemolithoautotrophic organism with a very low growth rate ($0.003\ h^{-1}$) and a conspicuous ultrastructure. It contains inside the cytoplasm so-called "anammoxosomes," membrane-bounded compartments that make up 30–60% of the cell volume and harbor an unusual hydroxylamine oxidoreductase (Jetten et al., 2001). The metabolic pathway of the so-called "ANAMMOX" process proposed for "*Candidatus* Brocadia annamoxidans" differs significantly from the known pathway of aerobic, lithotrophic ammonia oxidizer s, since ammonium is supposed to be oxidized with hydroxylamine to form hydrazine (N_2H_4), which is subsequently oxidized to dinitrogen, the main end product of the ANAMMOX process. The four reducing equivalents generated in this oxidation step are used for the initial reduction of nitrite to hydroxylamine (van de Graaf et al., 1997). Preliminary data suggest that the unusual hydroxylamine oxidoreductase of "*Candidatus* Brocadia annamoxidans" (Jetten et al., 2001), which has a smaller molecular mass than the respective enzyme of *Nitrosomonas* and contains several *c*-type cytochrome s, catalyzes the oxidation of hydrazine to dinitrogen. Anaerobic ammonia oxidation occurs only if the cell density of "*Candidatus* Brocadia annamoxidans" is higher than 10^{10} to 10^{11} cells ml^{-1}. The reason for this cell density dependent activity is not clear so far. The following equations (23–26) summarize the proposed pathway. The overall reaction resembles a process already proposed in 1977 based on theoretical considerations (Broda, 1977).



$$HNO_2 + 4[H] \rightarrow NH_2OH + H_2O \qquad (23)$$

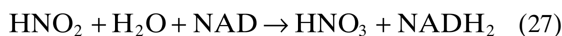
$$NH_2OH + NH_3 \rightarrow N_2H_4 + H_2O \qquad (24)$$

$$N_2H_4 \rightarrow N_2 + 4[H] \qquad (25)$$



$$HNO_2 + NH_3 \rightarrow N_2 + 2H_2O \qquad (26)$$

From equation 26, it is obvious that the overall reaction is balanced. No reducing power is gained which is essential for the fixation of carbon dioxide by autotroph ic organisms. To gain these reducing equivalents, the cells are required to oxidize nitrite to nitrate. Therefore, the amount of nitrite consumed is about 20% higher than one could expect from equation 26, and nitrate is additionally formed (van de Graaf et al., 1996):



$$HNO_2 + H_2O + NAD \rightarrow HNO_3 + NADH_2 \qquad (27)$$

The metabolic activity of "*Candidatus* Brocadia annamoxidans" is strongly inhibited by oxygen, phosphate, acetylene or shock loading. In addition, organic electron donors or high concentrations of nitrite are inhibitory (van de Graaf et al., 1996).

"*Candidatus* Brocadia annamoxidans" oxidizes ammonium anaerobically about 50-fold faster than *Nitrosomonas eutropha* with 25 ppm

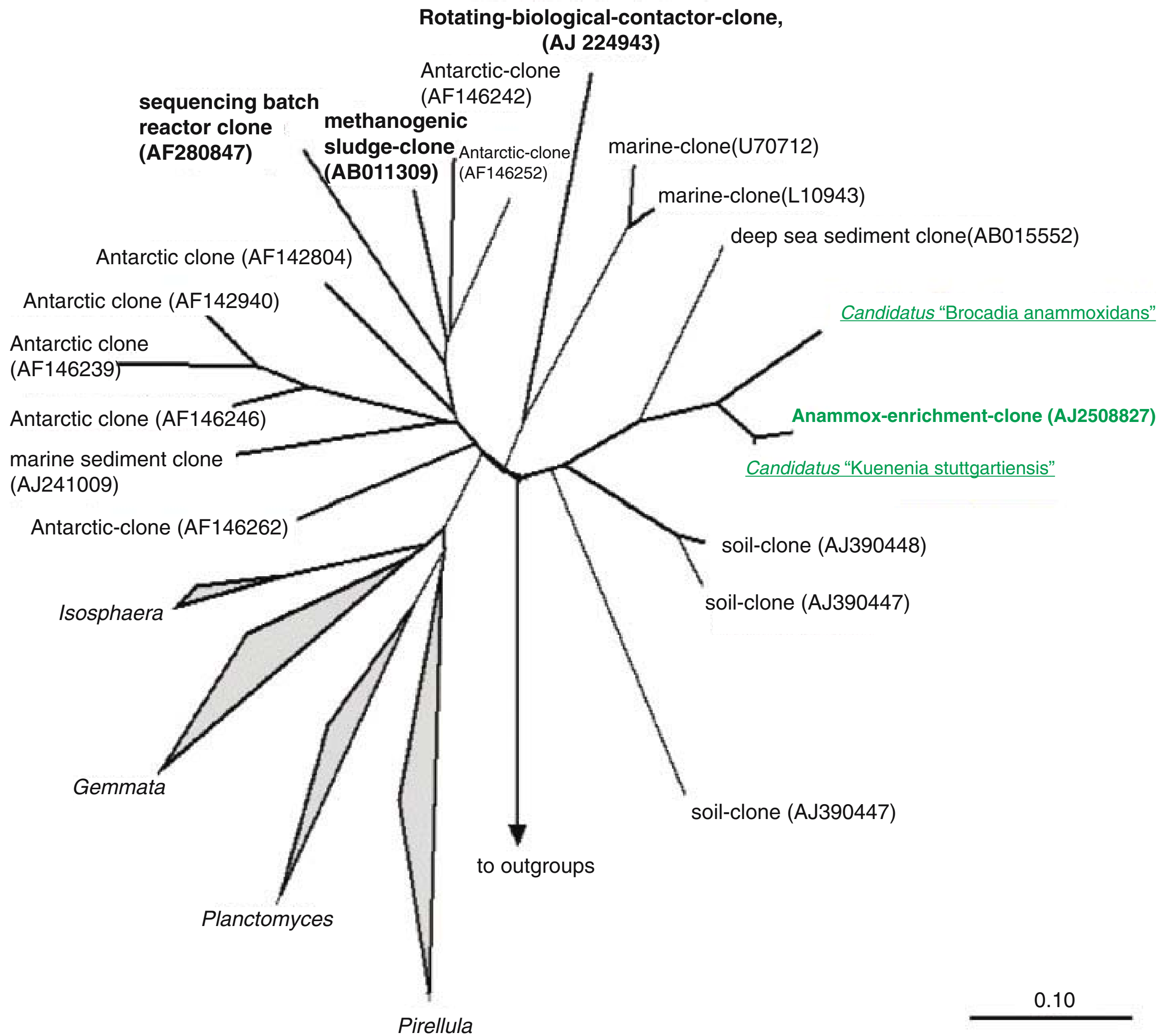


Fig. 18. 16S rDNA tree of the Planctomycetales. Anaerobic ammonium oxidizers are labeled green. The scale bar represents 10% estimated sequence divergence. Modified from Schmid et al. (2001).

of nitrogen dioxide in the absence of oxygen (Anaerobic ammonia oxidation catalyzed by Nitrosomonas eutropha). Since ANAMMOX was shown to exhibit rather efficient ammonium elimination rates of up to 3 kg of NH_4^+ m^{-3} day^{-1} (van de Graaf et al., 1996), it is suitable for the treatment of wastewater containing much ammonium and little organic chemical oxygen demand (COD). For such wastewater, it has been calculated that the replacement of the conventional nitrogen elimination steps by ANAMMOX would result in a reduction of the operational costs of up to 90% (Jetten et al., 2001). However, the presence of anaerobic ammonium oxidizers in addition to ammonium nitrite is required in the wastewater. Therefore, partial conventional nitrification, converting approximately half of the ammonium to nitrite, and ANAMMOX have been combined for efficient nitrogen removal from high-strength organic wastewater (summarized in Jetten et al., 2001). It should however be noted that very long lag-phases are required for obtaining ANAMMOX activity in such plants (Mulder et al., 1995; Jetten et al., 1997), which might be a disadvantage for applications.

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Prokaryotes (2006) 2:496–563
DOI: 10.1007/0-387-30742-7_17

CHAPTER 1.17

The H_2-Metabolizing Prokaryotes

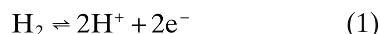
EDWARD SCHWARTZ AND BÄRBEL FRIEDRICH

Introduction

During the era of prebiotic evolution, which culminated in the appearance of cellular life forms, the earth had a reducing atmosphere. Fueled by volcanic activity and by magmatic outgassing, levels of atmospheric molecular hydrogen (H_2) may have been as high as 1% (Walker, 1977; Kasting, 1993). Various evolutionary scenarios envisage primeval life forms with H_2-based metabolism, e.g., ur-methanogens or ur-sulfate reducers (Stetter, 1992; Wächtershäuser, 1992; Edwards, 1998). With the advent of oxygenic photosynthesis (a process which may have originated as early as 3.5 billion years ago), oxygen began accumulating in the atmosphere and hydrogen levels decreased (Walker, 1977; Hayes, 1983; Blankenship, 1992; Kasting et al., 1992; Nisbet and Fowler, 1999). Concomitantly, strictly H_2-dependent organisms retreated to restricted habitats. Nevertheless, H_2 continues to be an important and widespread metabolite in both the archaeal and bacterial realms of the microbial world, in oxic as well as in anoxic habitats. The physiological role of H_2 in microbes is dual: Firstly, H_2 is a growth substrate, i.e., a source of energy and reductant. Prokaryotes of different metabolic types, such as methanogens, anoxygenic phototrophs, and aerobic knallgas bacteria, exploit H_2. Secondly, H_2 production is a means of dispersing excess reductant from fermentative metabolism. Hydrogen is among the fermentation products of both facultative fermenters, such as *Escherichia coli*, and obligate fermenters such as *Clostridium pasteurianum*.

This chapter surveys the H_2-metabolizing prokaryotes. It covers both H_2 evolution and H_2 consumption in archaea and bacteria. A glance at the list of H_2-metabolizing prokaryotes (Tables 1 and 2) will convince the reader that H_2 metabolism is not limited to specialized microbes grouped in a few taxonomic units. The comprehensive approach chosen here cuts across the boundaries of the physiological groups described in classical microbiology textbooks. This is necessary since the physiology of many H_2 metabolizers can only be explained in the context of syntrophic associations.

The common denominator of the disparate taxonomic and physiological groups treated here is hydrogenase, the enzyme responsible for reversibly catalyzing the reaction:



$$H_2 \rightleftharpoons 2H^+ + 2e^- \quad (1)$$

It is now agreed that presently known hydrogenases belong to three groups of independent phylogenetic origin. These groups are defined on the basis of their metal content: [FeFe] hydrogenases, [NiFe] hydrogenases (including [NiFe(Se)] hydrogenases), and [Fe] hydrogenases (formerly referred to as “metal-free hydrogenases”). Thus, hydrogenases constitute an example of convergent evolution. The [FeFe] and [NiFe] hydrogenases are particularly interesting in this context. Although the representatives of these two groups are not related, both the architectures of their active sites and the mechanisms of the chemical reaction catalyzed by them reveal striking similarities.

The past decade has seen rapid advances in our knowledge of the genetic basis of H_2 metabolism on the one hand and of the structure and catalytic mechanism of hydrogenases on the other. Accordingly, the major part of this chapter is devoted to an overview of these genetic, biochemical and spectroscopic studies. Hydrogen evolution and consumption is not catalyzed by hydrogenases alone. Nitrogenase, the enzyme which catalyzes the production of NH_3 from atmospheric dinitrogen, produces H_2 as a byproduct of N_2 fixation. Nitrogen-fixing prokaryotes are mentioned here because of their important contribution to the global H_2 flux and because N_2 reduction is an important physiological context of hydrogenase. An in-depth treatment of this subject is, however, beyond the scope of this article.

Hydrogenases are also found in eukaryotes. The hydrogenases of the chloroplasts of green algae, for instance, have attracted much attention in recent years (Happe et al., 1994; Florin et al., 2001). Furthermore, some eukaryotic microbes possess organelles called

Table 1. H_2-metabolizing bacteria.

Group	Order	Species[a]	E[b]	References
	Aquificales	*Aquifex aeolicus*	G	Deckert et al., 1998
		Aquifex pyrophilus	P	Huber et al., 1992
		Hydrogenobacter hydrogenophilus[c]	P	Kryukov et al., 1983
		Hydrogenobaculum acidophilum[d]	P	Shima and Suzuki, 1993
		Hydrogenobacter thermophilus	P	Kawasumi et al., 1984
				Shiba et al., 1984
		Hydrogenothermus marinus	P	Stöhr et al., 2001
		Thermocrinus ruber	P	Huber et al., 1998
Cyanobacteria	Chlorococcales	*Synechococcus*[e] PCC6301	P	Peschek, 1979
				Howarth and Codd, 1985
			A	Schmitz et al., 1995
				Schmitz and Bothe, 1996
		Synechococcus PCC6307	P	Howarth and Codd, 1985
		Synechocystis PCC6803	P	Howarth and Codd, 1985
		Synechocystis PCC6714	P	Howarth and Codd, 1985
		Synechocystis[f] PCC6308	P	Serebryakova et al., 1996
		Cyanothece PCC7822	P	van der Oost et al., 1989
		Microcystis PCC7820	P	Howarth and Codd, 1985
		Microcystis PCC7806	P	Moezelaar and Stal, 1994
	Nostocales	*Nostoc* sp. PCC73102	P	Lindblad and Sellstedt, 1990
		Nostoc sp.[g] PCC7937	PA	Mikheeva et al., 1995
				Serebryakova et al., 1994
			I	Serebryakova et al., 1996
		Nostoc muscorum PCC7120	PA	Houchins and Burris, 1981a
			I	Houchins and Burris, 1981b
		Anabaena cylindrica[h] PCC7122	PA	Bothe et al., 1977
				Lambert and Smith, 1980
			I	Ewart and Smith, 1989
	Stigonematales	*Fischerella muscicola*[i] PCC73103	A	Lambert and Smith, 1980
	Oscillatoriales	*Oscillatoria chalybea*	P	Bader and Abdel-Basset, 1999
		Oscillatoria limnosa	P	Heyer et al., 1989
		Microcoleus chthonoplastes	P	Moezelaar et al., 1996
Flexibacteria		*Chloroflexus aurantiacus*	PA	Holo and Sirevåg, 1986
Firmicutes	Actinomycetales	*Frankia* sp.	A	Sellstedt, 1989; Murry and Lopez, 1989
		Streptomyces thermoautotrophicus	P	Gadkari et al., 1990
	Bacillales	*Bacillus schlegelii*	P	Schenk and Aragno, 1979
		Bacillus tusciae	P	Bonjour and Aragno, 1984
	Clostridiales	*Clostridium pasteurianum*	P	Nakos and Mortenson, 1971
			I	Chen and Mortenson, 1974
				Chen and Blanchard, 1978
		Clostridium acetobutylicum	P	Gray and Gest, 1965
			I	Vasconcelos et al., 1994
		Clostridium kluyveri	P	Bornstein and Barker, 1948
		Clostridium tetanomorphum	P	Woods and Clifton, 1938
		Clostridium aceticum	P	Braun et al., 1981
				Braun and Gottschalk, 1981
		Clostridium mayombei	P	Kane et al., 1991
		Clostridium magnum	P	Bomar et al., 1991
		Clostridium scatologenes	PA	Küsel et al., 2000
		Eubacterium limosum	P	Sharak-Genthner et al., 1981
		Carboxydothermus	P	Svetlichny et al., 1991
		hydrogenoformans	I	Soboh et al., 2002
		Ruminococcus albus	P	Miller and Wolin, 1973
		Ruminococcus hydrogenotrophicus	P	Bernalier et al., 1996
		Ruminococcus productus	P	Lorowitz and Bryant, 1984
				Bernalier et al., 1996
		Ruminococcus hansenii	P	Bernalier et al., 1996
		Ruminococcus schinkii	P	Rieu-Lesme et al., 1996
		Syntrophomonas wolfei	PA	McInerney et al., 1979
				McInerney et al., 1981a
		Megasphaera elsdenii	I	van Dijk et al., 1979
				Atta and Meyer, 2000
		Desulfitobacterium dehalogenans	P	Utkin et al., 1994

(Continued)

Table 1. *Continued*

Group	Order	Species[a]	E[b]	References
		Rhodococcus opacus	P	Aggag and Schlegel, 1973
			I	
		Mycobacterium gordonae	P	Park and DeCicco, 1974
		Sporomusa termitida	P	Breznak et al., 1988
		Sporomusa sphaeroides	PA	Dobrindt and Blaut, 1996
		Sporomusa sylvacetica	P	Kuhner et al., 1997
		Acetonema longum	P	Kane and Brezhnak, 1991
		Desulfotomaculum orientis	P	Klemps et al.,1985
			A	Cypionka and Dilling, 1986
		Acetobacterium woodii	P	Balch et al., 1977
				Ljungdahl and Wood, 1982
			I	Ragsdale and Ljungdahl, 1984
		Acetobacterium bakii	P	Kotsyurbenko et al., 1995
		Acetobacterium paludosum	P	Kotsyurbenko et al., 1995
		Acetobacterium fimetarum	P	Kotsyurbenko et al., 1995
	Thermoanaero-bacteriales	*Thermoanaerobacter kivui*	PA	Leigh et al., 1981
				Daniel et al., 1990
				Pusheva et al., 1991
		Moorella thermoacetica[j]	PA	Fontaine et al., 1942
				Drake, 1982
				Daniel et al., 1990
			P	Pezacka and Wood, 1984
				Martin et al., 1983;
				Kerby and Zeikus, 1983
		Moorella thermoautotrophica	PA	Clark et al., 1982
				Wiegel et al., 1981
		Thermacetogenium phaeum	P	Hattori et al., 2000
	Halanaerobiales	*Halanaerobium alcaliphilum*	P	Tsai et al., 1995
		Acethalobium arabaticum	P	Zhilina and Zavarzin, 1990
	Bacterioidales	*Acetomicrobium flavidum*	P	Soutschek et al., 1984
			I	Mura et al., 1996
Proteobacteria	α Class	*Renobacter vacuolatum*	P	Malik and Schlegel, 1981
		Aquaspirillum autotrophicum SA32	P	Aragno and Schlegel, 1978
		Bradyrhizobium japonicum	P	Hanus et al., 1979
				Emerich et al., 1979
			A	McCrae et al., 1978
			I	Harker et al., 1984
		Paracoccus pantotrophus[k]	P	Kühnemund, 1971
			A	Schneider and Schlegel, 1977
			I	Knüttel et al., 1989
				Sim and Vignais, 1978
		Methylosinus trichosporium OB3b	A	Chen and Yoch, 1987
		Rhizobium leguminosarum	P	Dixon, 1968
				Nelson and Salminen, 1982
		Rhodobacter capsulatus	P	Yen and Marrs, 1977
				Madigan and Gest, 1978
				Madigan and Gest, 1979
			I	Colbeau et al., 1983
		Rhodobacter sphaeroides	P	Uffen and Wolf, 1970
		Rhodospirillum rubrum	P	Ormerod and Gest, 1962
				Gest, 1954
				Gorrell and Uffen, 1978;
				Voelskow and Schön, 1980
				Uffen, 1981
			I	Adams and Hall, 1979
		Rhodopseudomonas palustris	P	Qadri and Hoare, 1968
				Uffen and Wolf, 1970
		Thiorhodococcus minus	P	Guyoneaud et al., 1997
		Thiocystis violacea	P	Winogradsky, 1988
		Rhodomicrobium vannieli	P	Duchow and Douglas, 1949
		Xanthobacter flavus 301	P	Malik and Claus, 1979
		Xanthobacter autotrophicus	P	Schneider et al., 1973
				Baumgarten et al., 1974

Table 1. *Continued*

Group	Order	Species[a]	E[b]	References
			I	Schink, 1982
			A	Eberhardt, 1969
				Schneider and Schlegel, 1977
		Oligotropha carboxidovorans	P	Meyer and Schlegel, 1978
			I	Santiago and Meyer, 1997
		Azospirillum lipoferum	P	Malik and Schlegel, 1981
		Ancylobacter aquaticus	P	Malik and Schlegel, 1981
	β Class	*Acidovorax facilis*	P	Willems et al., 1990
		Acidovorax delafieldii	P	Willems et al., 1990
		Variovorax paradoxus	P	Davis et al. 1970
			A	Schneider and Schlegel, 1977
		Achromobacter ruhlandii	P	Packer and Vishniac, 1955
				Aragno and Schlegel, 1977
		Alcaligenes latus H-4	P	Palleroni and Palleroni, 1978
			I	Pinkwart et al., 1983
		Alcaligenes hydrogenophilus	P	Ohi et al., 1979
		Hydrogenophaga flava	P	Willems et al., 1989
		Hydrogenophaga pseudoflava	P	Willems et al., 1989
		Hydrogenophaga palleroni	P	Davis et al., 1970
			P	Willems et al., 1989
		Hydrogenophaga taeniospiralis	P	Lalucat et al., 1982
				Willems et al., 1989
		Ralstonia eutropha H16	P	Eberhardt, 1966
			I	Schneider and Schlegel, 1976
				Schink and Schlegel, 1979;
				Bernhard et al., 2001
		Ralstonia metallidurans CH34	PA	Mergeay et al., 1985
		Hydrogenophilus hirschii	P	Stöhr et al., 2001
		Hydrogenophilus thermoluteolus	P	Hayashi et al., 1999
		Rubrivivax gelatinosus[l]	P	Wertlieb and Vishniac, 1967
				Uffen, 1976
		Thiobacillus plumbophilus	P	Drobner et al., 1992
		Pseudomonas saccharophila	PA	Bone, 1960
				Bone et al., 1963
				Podzuweit et al., 1983
	γ Class	*Azotobacter chroococcum*	P	Lee and Wilson, 1943
			I	van der Werf and Yates, 1978
		Azotobacter vinelandii	P	Hyndman et al., 1953
				Wong and Maier, 1985
			I	Seefeldt and Arp, 1986
		Derxia gummosa	P	Pedrosa et al., 1980
		Acidithiobacillus ferrooxidans	P	Drobner et al., 1990
				Fischer et al., 1996
		Escherichia coli	P	Stephenson and Stickland, 1931
				Peck and Gest, 1957
			A	Krasna, 1980
				Krasna, 1984
			I	Adams and Hall, 1979
				Ballantine and Boxer, 1985
				Sawers and Boxer, 1986
		Salmonella typhimurium	A	Krasna, 1980
		Citrobacter freundii	A	Krasna, 1980
		Allochromatium vinosum	I	Gitlitz and Krasna, 1975
		Shewanella putrefaciens	P	Lovley et al., 1989
		Thiocapsa roseopersicina BBS	P	Bogorov, 1974
				Gogotov et al., 1974
			I	Zorin and Gogotov, 1975
				Gogotov et al., 1976
		Hydrogenovibrio marinus	P	Nishihara et al., 1990
				Nishihara et al., 1991
			I	Nishihara et al., 1997
		Methylococcus capsulatus Bath	P	Stanley and Dalton, 1982
			A	Hanczár et al., 2002

(Continued)

Table 1. *Continued*

Group	Order	Species[a]	E[b]	References
		Pseudomonas hydrogenovora	P	Kodama, T. et al., 1975
				Igarashi, Y. et al., 1980
	δ Class	*Desulfomicrobium norvegicum*[m]	P	Genthner et al., 1997
		Desulfomicrobium baculatum[n]	P	Rozanova et al., 1988
				Fauque et al., 1991
		Desulfomicrobium apsheronum	P	Rozanova et al., 1988
		Desulfobacterium autotrophicum	P	Brysch et al., 1987
		Desulfovibrio vulgaris	P	Hatchikian et al., 1976
				Badziong et al., 1978
				Traore et al., 1983
			I	Yagi, 1970
				van der Westen et al., 1978
		Desulfovibrio fructosovorans	P	Malki et al., 1997
		Desulfovibrio desulfuricans	P	Vosjan, 1975
				Tsuji and Yagi, 1980
		Desulfovibrio gigas	P	Hatchikian et al., 1976
				Hatchikian et al., 1978
		Desulfovibrio profundus	P	Bale et al., 1997
		Desulfovibrio senezii	P	Tsu et al., 1998
		Desulfobacter hydrogenophilus	P	Widdel, 1987
		Desulfobulbus propionicus	P	Laanbroek et al., 1982a
		Desulfonema limicola	P	Widdel et al., 1983
		Desulfuromonas acetoxidans	IA	Brugna et al., 1999
		Desulfurella multipotens	P	Miroshnichenko et al., 1994
		Desulfurella kamchatkensis	P	Miroshnichenko et al., 1998
		Hippea maritima	P	Miroshnichenko et al., 1999
		Pelobacter acetylenicus	P	Schink, 1985
		Syntrophus aciditrophicus	P	Jackson et al., 1999
		Syntrophus buswellii	PA	Mountfort et al., 1984
				Wallrabenstein and Schink, 1994
		Syntrophobacter wolinii	P	Boone and Bryant, 1980
				Wallrabenstein and Schink, 1994
		Syntrophobacter pfennigii	PA	Wallrabenstein et al., 1995
		Smithella propionica	P	Liu et al., 1999
	ε Class	*Helicobacter pylori*	A	Maier et al., 1996a
		Nautilia lithotrophica	P	Miroshnichenko et al., 2002
		Wolinella succinogenes	P	Wolin et al., 1961
			I	Aspen and Wolin, 1966
				Dross et al., 1992
		Campylobacter jejuni	PA	Laanbroek et al., 1982b
				Goodman and Hoffman, 1983
		Campylobacter sputorum	P	Schumacher et al., 1992
		Sulfurospirillum deleyianum	P	Schumacher et al., 1992
	Thermotogales	*Thermotoga maritima*	P	Huber et al., 1986
			I	Juszczak et al., 1991
		Thermotoga neapolitana	P	Jannasch et al., 1988
		Thermotoga thermarum	P	Windberger et al., 1989
		Fervidobacterium islandicum	P	Huber et al., 1990

Abbreviations: E, evidence; I, hydrogenase has been isolated; P, physiological evidence for hydrogenase activity; G, genetic evidence; and A, biochemical assay.

[a]Green: H_2 producer; brown: H_2 consumer; and blue: both.

[b]Type of evidence.

[c]Formerly *Calderobacterium hydrogenophilum*.

[d]Formerly *Hydrogenobacter acidophilus*.

[e]Formerly *Anacystis nidulans* (= SAG 1402-1).

[f]CCAP 1430/1, or CALU 743.

[g]CCAP 1403/13A, or ATCC 29413.

[h]CCAP 1403/2A, SAG 1403-2, or ATCC 27899.

[i]CCAP 1427/1.

[j]Formerly *Clostridium thermoaceticum*.

[k]Formerly *Paracoccus denitificans*.

[l]Formerly *Rhodocyclus gelatinosus*.

[m]Formerly *Desulfovibrio desulfuricans* Norway 4.

[n]Formerly *Desulfovibrio baculatus*.

Table 2. H_2-metabolizing archaea.

Group	Order	Species[a]	E[b]	References
Crenarchaeota	Desulfurococcales	*Pyrodictium brockii*	PA	Stetter et al., 1983
				Pihl et al., 1989
			I	Pihl and Maier, 1991
		Pyrodictium occultum	P	Stetter et al., 1983
				Fischer et al., 1983
		Pyrodictium abyssi	P	Pley et al., 1991
		Thermodiscus maritimus	P	Fischer et al., 1983
		Pyrolobus fumarii	P	Blöchl et al., 1997
		Hyperthermus butylicus	P	Zillig et al., 1990
		Ignicoccus islandicus	P	Huber et al., 2000
		Ignicoccus pacificus	P	Huber et al., 2000
		Stetteria hydrogenophila	P	Jochimsen et al., 1997
	Thermoproteales	*Thermoproteus tenax*	P	Zillig et al., 1981
				Fischer et al., 1983
		Thermoproteus neutrophilus	P	Fischer et al., 1983
		Pyrobaculum islandicum	P	Huber et al., 1987
		Pyrobaculum aerophilum	P	Völkl et al., 1993
	Sulfolobales	*Sulfolobus solfataricus*	P	Brock et al., 1972
		Sulfolobus acidocaldarius	P	Brock et al., 1972
		Metallosphaera sedula	P	Huber et al., 1989
		Acidianus brierleyi	P	Segerer et al., 1986
		Acidianus infernus	P	Segerer et al., 1986
		Stygiolobus azoricus	P	Segerer et al., 1991
Euryarchaeota	Archaeglobales	*Archaeoglobus fulgidus*	P	Stetter, 1988
		Archaeoglobus profundus	P	Burggraf et al., 1990
		Archaeoglobus lithotrophicus	P	Stetter et al., 1993
	Methanosarcinales	*Methanosarcina barkeri* Fusaro	I	Fiebig and Friedrich, 1989
			G	Künkel et al., 1998
				Vaupel and Thauer, 1998
				Meuer et al., 1999
		Methanosarcina barkeri DSM 800	P	Weimer and Zeikus, 1978
			I	Fauque et al., 1984
		Methanosarcina mazei Gö1	I	Mah, 1980
				Deppenmeier et al., 1992
			G	Deppenmeier et al., 1995
				Deppenmeier et al., 2002
	Methanomicrobiales	*Methanospirillum hungatei*	P	Ferry et al., 1974
			I	Sprott et al., 1987
		Methanocalculus halotolerans	P	Ollivier et al., 1998
		Methanomicrobium mobile	P	Paynter and Hungate, 1968
		Methanocorpusculum parvum	P	Zellner et al., 1989
		Methanocorpusculum sinense	P	Zellner et al., 1989
		Methanocorpusculum bavaricum	P	Zellner et al., 1989
		Methanogenium organophilum	P	Widdel et al., 1988
		Methanogenium marisnigri	P	Romesser et al., 1979
		Methanogenium cariaci	P	Romesser et al., 1979
		Methanogenium frigidum	P	Franzmann et al., 1997
		Methanoplanus limicola	P	Wildgruber et al., 1982
		Methanoplanus endosymbiosus	P	van Bruggen et al., 1986
	Methanobacteriales	*Methanothermobacter thermoautotrophicus*[c] ΔH	P	Zeikus and Wolfe, 1972
				Jacobson et al., 1982
			I	Kojima et al., 1983
				Fox et al., 1987
			G	Alex et al., 1990
				Smith et al., 1997
		Methanothermobacter marburgensis[d] DSM 2133	PA	Zirngibl et al., 1990
				Afting et al., 1998
			I	Setzke et al., 1994
			G	Tersteegen and Hedderich, 1999
		Methanobacterium formicicum sp.	P	van Bruggen et al., 1984
		Methanobacterium formicicum MF	IA	Jin et al., 1983

(Continued)

Table 2. *Continued*

Group	Order	Species[a]	E[b]	References
		Methanobacterium formicicum JF-1	IA	Baron and Ferry, 1989a; Baron and Ferry, 1989b; Baron et al., 1989 Baron et al., 1989
		Methanobacterium alcaliphilum	P	Blotevogel et al., 1985 Worakit et al., 1986
		Methanothermus fervidus	P	Stetter et al., 1981
		Methanothermus sociabilis	P	Lauerer et al., 1986
		Methanosphaera stadtmanae	P	Miller and Wolin, 1985
		Methanobrecibacter arboriphilicus	P	Zeikus and Henning, 1975; Zehnder and Wuhrmann, 1977
	Methanococcales	*Methanococcus janashii*	P	Jones et al., 1983a
			I	Shah, 1990
			G	Halboth and Klein, 1992 Bult et al., 1996
		Methanothermococcus thermolithotrophicus	P	Huber et al., 1982 Belay et al., 1986
		Methanococcus vanielli	I	Yamazaki, 1982
		Methanococcus voltae	I	Muth et al.,1987
		Methanococcus maripaludis	P	Jones et al., 1983b
		Methanococcus igneus	P	Burggraf et al., 1990
	Methanopyrales	*Methanopyrus kandleri*	P	Kurr et al., 1991
			G	Slesarev et al., 2002
	Thermococcales	*Pyrococcus furiosus*	P	Fiala and Stetter, 1986
			I	Bryant and Adams, 1989 Ma et al., 1993 Sapra et al. 2000; Silva et al., 2000
		Pyrococcus abyssi	P	Erauso et al., 1993
		Pyrococcus woesei	P	Zillig et al., 1987
		Thermococcus litoralis	P	Neuner et al., 1990
			I	Rákhely et al., 1999
		Thermococcus stetteri	P	Miroshnichenko et al., 1989 Pusheva et al., 1991
			I	Zorin et al., 1996
		Thermococcus celer	P	Zillig et al., 1983

Abbreviations: E, evidence; I, hydrogenase has been isolated; P, physiological evidence for hydrogenase activity; G, genetic evidence; and A, biochemical assay.

[a]Green: H_2 producer; brown: H_2 consumer; and blue: both.

[b]Letter codes for type of evidence.

[c]Formerly *Methanobacterium thermoautotrophicum* ΔH.

[d]Formerly *Methanobacterium thermoautotrophicum* Marburg.

"hydrogenosomes" which contain hydrogenases and engage in H_2 metabolism (Müller, 1993). Since organelles are descendents of prokaryotic cells, these organisms will be mentioned briefly but not treated in depth.

A number of review articles have been published on various aspects of H_2 metabolism and hydrogenases (Adams, 1990; Wu and Mandrand, 1993; Sasikala et al., 1993; Albracht, 1994; Vignais and Toussaint, 1994; Fontecilla-Camps, 1996; Maier and Böck, 1996; Frey, 1998; Nandi and Sengupta, 1998; Peters, 1999; Casalot and Rousset, 2001; Vignais et al., 2001; Horner et al., 2002). The reader is also referred to reviews on various groups of H_2-metabolizing prokaryotes including sulfate reducers (Fauque et al., 1988; Voordouw, 1995), aerobic, H_2-oxidizing bacteria (Aragno and Schlegel, 1992; Friedrich and Schwartz, 1993), methanogens (Sorgenfrei et al., 1997; Thauer, 1998), homoacetogens (Drake, 1994) and cyanobacteria (Tamagnini et al., 2002).

Ecology

The term "H_2-metabolizing prokaryotes" lumps together many taxonomically and physiologically unrelated organisms. Accordingly, the representatives of this group are found in a range of very different habitats. This section does not

attempt to describe exhaustively the biotopes of H_2-metabolizing prokaryotes. The emphasis, rather, is on the interactions between H_2-producers and H_2-consumers, which are important for global geochemical cycles.

The Global H_2 Budget

Before considering the ecophysiology of the various groups of H_2-metabolizing prokaryotes and the microbial communities they belong to, it is appropriate to briefly discuss the global budget of atmospheric H_2. Three types of processes impact the pool of atmospheric H_2: anthropogenic, biogenic and geochemical. As will be discussed later, substantial amounts of H_2 are turned over within microbial ecosystems without, however, affecting the atmospheric H_2 budget. Conrad has summarized the numerous studies on atmospheric H_2 (Conrad, 1988). Anthropogenic activities are a major source of atmospheric H_2. Equally important is the production of H_2 by the oxidation of atmospheric methane (CH_4) and nonmethane hydrocarbons. This entails chemical reactions with photochemically produced hydroxyl radical. Photochemical evolution of H_2 accounts for 30–40% of the total production. In contrast, the total biospheric emission, i.e., the contribution of all ocean, lake and soil biota to the global H_2 pool is only 7–11%. Several studies have addressed the sources of biogenic H_2 emission breaking it down into its marine, lacustrine and terrestrial components. The major part of the euphotic surface layer of the world's oceans are supersaturated for H_2. Thus, a net emission of H_2 from the surface water to the atmosphere must take place. While experimental data are still lacking, the main source of this H_2 is probably the N_2-fixing cyanobacteria and prochlorophytes. The same probably holds true for H_2 production in the oxic layers of freshwater lakes. The H_2 concentration of the epilimnion reaches levels of 0.5–50 nM (Schropp et al., 1987). Studies have shown that the H_2 concentration of lake water correlates with cell counts of cyanobacteria on the one hand and with N_2 fixation rates on the other (Conrad et al., 1983a; Schmidt and Conrad, 1993; Schütz et al., 1988). The results refute the older notion that H_2 arises by fermentation in the anoxic sediment and diffuses up into the oxic zone. Almost all of the H_2 evolved in anoxic sediments is also consumed there.

The contribution of soil to the atmospheric H_2 pool is more complex. Most soils do not emit H_2 but, on the contrary, consume it (Seiler, 1978; Conrad and Seiler, 1981; Conrad and Seiler, 1985). An exception to this are soils in areas where leguminous plants grow. Symbiotic rhizobia in root nodules produce H_2 in conjunction with N_2 fixation. An estimated 1 million tons of H_2 is produced by nodule bacteria annually (Evans et al., 1987). During the vegetation period, the rates of H_2 production by root nodules are high enough to lead to a net increase in the H_2 concentration in the soil. A portion of this H_2 escapes to the atmosphere. Thus, in all three major environmental zones, release of H_2 into the atmosphere is a result of N_2 fixation. The production of H_2 by microbial fermentation processes in the gut of termites may be an exception to this generalization. It has been estimated that as much as 10^{14} g of H_2 could be released into the atmosphere annually by these microbes (Zimmerman et al., 1982). Experimental data on this question are inconclusive. Laboratory experiments with termites pointed to a significant release (Zimmerman et al., 1982). On the other hand, measurements made in the field on actual termite mounds showed that there was no measurable release of H_2 at all (cited by Conrad [1988] as W. Seiler and R. Conrad, unpublished observation). On the consumption side of the balance sheet, chemical processes in the atmosphere are responsible for only a small fraction of H_2 decomposition. By far the most important global sink for H_2 is the soil, which accounts for over 90% of the total global consumption. Remarkably, consumption of atmospheric H_2 in soils may not be directly attributable to the activity of microorganisms. Rather, it appears that H_2-oxidizing activity associated with soil particles is the basis of H_2 consumption by soil (reviewed by Conrad, 1996). Various lines of evidence support this conclusion: 1) For an organism to utilize atmospheric H_2, it must have a K_m for H_2 in the range of 5–80 nM (Conrad, 1984). Several characterized strains of chemolithotrophic bacteria have K_m values for H_2 above 0.5 μM. 2) Suspensions of H_2-oxidizing chemolithotrophs provided with a mixture of H_2 and air as growth substrate consume H_2 down to a certain concentration. After this point, no more H_2 is utilized and growth ceases. The critical concentration for H_2 uptake is known as the threshold value. The threshold for H_2 utilization for various H_2-oxidizing laboratory strains is significantly higher than the concentration of H_2 in the atmosphere (Conrad and Seiler, 1979; Conrad et al., 1983b; Schuler and Conrad, 1990). 3) The H_2-oxidizing activity of the soil is destroyed by boiling and autoclaving and has an optimum of 25–40°C (Seiler, 1978; Fallon, 1982; Schuler and Conrad, 1991a). 4) The H_2-oxidizing activity associated with size-fractioned soil particles is not correlated with parameters indicative of microbial biomass (ATP content and microscopic cell counts; Häring et al., 1994). Taken together, these results suggest that hydrogenase enzymes released from lysed bacteria and immobilized on

soil particles or persisting in dead cells are responsible for oxidation of atmospheric H_2 in the soil.

The above findings are paradoxical, since H_2-oxidizing chemolithoautotrophs are readily isolated from a variety of soil biotopes. Why are chemolithoautotrophs widespread in soils if they are not able to metabolize atmospheric H_2? The following aspects are important in this context and may be at least part of the explanation. 1) Most of the classical, mesophilic H_2-oxidizers (knallgas bacteria) isolated from soils are facultative chemolithoautotrophs that also thrive organotrophically (Aragno and Schlegel, 1992). Such organisms are predestined to utilize H_2 that is transiently available in biologically relevant concentrations. It is well known that soils rapidly become anoxic when they are waterlogged. This can lead to a transient production of H_2 when soil microbes shift to fermentation. 2) Various soil microenvironments, e.g., the vicinity of root nodules, may provide high local concentrations of H_2 which could at least transiently support the growth of H_2-oxidizing chemolithotrophs. 3) Syntrophic associations between H_2 producers and H_2 consumers may be a widespread phenomenon. Juxtaposition of individual cells would, in effect, provide the consumer with a high local concentration of H_2. This will be discussed in more detail below.

Habitats of H_2-Evolving Prokaryotes

Anaerobic food chains which degrade organic material via the various fermentation processes outlined above are a major source of H_2 in the biosphere (Fig. 1). Fermenting organisms are limited to anoxic zones rich in organic substance. Marine and lacustrine sediments are the most important biotopes of this sort. These sediments are fed by a constant influx of organic material derived from photosynthetic primary producers and from the ensuing food chains. The upper, oxic layer of the sediment varies in depth both in marine and freshwater sediments. Below this layer is the zone of anoxic decomposition. In this stratum, H_2 is evolved as a product of fermentation. The H_2 produced neither accumulates nor does it escape in significant quantities to the oxic zone. If H_2 were to accumulate, the fermentative metabolic processes would soon come to a halt, since these are inhibited by relatively low concentrations of H_2 in the environment. The inhibitory concentrations vary for the different fermentative reactions, depending on their energetics. Fermentation of fatty acids to acetate, H_2 and CO_2, for instance, is more endergonic than the fermentation of ethanol to acetate and H_2 and the former process ceases at a much lower concentration of external H_2 than the latter (See

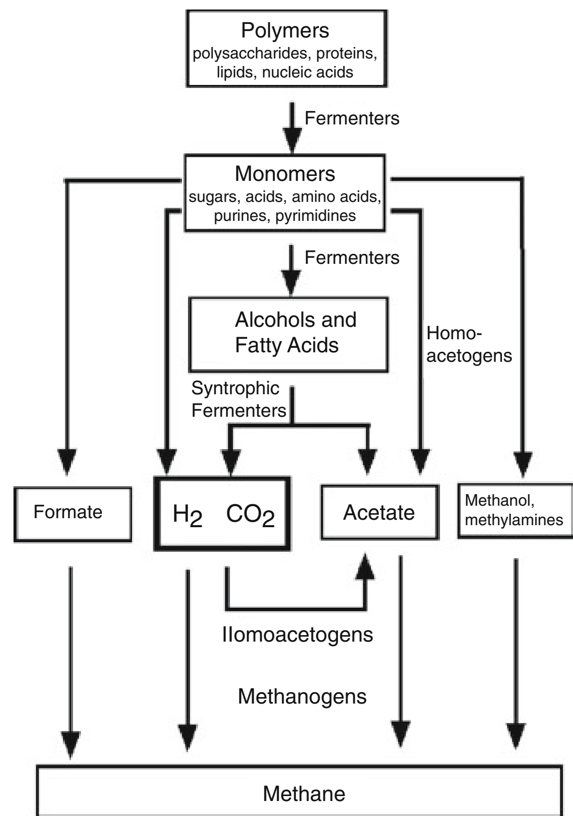


Fig. 1. Schematic overview of the flux of matter in the anaerobic zone of freshwater sediments. The major pathways in the mineralization of organic substance and the microbial groups responsible for them are given (after The Anaerobic Way of Life in this Volume).

Syntrophism among Prokaryotes in this Volume). Studies on the sediment of a eutrophic lake showed that fermentation of butyrate and propionate were inhibited by H_2 concentrations of 100 nM and 20 nM, respectively (Conrad et al., 1986). The concentration of H_2 is kept at a low level, i.e., 5–30 nM in marine sediments and 1–150 nM in lacustrine sediments, due to constant depletion by H_2-consuming organisms (reviewed by Jørgensen, 1989; see also Strayer and Tiedje, 1978; Lovley and Klug, 1982a; Robinson and Tiedje, 1982; Phelps and Zeikus, 1984; Conrad et al., 1985). High turnover rates for H_2 have been measured. In a study on a lake sediment, the turnover time of the pool of free H_2 was estimated to be 2 min (Conrad et al., 1985).

Two minor habitats of fermenting bacteria deserve mention. The stomach of ruminants and the gut of xylophagous arthropods including termites (Breznak, 1982; Zimmerman et al., 1982; Leschine, 1995; Ricke et al., 1996; Flint, 1997). The rumen harbors a rich microbial flora. Among these microorganisms are bacteria such as *Ruminococcus albus* and *Butyrovibrio fibrisol-*

vens which ferment cellulose to organic acids, H_2 and CO_2.

Hydrogen is generated as a byproduct of N_2 fixation in both oxic and anoxic environments. Cyanobacteria and prochlorophytes are probably the most widespread diazotrophs on earth. These organisms inhabit the upper, oxic zones of oceans and lakes. The anoxygenic photosynthetic bacteria, which occupy deeper zones depleted for O_2, also engage in N_2 fixation. These and other diazotrophs contain uptake hydrogenases and, hence, are capable of exploiting at least a part of the H_2 generated by nitrogenase. However, hydrogenase-free strains abound in nature resulting in the liberation of large quantities of H_2. The third important group of diazotrophs are the endosymbiotic rhizobia. The role of these organisms in rhizospheric H_2 production has been mentioned above. A special cyanobacterial habitat is the extensive mats found in coastal areas. The mats consist of a gelatinous mass produced by the microbes. The generation of H_2 leads to the formation of bubbles in this viscous matrix. The mats formed by different microbial communities differ in their consistency. This, in turn, determines whether the H_2 is retained or released into the atmosphere (Hoehler et al., 2001).

Habitats of H_2-Consuming Prokaryotes

Both biogenic and abiogenic H_2 production can support growth of H_2-utilizing prokaryotes. Many H_2-utilizing species profit from fermentative H_2 production in anoxic sediments. The two most important groups of H_2-consumers in such biotopes are the methanogenic archaea and the sulfate-reducing bacteria. It has been known for some time that methanogens and sulfate reducers compete for H_2 (Winfrey and Zeikus, 1977; Winfrey et al., 1977; Abram and Nedwell, 1978; Oremland and Polcin, 1982; Lovley et al., 1982; Lovley and Klug, 1983). Sulfate reducers outcompete the methanogens in the presence of sulfate, because the former have a higher affinity for H_2 and a higher growth yield (Kristjansson et al., 1982; Schönheit et al., 1982). In studies using pure cultures, K_m and Y_{H2} values of 5.0 ± 0.5 μM and 0.2 g protein/mol H_2, respectively, were determined for *Methanospirillum hungatei* JF-1 versus 1.1 ± 0.1 μM and 0.85 g protein/mol H_2, respectively, for *Desulfovibrio* strain G11 (Robinson and Tiedje, 1984). The key factor determining which of the two terminal degradation processes—sulfidogenesis or methanogenesis—prevails in a given habitat is SO_4^{2-} concentration. In anoxic marine sediments, where there is an abundant supply of SO_4^{2-}, sulfate reduction is the dominant process, consuming most of the available H_2 and acetate.

The sulfate level in lakes varies depending on their trophic state, but in general is lower than in seawater. The thickness of the zone of sulfate reduction varies accordingly. In eutrophic lakes the sulfate concentration in the sediment drops of sharply. Here the zone of sulfate reduction is only a few centimeters thick. In lakes with lower nutrient contents the zone of sulfate reduction extends deeper into the sediment (Lovley and Klug, 1983). Competition for H_2 does not mean that sulfate reduction and methanogenesis are mutually exclusive processes. Various methanogens can exploit substrates, e.g. methylamine, that are not utilized by sulfate reducers (Oremland and Polcin, 1982; Winfrey and Ward, 1983). Therefore, the two groups of organisms can coexist in the same biotope, as has been shown for instance for estuarine sediments. According to one estimate based on sediment from an oligotrophic lake, the fraction of the total flux of electrons and carbon routed through sulfate reduction is between 30 and 81% of the total terminal metabolism (Lovley and Klug, 1983).

Sulfate reduction is also an important process in extreme environments, such as the anaerobic sediments of soda lakes. Among the specialized, H_2-utilizing, sulfate-reducing bacteria found in such sediments are the alkaliphilic lithoheterotroph *Desulfonatronovibrio hydrogenovorans* and the alkaliphilic lithoautotroph *Desulfonatronum lacustre* (Zhilina et al., 1997; Pikuta et al., 1998).

The role of soils as habitats for facultative H_2-oxidizing chemolithotrophs has been discussed above. Two niches are especially important in this context: the rhizosphere in the vicinity of nodulated plants, and the interface of anoxic enclaves where H_2 is evolved and can diffuse into the surrounding oxic zone.

A few obligately chemolithoautotrophic bacteria, which oxidize either H_2 or sulfur, have been described. These include both mesophilic and thermophilic forms. The latter are mostly confined to special niches such as hot springs and hydrothermal vents. *Hydrogenobacter thermophilus* and *Hydrogenobacter halophilus* inhabit freshwater and saline hot springs, respectively (Kawasumi et al., 1984; Nishihara et al., 1989; Nishihara et al., 1990). The hyperthermophilic H_2-oxidizing bacterium *Aquifex pyrophilus* grows in hot marine sediments (Huber et al., 1992). Several obligately chemolithotrophic archaea have been identified. *Thermodiscus maritimus* and *Pyrodictium occultum* inhabit submarine solfataric springs (Fischer et al., 1983). Two obligate H_2-oxidizing, sulfate-reducing archaea, *Ignicoccus islandicus* and *Ignicoccus pacificus*, were enriched from hot marine sediments and from the orifice of a deepsea vent, respectively (Huber et al., 2000). These organ-

isms utilize the H_2, CO_2 and sulfate dissolved in the hydrothermal fluid for growth. Thus, they are chemolithoautotrophic primary producers and form the basis of food chains in their respective habitats (Jannasch and Mottl, 1985). The hyperthermophile *Archaeoglobus profundus* is another example of obligate H_2-based lithotrophy (Burggraf et al., 1990b). However, this organism requires organic carbon sources.

Syntrophy and Interspecies H_2 Transfer

Syntrophy is the mutual metabolic dependence of two different types of prokaryotes (Syntrophism among Prokaryotes in this Volume). The first obligately syntrophic relationship involving an exchange of H_2 between the partner organisms was recognized by Bryant. He discovered that the ethanol-degrading "bacterium" *Methanobacillus omelianskii* was in fact a coculture of a so-called "S-organism," which was the actual ethanol-degrader, and a methanogen. The ethanol-degrader was strictly dependent on the methanogen, because the latter consumed H_2, thereby "pulling" the otherwise thermodynamically unfavorable oxidation of ethanol. Wolin reported new examples of microbial H_2 exchange and introduced the phrase "interspecies hydrogen transfer" to describe this general phenomenon (Scheifinger et al., 1975; Wolin, 1976, 1982). Based on quantitative studies on freshwater sediments, Conrad proposed the juxtaposition of H_2-producers and H_2-consumers (Conrad et al., 1985). He suggested that the two types of cells are in close, physical contact in particles or flocs. As a result of juxtaposition, a major fraction of the H_2 produced would never enter the pool of dissolved H_2 but rather be transferred directly from cell to cell. This would explain the discrepancy between turnover rates in the extracellular H_2 pool and growth yield of the population of methanogens.

The interspecies transfer of H_2 between juxtaposed cells of H_2-producers and H_2-consumers is especially important for organisms which ferment fatty acids, such as butyrate and propionate, since for thermodynamic reasons these processes are inhibited by low levels of H_2 (Boone and Bryant, 1980). Numerous syntrophic H_2-producing strains have been identified as partners in interspecies H_2 transfer. These include strains such as *Desulfovibrio vulgaris*, *Thermoanaerobacter brockii* and *Pelobacter venetianus* which ferment primary alcohols (Bryant et al., 1977; Ben-Bassat et al., 1981; Schink and Stieb, 1983), the butyrate and propionate fermenters *Syntrophomonas wolfei*, *Syntrophomonas bryantii*, *Syntrophobacter wolinii*, *Syntrophobacter pfennigii* and *Smithella propionica* (McInerney et al., 1979; Boone and Bryant, 1980; Stieb and Schink, 1985; Wallrabenstein et al., 1995; Liu et al., 1999), the acetate oxidizer *Thermoacetogenium phaeum* (Hattori et al., 2000), and the oligosaccharide-fermenting strain Thermicanus aegypticus (Gössner et al., 1999). Interestingly, the propionate fermenters isolated so far are all capable of reducing sulfate (Harmsen et al., 1995; Conrad, 1999). On the other hand, there is important indirect evidence that a significant portion of the syntrophic population of some sediments is made up of sulfate reducers. Addition of sulfate to freshwater methanogenic sediments caused an immediate cessation of methane production (Lovley et al., 1982b; Conrad et al., 1987). The most likely explanation for this observation is that sulfate reducers, which at low-sulfate concentrations obtain energy mainly via fermentation and therefore release H_2 to rid themselves of excess reductant, immediately switch to respiration when sulfate is available as the terminal electron acceptor. In doing so, they cut off the supply of H_2 to the methanogens. A similar effect has been observed in methanogenic soils of rice paddies (Achtnich et al., 1995a; Achtnich et al., 1995b; Krylova et al., 1997). Sulfate reducers are probably predestined to participate in syntrophic associations because of the ambivalent nature of their H_2-metabolism: They can take on the role of H_2-consumer or H_2-producer, depending on the sulfate concentration in the environment (Boone and Bryant, 1980; McInerney et al., 1981b; Traore et al., 1981).

Physiology—Varieties of H_2 Metabolism

This section is a brief summary of the major metabolic activities involving H_2 in prokaryotes. In light of the diversity of the metabolic types, it is obvious that this discussion is intended as an overview emphasizing the various physiological roles assumed by hydrogenases.

H_2-Evolving Processes

Fermentation The decomposition of organic matter via fermentation is one of the major biotic energy-yielding processes in anaerobic habitats (reviewed by The Anaerobic Way of Life in this Volume). Various types of fermentation result in the formation of H_2 as a terminal product and hence constitute a substantial contribution to the global H_2 balance. Both obligate and facultative fermenters produce H_2. One of the best-studied representatives of the former group is *Clostridium pasteurianum*. This bacterium ferments glucose and other substrates and evolves H_2 as a means of dispersing excess reductant. Hydrogen

production is catalyzed by two monomeric [FeFe] hydrogenases (Mortenson and Chen, 1974; Adams et al., 1989; Adams, 1990). A classical example of fermentative H_2 production in a facultative fermenter is mixed-acid fermentation in *E. coli*. This organism produces H_2 via the formate hydrogenlyase reaction (reviewed by Sawers [1994]). The hydrogenase 3, one of four [NiFe] hydrogenase isoenzymes in *E. coli*, is part of a membrane-bound complex containing the enzyme formate dehydrogenase (Sawers et al., 1985; Sawers and Boxer, 1986a; Böhm et al., 1990; Sauter et al., 1992). The formate hydrogenlyase complex converts formate, an intermediary fermentation product, to the gaseous products CO_2 and H_2 (Fig. 2).

Fermentative bacteria constitute a major group of rumen flora, and as such, they are instrumental in the breakdown of cellulose and other biopolymers (reviewed by Hungate [1966] and Flint [1997]). Representatives of this group such as *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Megasphaera elsdenii* and *Eubacterium limosum* ferment various substrates to organic acids, CO_2 and H_2 (Miller and Wolin, 1973; Joyner et al., 1977; Miller and Wolin, 1979). Hydrogen does not accumulate in the digestive tract, however, since it is immediately consumed by methanogens and/or homoacetogens (Sharak-Genthner and Bryant, 1987). *Megasphaera elsdeni* is with respect to H_2 metabolism the best-studied organism of this group. It produces a monomeric, [FeFe] hydrogenase which is one of the smallest of all (Van Dijk et al., 1979; Filipiak et al., 1989; Atta and Meyer, 2000).

The thermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis* obtain energy via a strictly fermentative metabolism and dispose of surplus reducing equivalents in the form of H_2 or H_2S (Bryant and Adams, 1989; Schäfer and Schönheit, 1991; Pedroni et al., 1995; Rákhely et al., 1999). These organisms contain specialized cytoplasmic, tetrameric [NiFe] hydrogenases called "sulfhydrogenases." The role of this type of hydrogenase could be to transfer electrons from a donor (reduced nicotinamide adenine dinucleotide phosphate [$NADPH_2$] in the case of *P. furiosus* or reduced ferredoxin in the case of *T. litoralis*) to protons or, in the presence of S^0, to sulfur, thereby catalyzing the formation of H_2 or H_2S, respectively (Malik et al., 1989; Blumentals et al., 1990; Ma et al., 1993; Ma and Adams, 1994; Rákhely et al., 1999). *Pyrococcus furiosus* synthesizes two sulfhydrogenase isoenzymes encoded by duplicated genes (Bryant and Adams, 1989; Ma et al., 1993; Ma et al., 2000) and a third hydrogenase that is clearly related to the multisubunit [NiFe] hydrogenase 3 of *E. coli* (Sapra et al., 2000). The physiological role of the latter enzyme is supposedly comparable to that of the sulfhydrogenases: evolution of H_2 to dispose of reducing equivalents during fermentation. A complex isolated from *P. furiosus* membranes under mild conditions was shown to possess H_2-evolving activity using reduced *P. furiosus* ferredoxin as electron donor (Silva et al., 2000). The fact that this activity is sensitive to inhibition by a complex I inhibitor suggests that the complex engages in proton pumping.

Another example of fermentative H_2 production is in *Thermotoga maritima*, a thermophilic, strictly anaerobic bacterium found in warm marine sediments (Huber et al., 1986). Fermentative H_2 evolution in *T. maritima* is catalyzed by a heterotrimeric cytoplasmic [FeFe] hydrogenase (Verhagen et al., 1999). The physiological redox carrier of *T. maritima* hydrogenase is not known. Neither reduced ferredoxin nor $NADPH_2$ func-

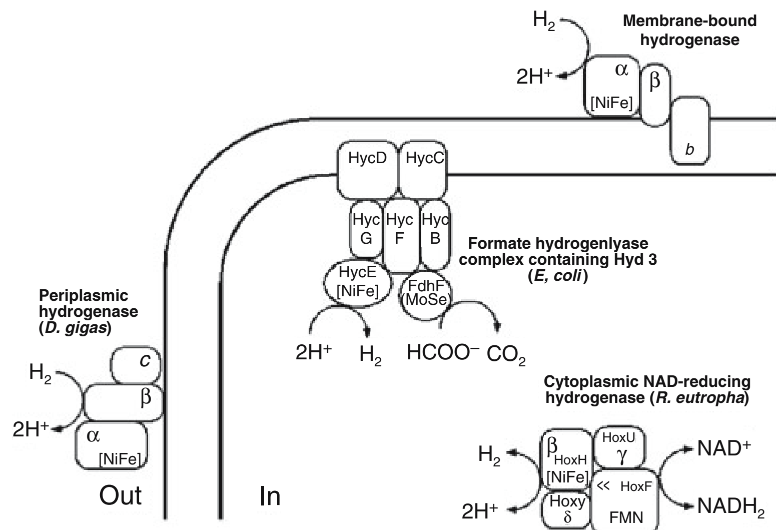
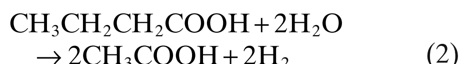


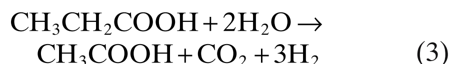
Fig. 2. Representatives of major classes of bacterial hydrogenases: subunit composition, catalytic activity, and cellular location. See text for details.

tioned as electron donors in vitro (Blamey et al., 1994; Verhagen et al., 1999).

Another group of specialized fermenters are the syntrophic, fatty-acid oxidizers which inhabit anaerobic sediments (reviewed by Syntrophism among Prokaryotes in this Volume). These bacteria ferment butyrate and propionate via reactions that are endergonic under standard conditions (Thauer et al., 1977):



$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \quad (2)$$



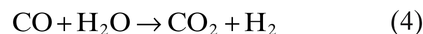
$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \quad (3)$$

To solve the thermodynamic problem inherent in these reactions, the syntrophic fermenters live in aggregates with H_2-utilizing organisms such as methanogens. Hydrogen is consumed as rapidly as it is produced, preventing its accumulation in the milieu. Thus, the reactions are "pulled" in the direction of the oxidized products (cf., the discussion of syntrophic associations in the section Syntrophy and Interspecies H_2 Transfer in this Chapter).

The cyanobacteria are phototrophic organisms. However, many cyanobacterial strains switch to a fermentative metabolism during periods of darkness when O_2 of the milieu is exhausted by respiration (reviewed in Stal and Moezelaar, 1997). In this metabolic state, they usually consume endogenous reserves (such as glycogen) that accrue during photosynthetic growth. In *Microcystis* PCC7806 and *Cyanothece* PCC7822, for instance, endogenous glycogen is fermented to ethanol, acetate, lactate, CO_2 and H_2 via a mixed acid pathway using ferredoxin as oxidant (van der Oost et al., 1989; Moezelaar and Stal, 1994). Hydrogenase couples H_2 production to ferredoxin reoxidation. *Microcoleus chthonoplastes* employs a similar mixed acid-type fermentation to generate energy under anoxic conditions (Moezelaar et al., 1996). However, this organism produces hydrogen from formate via a formate hydrogenlyase reaction.

Many anoxygenic phototrophs ferment endogenous reserves in the dark and thereby produce H_2 as one of the fermentation products. *Rhodospirillum rubrum* grows anaerobically in the dark on fructose or pyruvate (Schön, 1968; Uffen and Wolfe, 1970; Schön and Biedermann, 1973; Uffen, 1973a; Uffen, 1973b; Jungermann and Schön, 1974). The key enzyme for the fermentation of pyruvate is pyruvate-formate lyase (Gorrell and Uffen, 1977; Gorrell and Uffen, 1978). Formate hydrogenlyase catalyzes the evolution of CO_2 and H_2 from formate (Kohlmiller and Gest, 1951). *Rhodobacter capsulatus* ferments fructose to succinate, lactate, acetate, CO_2 and H_2 (Yen and Marrs, 1977; Madigan and Gest, 1978; Schultz and Weaver, 1982). Also capable of fermentation are *Rhodopseudomonas palustris*, *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* (Uffen and Wolfe, 1970).

Anaerobic CO Oxidation The anoxygenic photosynthetic bacterium *Rhodospirillum rubrum* can grow anaerobically in the dark on CO as the sole source of energy (Uffen, 1976). Under these conditions, it forms a membrane-bound enzyme complex consisting of carbon monoxide dehydrogenase (CODH) and a CO-insensitive [NiFe] hydrogenase (Fox et al., 1996a; Fox et al., 1996b). Together these enzymes catalyze the following net reaction:



$$CO + H_2O \rightarrow CO_2 + H_2 \quad (4)$$

Like the hydrogenase 3 of *E. coli*, this multienzyme complex includes other proteins related to reduced nicotinamide adenine dinucleotide (NADH):quinone dehydrogenase (complex I; Fox et al., 1996a). The fact that CO supports growth of *R. rubrum* in the dark argues for an energy conserving function of the CO dehydrogenase-hydrogenase complex. Another well-studied example of carboxydotrophy is the Gram-positive bacterium *Carboxydothermus hydrogenoformans* (Svetlichny et al., 1991; Soboh et al., 2002).

Production of H_2 as a Byproduct of N_2 Fixation Nitrogen fixation is one of the main processes of biogenic H_2 production but is unique for the reason that it does not involve a specialized H_2-forming enzyme, i.e., a hydrogenase, but rather nitrogenase. Although beyond the scope of a review dedicated to hydrogenases, nitrogenase-mediated H_2 production deserves mention on account of its global dimension. About 30–50% of the total reducing power consumed by nitrogenase is side-tracked into the formation of H_2 (Schubert and Evans, 1976; Brewin, 1984; Evans et al., 1987). This phenomenon, a paradox considering the efficiency of other enzyme systems, raises the question of a biological role for this "side effect." However this may be, N_2-fixing microbes often possess uptake hydrogenases which permit them to recover at least a part of the energy flowing into nitrogenase-mediated H_2 production. Surprisingly, however, this is by no means a universal feature of diazotrophs. In many soil habitats, both H_2-oxidizing and H_2-nonoxidizing strains of the same diazotrophic species can be isolated. Therefore, significant quantities of H_2 must escape into the environment (reviewed by Evans et al., 1987). This could be a major source of nutrient for H_2-utilizing microbes such as the aerobic H_2-oxidizing bacteria (La Favre and Focht, 1983; Schuler and Conrad, 1991b).

The capacity to reduce atmospheric dinitrogen is found both in archaea and bacteria, in aerobic as well as in anaerobic organisms (see Dinitrogen-Fixing Prokaryotes in this Volume for a list). Among the best-studied diazotrophs are the rhizobia including *Bradyrhizobium japonicum*, *Sinorhizobium meliloti* and *Rhizobium leguminosarum* (reviewed in Hennecke [1990], Agron et al. [1994], Stacey et al. [1995], van Rhijn and Vanderleyden [1995], Ovtsyna et al. [2000], and Spaink [2000]), strains of *Azotobacter* (reviewed by Peters et al., 1995) and various cyanobacteria (Fay, 1992; Haselkorn and Buikema, 1992). Many oxygenic and anoxygenic phototrophs fix dinitrogen and produce H_2 concomitantly. Gest and Kamen discovered that cultures of *R. rubrum* grown in the light under nitrogenase-inducing conditions evolved significant amounts of H_2 (Gest and Kamen, 1949a; Gest and Kamen, 1949b). This process was referred to as "photoproduction" of H_2. Subsequently, H_2 photoproduction was observed in other phototrophic bacteria including *R. capsultus*, *R. gelatinosus* and *R. palustris*. Later investigations revealed that nitrogenase-catalyzed reduction of protons is the basis of H_2 photoproduction (Bulen et al., 1965a; Bulen et al., 1965b). Recently, a different kind of photoproduction has been postulated. A hypothesis has been put forward that hydrogenases may act as redox buffers for the photosynthetic apparatus during transition from darkness to light (Appel and Schulz, 1998; Appel et al., 2000). During such a transition, electrons from the photosynthetic apparatus could be channeled to H_2 via a hydrogenase. Indeed, in *Synechocystis*, a darkness-to-light transition triggers a transient production of H_2 (Abdel-Basset and Bader, 1998). This hypothesis awaits confirmation by additional experimental studies.

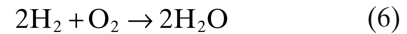
Production of H_2 as a Byproduct of Phosphite Oxidation Recently, a novel pathway for phosphite oxidation was discovered in *E. coli* (Yang and Metcalf, 2004). The well known periplasmic enzyme alkaline phosphatase (BAP) turned out to be the sole enzyme of this pathway, which catalyzes the following reaction:

$$H_3PO_3 + H_2O \rightarrow H_3PO_4 + H_2 \quad (5)$$

Thus, BAP is an H_2-evolving hydrogenase. To date, BAP is the only microbial phosphatase show to have this activity.

H_2-Consuming Processes

Aerobic H_2 Oxidation The aerobic hydrogen-oxidizing (knallgas) bacteria attracted the attention of microbiologists early on (Kaserer, 1906; Niklewski, 1910). These organisms utilize H_2 as a source of energy via the oxyhydrogen reaction:



$$2H_2 + O_2 \rightarrow 2H_2O \quad (6)$$

The first part of the above net reaction, a heterolytic cleavage of H_2, is catalyzed by various types of [NiFe] hydrogenases. It turned out that the first knallgas bacteria to be isolated and systematically studied were facultative H_2 chemolithoautotrophs, which in the presence of sugars or organic acids grew organoheterotrophically. Furthermore, many of these organisms utilized H_2 and organic substances mixotrophically (reviewed by Aragno and Schlegel [1992]). The first obligate H_2 chemolithotrophs were discovered relatively recently. The aerobic thermophile *Hydrogenobacter thermophilus* is an obligate chemolithoautotroph (Kawasumi et al., 1984). The bacterium can, however, use H_2 or elemental sulfur alternatively as electron donors. The marine bacterium *Hydrogenovibrio marinus* is an obligate H_2 oxidizer (Nishihara et al., 1991; Nishihara et al., 1997). Mixotrophic H_2 utilization probably plays an important role in pathogens of the human gastrointestinal tract. In the stomach pathogen *Helicobacter pylori*, for instance, hydrogenase significantly effects the efficiency of colonization of the gastric mucosa in the mouse experimental system (Olson and Maier, 2002).

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) is one of the classical knallgas bacteria (Wilde, 1962) and is now one of the best-studied H_2 oxidizers. *Ralstonia eutropha* thrives on mixtures of H_2 and CO_2 but can, alternatively, utilize a broad spectrum of organic compounds. Moreover, it can also utilize H_2 and organic substrates simultaneously. The bacterium contains two energy-generating [NiFe] hydrogenases: A membrane-bound type and a tetrameric, cytoplasmically localized species (Schneider and Schlegel, 1976; Schink and Schlegel, 1979). The membrane-bound enzyme is anchored to the periplasmic face of the cytoplasmic membrane and feeds electrons into a respiratory chain via a *b*-type cytochrome (Fig. 2). This type of hydrogenase is very widespread and is the basis of H_2 oxidation in most of the aerobic H_2 oxidizers examined so far. The soluble, tetrameric hydrogenase of *R. eutropha* was the first of its kind to be characterized genetically (Tran-Betcke et al., 1990). It consists of a hydrogenase moiety complexed with a NADH oxidoreductase module (Fig. 2). The enzyme couples the oxidation of H_2 to the reduction of NAD^+. A similar tetrameric [NiFe] hydrogenase is found in the Gram-positive, facultative H_2-oxidizer *Rhodococcus opacus* (formerly *Nocardia opaca*; Schneider et al., 1984a, 1984b; Grzeszik et al., 1997b).

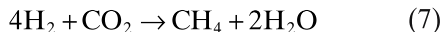
Some diazotrophs are facultative chemolithoautotrophs. This dual strategy makes sense for inhabitants of the rhizosphere, since they

often form hydrogenase to recycle H_2 produced during N_2 fixation and hence have the enzymatic tools to exploit H_2 produced externally by other diazotrophs. The endosymbiotic N_2-fixer *Bradyrhizobium japonicum* is an example for this group (Hanus et al., 1979). Outside the root nodule, *B. japonicum* is able to grow on H_2 and CO_2 as sole sources of energy and carbon. *Azotobacter vinelandii* is a free living, strictly aerobic diazotroph. It is not an autotroph, but thrives mixotrophically on H_2 in the presence of organic substrates (Wong and Maier, 1985).

In the aerobic carboxydotrophs, CO can serve as the sole source of energy and carbon (reviewed in Meyer, 1989). Many of these bacteria also thrive on H_2 and CO_2, and mixotrophic growth on H_2 or CO in the presence of organic acids has also been reported (Kiessling and Meyer, 1982). In *Oligotropha carboxydovorans*, oxidation of H_2 is catalyzed by a membrane-bound hydrogenase (Santiago and Meyer, 1997; Fig. 2).

Some phototrophs are facultative H_2 chemolithotrophs (Bogorov, 1974; Madigan and Gest, 1979; Siefert and Pfennig, 1979; Kämpf and Pfennig, 1980; Kämpf and Pfennig, 1986). This has been shown for *Rhodobacter capsulatus*, *Rhodobacter sulfidophilus* and *Rhodopseudomonas acidophila*, which grow in the dark on H_2 as sole source of energy and reducing power.

METHANOGENESIS Methanogenesis is one of the major H_2-consuming processes in the biosphere. The global, annual rate of natural CH_4 production has been estimated to be 190 ± 70 Tg (Lelieveld et al., 1998). Hydrogenotrophic methanogens are true lithotrophs that convert H_2 and CO_2 to CH_4 according to the following net reaction:



$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (7)$$

Other methanogens can utilize partially reduced forms of carbon such as methanol:

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \quad (8)$$

Whatever carbon compound is used as substrate, H_2 is the source of reductant in most methanogens and, hence, hydrogenases participate at different stages of methanogenesis. The enzymatic pathway varies depending on the carbon substrate and organism (reviewed in Thauer [1998] and Keltjens and Vogels [1993]). The production of CH_4 from CO_2 proceeds via an initial reaction in which *N*-formylmethanofuran is formed. In *Methanosarcina barkeri*, this reaction is dependent on reducing equivalents generated by the *Escherichia coli* hydrogenase3-like hydrogenase (Ech) (Meuer et al., 2002; Fig. 3). Similar membrane-bound complexes are thought to fulfill the same function in species of *Methanothermobacter* (Tersteegen and Hedderich, 1999). Following the formation of *N*-formylmethanofuran, the C_1 unit is transferred to another carrier, tetrahydromethanopterin (H_4MPT), and reduced in a stepwise fashion: N^5, N^{10}-methenyl-H_4MPT is first converted to N^5, N^{10}-methylene-H_4MPT. Subsequently N^5, N^{10}-methylene-H_4MPT is reduced to N^5-methyl-H_4MPT. A cytoplasmic [NiFe] hydrogenase, the trimeric F_{420}-reactive hydrogenase present in two isoenzymes, contributes reducing equivalents in the form of reduced cofactor F_{420} which drive these reactions (Fig. 3). When deprived of nickel, *M. thermoautotrophicus* and some other methanogens produce high levels [Fe] hydrogenase, the so-called "H_2-forming H_4MPT dehydrogenase." Hydrogen-forming H_4MPT dehydrogenase, also called "Hmd hydrogenase," is a cytoplasmic homodimer. This enzyme apparently substitutes for the nickel-containing F_{420}-reactive hydrogenase, catalyzing the reduction of coenzyme F_{420}. In the final stage of methanogenesis, CH_3-S-CoM and HS-CoB are oxidized to the heterodisulfide CoM-S-S-CoB liberating CH_4 (Fig. 3). The enzyme heterodisulfide reductase then regenerates the reduced forms of coenzyme M and coenzyme B via the reduction of CoM-S-S-CoB using reducing equivalents generated by hydrogenase. The heterodisulfide reductase differs from species to species. In strains of *Methanosarcina*, the enzyme is membrane-bound. It receives its reducing power from a hydrogenase attached to the outer surface of the cytoplasmic membrane via the electron carrier methanophenazine (Fig. 3). The reduction of CoM-S-S-CoB results in the formation of scalar protons and, hence, a gradient across the membrane which couples H_2 oxidation to phosphorylation of ADP. In other species (e.g., strains of *Methanothermobacter*, *Methanococcus* and *Methanopyrus*), the heterodisulfide reductase and an F_{420}-nonreducing hydrogenase form a soluble complex located in the cytoplasm (Setzke et al., 1994).

Methanosarcina barkeri, *M. mazei* and other methylotrophic methanogens can grow on substances such as methanol, methylamines and acetate in addition to H_2 and CO_2 (Keltjens and Vogels, 1993; Deppenmeier et al., 1999). Regardless of what substrate is utilized, CH_4 production leads to the formation of the heterodisulfide CoM-S-S-CoB and the pools of coenzymes HS-CoM and HS-CoB are replenished by the action of the heterodisulfide reductase. If adequate amounts of H_2 are available, the reducing power for this reaction is supplied by membrane-bound methanophenazine-reducing hydrogenases. In the absence of H_2, reducing power comes from a membrane-bound $F_{420}H_2$-dehydrogenase, which oxidizes coenzyme F_{420} in an energy-conserving manner. Both hydrogenase and

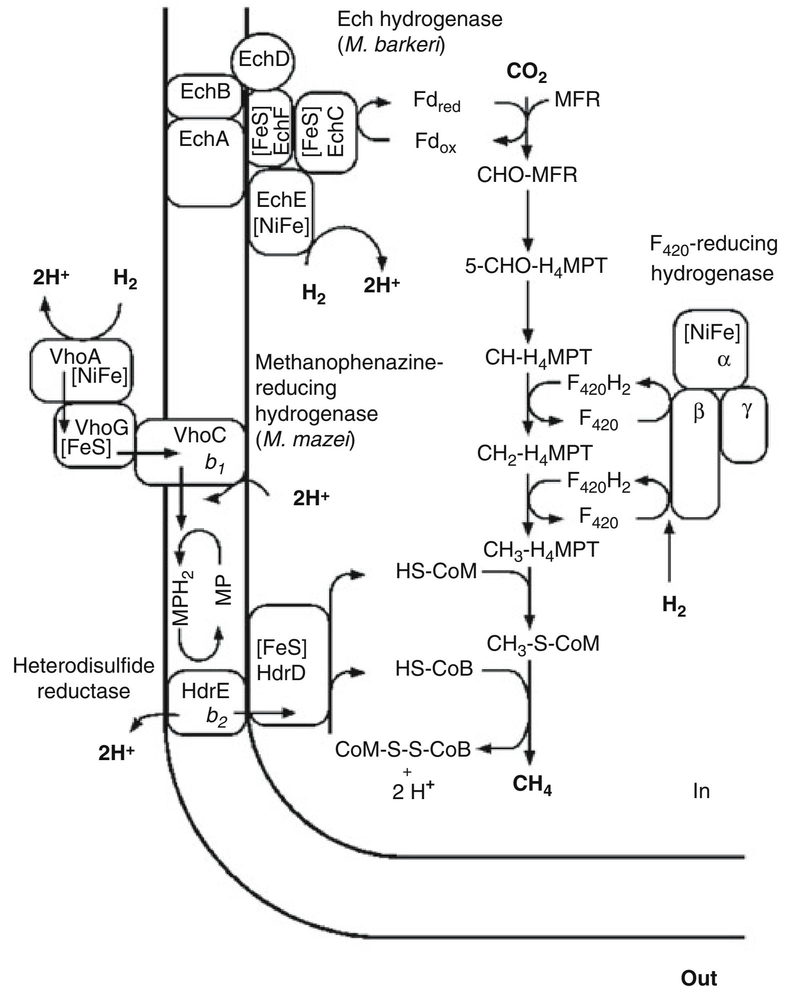


Fig. 3. Archaeal hydrogenases of *Methanosarcina* strains: subunit composition, catalytic activity and cellular location. MP, Methanophenazine; MFR, methanofuran; CHO-MFR, formyl-methanofuran; HS-CoB. Coenzyme B; HS-CoM, coenzyme M; Fd_{red}/Fd_{ox}, reduced/oxidized ferredoxin; b_1, and b_2, cytochromes b_1, and b_2, respectively. See text for additional details.

$F_{420}H_2$-dehydrogenase are coupled to the heterodisulfide reductase via the redox intermediate methanophenazine (Deppenmeier, 1995a).

The Ech hydrogenase of *Methanosarcina barkeri* is part of a multisubunit membrane-bound complex related to hydrogenase 3 of *E. coli* (Künkel et al., 1998). Recent studies point to multiple metabolic roles for the Ech hydrogenase (Meuer et al., 2002). During growth on H_2 and CO_2, Ech oxidizes H_2, transferring the electrons to ferredoxin. Reduced ferredoxin provides the reducing power for the first step of methanogenesis, the reduction of CO_2 to formylmethanofuran. In acetoclastic methanogenesis, Ech couples the oxidation of reduced ferredoxin (arising from the oxidation of the carbonyl group of acetate) to the production of H_2. Finally, during growth on methanol, H_2 and CO_2, Ech seems to have a biosynthetic role.

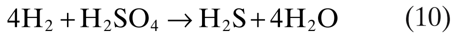
Acetogenesis When organisms such as *Acetobacterium woodii*, *Moorella thermoacetica*, *Moorella thermoautotrophica* and *Clostridium aceticum* grow on CO_2 and H_2, CO_2 is converted to acetate via the Ljungdahl-Wood pathway (see Acetogenic Prokaryotes in this volume). This reaction proceeds via the following stoichiometry:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (9)$$

The reducing power for this reaction is ultimately derived from H_2 by the action of hydrogenases (Drake, 1982; Pezacka and Wood, 1984). *Acetobacterium woodi* contains a soluble hydrogenase of the [FeFe] type (Ragsdale and Ljungdahl, 1984). *Sporomusa sphaeroides* oxidizes H_2 with the help of a dimeric, membrane-bound, [NiFe] hydrogenase (Dobrindt and Blaut, 1996).

Sulfate and Sulfur Reduction The different strains of sulfate-reducing bacteria and archaea use a spectrum of electron donors including alcohols, H_2 and organic acids such as acetate, lactate, malate and pyruvate (reviewed by Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes in this Volume). The electron accep-

tor, sulfate, sulfite, thiosulfate or elemental sulfur, is reduced to sulfide. via the following net reactions:



$$4H_2 + H_2SO_4 \rightarrow H_2S + 4H_2O \quad (10)$$

$$3H_2 + H_2SO_3 \rightarrow H_2S + 3H_2O \quad (11)$$

$$4H_2 + H_2S_2O_3 \rightarrow 2H_2S + 3H_2O \quad (12)$$

$$H_2 + S \rightarrow H_2S \quad (13)$$

Thus, the oxidation of an organic compound or H_2 is coupled to the reduction of a sulfur compound. This process entails the generation of a proton gradient across the cytoplasmic membrane. Energy is conserved via a chemiosmotic mechanism, for which reason the process is loosely called "anaerobic respiration" (Brandis and Thauer, 1981). Many sulfate reducers thrive on H_2 as the sole source of energy and reductant. This group includes strains of *Desulfovibrio*, *Desulfomicrobium*, *Desulfobacter*, *Desulfobacterium*, *Desulfonema*, *Desulfobulbus*, *Desulfosarcina*, *Thermodesulfovibrio* and *Thermodesulfobacterium* (see Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes in this Volume). Several of these strains can grow lithoautotrophically on H_2 and CO_2 (Jansen et al., 1984; Schauder et al., 1987; Schauder et al., 1989). Autotrophic growth on H_2 and CO_2 has been documented for *Desulfovirio fructosovorans*, *Desulfomicrobium apsheronum* (Rozanova et al., 1988), *Desulfobacterium autotrophicum* (Brysch et al., 1987), *Desulfotomaculum geothermicum* (Daumas et al., 1988), *Desulfotomaculum kuznetsovii* (Rozanova et al., 1988), *Desulfobacter hydrogenophilus*, *Desulfomonas limicola* and *Desulfosarcina variabilis* (Widdel, 1988a). Perhaps the most remarkable property of sulfate reducers is their mixotrophic metabolism, i.e., their ability to utilize H_2 and organic compounds simultaneously. In this case, energy is generated by two different modes operating at the same time: 1) electron-transport phosphorylation driven by the hydrogenase-dependent respiratory chain and 2) substrate-level phosphorylation coupled to the oxidation of an organic substrate. Hydrogen is one of the products of the latter process. If sufficient sulfate is present, net production of H_2 seldom occurs. Hydrogen produced via fermentation is reoxidized via the hydrogenase-dependent respiratory chain (Tsuji and Yagi, 1980). Under low-sulfate conditions, H_2 is produced and released into the environment (Postgate, 1952; Vosjan, 1975; Hatchikian et al., 1976; Traore et al., 1981).

Extensive studies on sulfate-reducing bacteria have revealed the existence of multiple hydrogenases in one and the same organism (see Voordouw [1992] for a list). Various strains of *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*, for instance, form three different hydrogenases, one representative each of the [FeFe], [NiFe] and [NiFe(Se)] types. The assignment of physiological functions to the hydrogenases is difficult in such cases. One approach to the problem is the analysis of mutants with defined genetic lesions. *Desulfovibrio fructosovorans* forms three different hydrogenases: Two of these, an [FeFe] and a [NiFe] enzyme, are periplasmic. The third hydrogenase is a tetrameric, cytoplasmic, NADP-dependent [FeFe] enzyme (Rousset et al., 1990; Malki et al., 1995; Malki et al., 1997). The former enzymes are probably part of an H_2-dependent respiratory chain. The latter could couple H_2 oxidation directly to the generation of reducing equivalents. Mutants defective for the [NiFe] hydrogenase or the cytoplasmic hydrogenase or both still grew well on H_2 and sulfate. During growth on fructose, lactate and pyruvate, the mutants behaved differently: The strain defective for the [NiFe] enzyme grew as well as the wildtype. Growth of the strains lacking a functional cytoplasmic hydrogenase was significantly curtailed (Malki et al., 1997). Thus, the bioenergetic contributions of the three hydrogenases are not sharply defined and seem to allow for a certain degree of mutual compensation. However, the cytoplasmic [FeFe] hydrogenase is evidently more important during growth involving fermentative utilization of organic substrates. *Desulfovibrio vulgaris* Hildenborough harbors a soluble periplasmic [FeFe] hydrogenase and two membrane-bound enzymes: a [NiFe] hydrogenase and a [NiFe(Se)] hydrogenase. The genome sequence of this organism indicates the presence of a fourth hydrogenase akin to hydrogenase 3 of *E. coli* (Pohorelic et al., 2002). A *Desulfovibrio vulgaris* mutant defective for the [FeFe] hydrogenase showed reduced growth both on H_2 and on lactate (Pohorelic et al., 2002). Under the latter conditions, the mutant also liberated much more H_2 than the wildtype. Thus, the [FeFe] hydrogenase is involved in the utilization both of external H_2 and of internally generated H_2 arising from the fermentation of organic substrates. The respiration of sulfate with H_2 as electron donor has been reported for other groups of bacteria aside from the classical sulfate reducers. One example of this is *Allochromatium minutissimum* (Nakamura, 1939; Nakamura, 1941), which grows anaerobically in the dark on H_2 and sulfate.

In some anaerobic H_2-oxidizers, sulfur takes the place of sulfate as electron acceptor in the oxidation of H_2 (see Hedderich et al. [1999] for a list). These organisms are typically found in hot, marine solfataras. The marine thermophilic archaeon *Thermoproteus neutrophilus* is an obli-

gate chemolithoautotroph, growing on H_2 and elemental sulfur with CO_2 as sole carbon source (Fischer et al., 1983). Its relatives, *Thermoproteus tenax* and *Pyrobaculum islandicum*, are H_2-utilizing chemolithotrophs that can grow in a facultative chemoorganotrophic mode, respiring sulfur (Fischer et al., 1983). *Pyrodictium occultum* and *Pyrodictium brockii* are both hyperthermophilic archaea that obtain energy via H_2/S chemolithotrophy (Fischer et al., 1983; Stetter and Gaag, 1983a). In the latter organism, H_2 oxidation is catalyzed by a dimeric, membrane-bound [NiFe] hydrogenase (Pihl et al., 1989; Pihl and Maier, 1991). The moderately thermophilic, strictly anaerobic bacterium *Desulfurella multipotens* is a facultative chemolithotroph growing on H_2 and elemental sulfur (Miroshnichenko et al., 1994).

Mixotrophic growth has been reported for another member of the genus *Pyrodictium*: The marine hyperthermophile *Pyrodictium abyssi* (Pley et al., 1991). Unlike the other two species, it is a heterotroph, growing by fermentation of organic compounds. The addition of elemental sulfur to heterotrophic cultures has little effect on growth. However, when H_2 and S are provided in addition to organic substrates, growth is markedly stimulated and sulfur is reduced to H_2S. Perhaps the most impressive examples of the metabolic versatility of the sulfur-reducers are the archaeon *Acidianus infernus* and its relatives (Segerer et al., 1986). Under anaerobic conditions these organisms grow by H_2/S chemolithotrophy. They are, however, facultative aerobes: In the presence of O_2 they oxidize sulfur to sulfuric acid. The exploitation of a variety of electron acceptors for the oxidation of H_2 is not limited to archaea. The bacterium *Sulfurospirillum deleyianum* (Schumacher et al., 1992; Eisenmann et al., 1995) can grow on H_2 or elemental sulfur as sole source of energy and reducing power. It utilizes a palette of electron acceptors including oxygen, nitrate, nitrite, sulfur compounds, and organic acids. The hyperthermophile *Aquifex pyrophilus* (Huber et al., 1992) grows on H_2 in the presence of O_2 just like a conventional knallgas bacterium. Alternatively, both S^0 and $S_2O_3^{-2}$ can serve as electron donors in place of H_2. Under anoxic conditions, *A. pyrophilus* can grow on H_2, S^0 or $S_2O_3^{-2}$ using nitrate as the terminal electron acceptor. Furthermore, in the late stage of exponential growth on H_2 under oxic conditions, *A. pyrophilus* switches from O_2 to S^0 as the terminal electron acceptor and begins producing H_2S.

The model organism for biochemical and molecular studies on anaerobic respiration is *Wolinella succinogenes* (Hedderich et al., 1999; Lancaster, 2001). This anaerobic, rumen organism oxidizes H_2 using sulfur in the form of polysulfide as the terminal electron acceptor (Jacobs and Wolin, 1963; Kröger and Innerhofer, 1976; Bronder et al., 1982; Jankielewicz et al., 1995). The membrane-bound [NiFe] hydrogenase is coupled to the membrane-bound polysulfide reductase via the redox carrier menaquinone (Dross et al., 1992).

Fe(III) Reduction Many strains of Fe(III) reducing bacteria are capable of using H_2 as an electron donor. The coupling of H_2 oxidation to the reduction of Fe(III) is an important energy-yielding process in subsurface microbial communities. Members of the family Geobacteriaceae are also capable of reducing other metals such as Mn(IV) and U(VI). Both *Geobacter hydrogenophilus* and *Geobacter sulfurreducens* grow on H_2 (Coates et al., 1991; Caccavo et al., 1994). In *Geobacter sulfurreducens*, H_2 oxidation is catalyzed by a membrane-bound hydrogenase (Coppi et al., 2004).

Dehalorespiration Certain bacteria exploit specialized respiratory chains, in which the oxidation of H_2 or organic acids is coupled to the dehalogenation of haloaliphatic or haloaromatic compounds. This type of energy metabolism is known as dehalorespiration and has been found in both gram-negative and gram-positive bacteria. Hydrogenases and reductive dehalogenases are key components of these pathways. Among the representatives of this group are both strict H_2 oxidizers, such as *Dehalobacter restrictus* (Holliger et al., 1998), and organisms, such as *Halospirillum multivorans* (Scholz-Muramatsu et al., 1995), which can utilize a variety of electron donors. Both of these strains contain membrane-bound hydrogenases.

Anoxygenic Photosynthesis In the versatile metabolism of the anoxygenic phototrophic bacteria, H_2 has different roles depending on the growth conditions (Vignais et al., 1985). Two of the metabolic functions of H_2 have been discussed above: H_2 is consumed as a source of energy and reductant during aerobic, chemolithoautotrophic growth and is produced as a fermentation product during anaerobic, heterotrophic growth. A third role of H_2 is linked to anaerobic growth in the light: Many anoxygenic phototrophs can utilize H_2 as an electron donor for photoautotrophic growth. This was first shown for the purple sulfur bacterium *Allochromatium minutissimum* (Roelofsen, 1934; Gaffron, 1935). Photosynthetic H_2 oxidation is dependent on uptake hydrogenases (Gest, 1951). Both purple nonsulfur and purple sulfur bacteria are capable of photosynthetic growth on H_2 (see

Drews and Imhoff [1991] for a list). Included in this group are *Rhodospirillum rubrum* (Ormerod and Gest, 1962; Anderson and Fuller, 1967), *Rhodopseudomonas palustris* (Qadri and Hoare, 1968), *Rubrivivax gelatinosus* (Wertlieb and Vishniac, 1967), *Rhodobacter capsulatus* (Klemme and Schlegel, 1967), *Allochromatium vinosum* (Gitlitz and Krasna, 1975) and *Thiocapsa roseopersicina* (Gogotov, 1968). In some if not all of the above-named organisms, oxidation of H_2 for photosynthetic growth is catalyzed by membrane-bound [NiFe] hydrogenases (Gitlitz and Krasna, 1975; Adams and Hall, 1977; Bagyinka et al., 1982; Kondratieva and Gogotov, 1983; Kovacs et al., 1983; Gogotov, 1984; Zorin et al., 1996; Fig. 2).

The purple sulfur bacterium *Thiocapsa roseopersicina* contains at least three hydrogenases (Colbeau et al., 1994; Rákhely et al., 1998; Kovács et al., 2002; Rákhely et al., 2004). Two of these hydrogenases are dimeric, membrane-bound enzymes; the third is a cytoplasmic, pentameric hydrogenase. While the precise physiological roles of the three species remain to be delineated, one of the membrane-bound enzymes (HupSL) seems to be involved in the recycling of H_2 produced by nitrogenase (Kovács and Bagyinka, 1990). The soluble, NAD-reducing hydrogenase catalyzes the evolution of H_2 in vivo. The physiological significance of the later reaction is unclear.

Ancillary Processes This category comprises H_2-consuming metabolic activities that are allied to and dependent upon H_2-evolving processes in one and the same cell. Here we are dealing with hydrogenases whose primary purpose is to consume internally produced H_2. Hydrogenase-free strains of the organisms in question are readily isolated from natural habitats and thrive despite the lack of H_2-activating enzymes. The foremost activity of this kind is the so-called “hydrogen recycling” observed in many diazotrophs. This is the role of the dimeric, membrane-bound, [NiFe] hydrogenases in the endosymbiotic N_2-fixers such as *Rhizobium leguminosarum* (Brewin, 1984). A similar function can be ascribed to free-living N_2-fixers such as *Azotobacter chroococcum* (Ford et al., 1990), *Bradyrhizobium japonicum* (Harker et al., 1984; Sayavedra-Soto et al., 1988), *Rhodobacter capsulatus* (Willison et al., 1983; Leclerc et al., 1988) and *Rhodocyclus gelatinosus* (Uffen et al., 1990). In the latter three cases hydrogenases can also catalyze the oxidation of externally available H_2 and hence support facultative chemolithotrophic growth. Another example which falls into this category is the hydrogenase-mediated consumption of H_2 which is evolved as a terminal product of fermentation. This is probably the role of one or both of the hydrogenase isoenzymes 1 and 2 of *E. coli* (overview in Sawers, 1994). These two membrane-bound uptake hydrogenases are formed under anoxic conditions, and may act to conserve energy by oxidizing H_2 generated by the formate hydrogen lyase complex. *Desulfovibrio vulgaris* and other sulfate reducers evolve H_2 both during growth on SO_4^{-2} and during fermentation. A portion of this H_2 is reoxidized by hydrogenase in an energy-conserving mechanism called “hydrogen cycling” (Odom and Peck, 1981; Lupton et al., 1984). The methanogen *M. barkeri* deserves mention in this context. During growth on acetate, H_2 is produced by the Ech hydrogenase (Meuer et al., 2002). The internally produced H_2 is consumed by the methanophenazine-reducing hydrogenases.

Classification of Hydrogenases

The first hydrogenases to be isolated and characterized biochemically were the iron-containing enzymes of sulfur-reducing bacteria. Later on, it was discovered that some hydrogenases contain nickel in addition to iron (Friedrich et al., 1981b; Graf and Thauer, 1981). The preponderance of hydrogenases that have been characterized to date are of this type. Some of these enzymes contain selenium in the form of the unusual amino acid selenocysteine (Rieder et al., 1984; He et al., 1989). Finally, [Fe] hydrogenases found in methanogens are a relatively recent discovery (Thauer et al., 1996). These enzymes, called “H_2-evolving N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenases” or “Hmd hydrogenases,” are neither mechanistically nor structurally related to other hydrogenases. Originally classified as “metal-free hydrogenases,” the Hmd’s were subsequently shown to contain iron. Unlike the classical iron hydrogenases, however, the Hmd’s harbor cofactor-bound, redox-inactive iron, but no iron-sulfur centers (Lyon et al., 2004).

Early classification schemes, which had mainly biochemical data to go on, grouped hydrogenases on the basis of metal content or redox cofactors (Fauque et al., 1988; Przybyla et al., 1992). A rapidly growing base of nucleotide sequence data prompted Voordouw to attempt a classification on the basis of comparisons of deduced amino-acid sequences (Voordouw, 1992). In a similar study, Wu and Mandrand (1993) went a step further. These authors generated multiple alignments for full-length amino acid sequences and performed cluster analysis on the pairwise alignment scores. On the basis of the resulting dendrograms, 30 hydrogenases were grouped in six classes. Recently, Vignais and coworkers have refined and extended this classification system,

including a greatly expanded database (Vignais et al., 2001). They carried out thorough cluster analyses using both complete amino acid sequences and segments corresponding to functional domains. The results of this important study support the notion that hydrogenases belong to three phylogenetically distinct groups: [FeFe] hydrogenases, [NiFe] hydrogenases (including [NiFe(Se)] hydrogenases), and [Fe] hydrogenases. Thus, the original, pragmatic classification now has a foundation in molecular phylogenetics.

[NiFe] Hydrogenases

The revised system of Vignais and coworkers subdivides the [NiFe] hydrogenases into four groups (see Vignais et al. [2001] for dendrograms):

Group 1. Energy-transducing hydrogenases Enzymes which couple the oxidation of H_2 to electron-transport phosphorylation. This group includes both membrane-bound hydrogenases attached to the periplasmic side of the cytoplasmic membrane and nonmembrane-bound, periplasmic hydrogenases. The group breaks down into two subclusters. One contains the membrane-bound hydrogenases of the proteobacteria, and the other, archaeal membrane-bound hydrogenases.

Group 2. Sensory hydrogenases These soluble proteins are components of signal-transmitting circuits governing the expression of hydrogenase genes. Specialized hydrogenases of this type have to date been identified in *Bradyrhizobium japonicum*, and strains of *Ralstonia* and *Rhodobacter*. The group includes a second subcluster containing cyanobacterial uptake hydrogenases.

Group 3. Multimeric cytoplasmic hydrogenases These are enzymes of complex subunit composition, which interact with soluble cofactors. The four subclusters contain F_{420}-reducing hydrogenases of methanogenic archaea, F_{420}-nonreducing hydrogenases of *Methanothermobacter* and *Methanococcus* strains, sulfhydrogenases of thermophilic archaea, and cytoplasmic NAD-reducing hydrogenases of *R. eutropha*, *R. opacus* and cyanobacteria.

Group 4. *Escherichia coli* hydrogenase 3 and relatives *Escherichia coli* hydrogenase 3 is an H_2-evolving enzyme, which is part of the multisubunit formate-hydrogen lyase complex. Similar enzymes have been identified in various bacteria and archaea including *R. rubrum*, *C. hydrogenoformans*, *M. barkeri*, *M. thermoautotrophicus* and *P. furiosus*.

[FeFe] Hydrogenases

The [FeFe] hydrogenases are quite heterogeneous in quaternary structure and domain organization (Vignais et al., 2001). Monomeric, dimeric, trimeric and tetrameric enzymes have been described. Moreover, the basic hydrogenase domain is coupled to various other functional modules in one and the same subunit. Even analyses confined to partial sequences correponding to the conserved hydrogenase catalytic site (H-cluster) fail to show well-separated subgroups (Vignais et al., 2001). Thus, a subdivision of the [FeFe] hydrogenases is not feasable at present.

[Fe] Hydrogenases

A third class of hydrogen-activating enzymes consists of the Hmd's. Originaly discovered in *Methanothermobacter marburgensis*, Hmd enzymes have been found in other methanogenic archaea including *Methanococcus voltae* and *Methanopyrus kandleri* (Thauer et al., 1996). The extensive sequence identity in this class of enzymes shows that they are highly conserved.

Biochemistry

The old observation that knallgas bacteria require Ni for growth on H_2, O_2 and CO_2 (Bartha and Ordal, 1965; Tabillion et al., 1980) took on new significance when it was discovered that Ni is essential for the biosynthesis of active hydrogenase in *Ralstonia eutropha* (Friedrich et al., 1981b) and that hydrogenase purified from *Methanothermobacter marburgensis* contains the transition metal Ni (Graf and Thauer, 1981). Since then, extensive biochemical, genetic and spectroscopic analyses have shed light on the molecular structure and catalytic mechanism of the three classes of hydrogenase: [NiFe], [FeFe], and [Fe] hydrogenases.

Three-dimensional structures are now available for both the [NiFe] and [FeFe] hydrogenases. This section focuses on the structure and function of the dinuclear metal site and its interaction with various prosthetic groups either embedded in the same polypeptide or located on other subunits.

[NiFe] Hydrogenases

The Basic Module The basic module of a catalytically active [NiFe] hydrogenase consists of two heterologous subunits of approx. 60 and 30 kDa (reviewed by Albracht [2001] and Fontecilla-Camps et al. [2001]). Three-dimensional structures of [NiFe] hydrogenases

from anaerobic sulfate-reducing bacteria, including *Desulfovibrio gigas* (Volbeda et al., 1995; Volbeda et al., 1996), *Desulfovibrio vulgaris* (strain Miyazaki F; Higuchi et al., 1997), *Desulfovibrio fructosovorans* (Montet et al., 1997), *Desulfovibrio desulfuricans* (Matias et al., 2001) and of the [NiFe(Se)] hydrogenase from *Desulfomicrobium baculatum* (formerly *Desulfovibrio baculatus*; Garcin et al., 1999) have been solved. In the following, the structure of the prototypic [NiFe] hydrogenase of *D. gigas* is described in detail and compared with other representatives of this group of enzymes.

The *D. gigas* hydrogenase contains one nickel and twelve iron atoms. Eleven of the Fe atoms are assigned to three iron-sulfur clusters in the small subunit. The twelfth Fe atom and the Ni atom are located in the large subunit (Hatchikian et al., 1978; Cammack et al., 1982; Huynh et al., 1987). The [NiFe] active site is coordinated by an N-terminal and a C-terminal pair of cysteines (Fig. 4). Two of the thiolate groups (provided by Cys68 and Cys533) form a bridge between the two metals (Volbeda et al., 1995). In the case of the [NiFe(Se)] hydrogenase of *Desulfomicrobium baculatum*, the nickel ligating cysteine residue (Cys530) is occupied by a selenocysteine (Garcin et al., 1999). Electron density maps of the oxidized catalytically inactive [NiFe] hydrogenase of the sulfate-reducing bacteria show μ-oxo or hydroxo (*D. gigas*) and sulfur species (*D. vulgaris*) as bridging ligands between the Ni and the Fe atom. This bridging ligand disappears upon reduction, a process which correlates with the onset of the catalytic cycle (Volbeda et al., 1995; Higuchi et al., 1997).

Characteristic electron density peaks were observed near the Fe atom. Fourier-Transform Infrared (FTIR) spectroscopy, conducted with the [NiFe] hydrogenase of *Allochromatium vinosum* (formerly *Chromatium vinosum*), uncovered three distinct infrared bands in the high stretching frequency region (Bagley et al., 1994; Bagley et al., 1995). These bands were subsequently ascribed to three intrinsic diatomic nonprotein ligands, one CO and two CN^- ligands, bound to the Fe at the heterodinuclear site (Happe et al., 1997; Pierik et al., 1999). FTIR studies on the *D. gigas* [NiFe] hydrogenase confirmed the assignment of one CO and two CN^- ligands (Fig. 4). Modelling predicts that the two CN^- ligands accept hydrogen bonds from the two protein-borne amino acids Arg463 and Ser486 and that the CO is completely surrounded by hydrophobic residues (Volbeda et al., 1995; Volbeda et al., 1996). In the *D. vulgaris* enzyme, one of the three nonprotein ligands was modeled as SO (Higuchi et al., 1997). This assignment was confirmed by pyrolysis spectrometry (Higuchi et al., 2000) pointing to a mixed population of the diatomic ligands SO/ CN^- and CO.

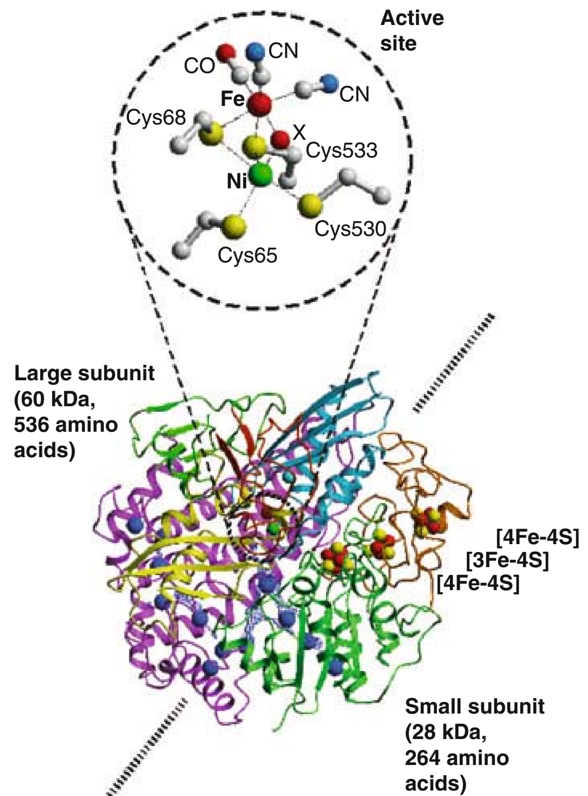


Fig. 4. Three-dimensional structure of the *Desulfovibrio gigas* [NiFe] hydrogenase. The structure of the active site is shown as a blow-up. The plane of the interface between the two subunits is indicated by a thick, dashed line. The large subunit (to the left and above the dashed line) harbors the active site. The small subunit coordinates three iron-sulfur clusters (red: iron; yellow: sulfur). Cysteine residues which participate in coordinating the active-site metals are labeled. The xenon atoms used to probe for tunnels in the protein are colored blue. A blue grid indicates the inferred gas channel (Montet et al., 1997). The blow-up shows the three diatomic ligands and the bridging ligand (X) present in the oxidized form of the enzyme. (Based on Frey et al. [2000] and Fontecilla-Camps et al. [2001]. Reproduced with permission.)

The heterodinuclear site might also differ in the number of diatomic ligands. FTIR data combined with chemical analysis suggest that the structure of the active site in the cytoplasmic NAD-reducing [NiFe] hydrogenase of the aerobically H_2-oxidizing *Ralstonia eutropha* is somewhat different. The data point to the existence of two extra CN^- ions, one bound to the Fe and one bound to the Ni atom (Happe et al., 2000). This special architecture, which may account for the O_2 and CO insensitivity of this particular hydrogenase, has yet to be confirmed by X-ray crystallography.

On the basis of the biochemical and structural data, it can be concluded that the standard type

of [NiFe] hydrogenase forms a globular heterodimer with a radius of 3 nm. The two subunits make contact over a large planar surface (Fig. 4) and are held together by intersubunit ion pairs, which are important for enzyme stability (Szilágyi et al., 2002). The catalytic site and the proximal [4Fe-4S] cluster of the small subunit are buried deep in the protein (Volbeda et al., 1995). Spectroscopic data and the spatial arrangement of the proximal [4Fe-4S], the medial [3Fe-4S], and the distal [4Fe-4S] cluster, all of which are separated from each other by 1.0–1.5 nm, suggest that the iron-sulfur clusters function as an "electrical wire." The proximal [4Fe-4S] cluster could directly exchange electrons with the catalytic site (Fig. 5). The N-terminal part of the small subunit is composed of a highly conserved flavodoxin domain and the binding site for the proximal [4Fe-4S]. These two elements are obviously essential features of all [NiFe] hydrogenases. The involvement of the medial [3Fe-4S] in the redox reaction with H_2 is still a matter of debate since its redox potential is too high relative to that of the H_2 reactive site (Albracht, 2001; Fontecilla-Camps et al., 2001). The distal [4Fe-4S] cluster of the *D. gigas* small subunit is coordinated by three cysteines and one histidine residue. The solvent-exposed histidine appears to be essential for the electronic exchange between the hydrogenase and its corresponding redox partner, e.g., a multiheme cytochrome c_3 (Rousset et al., 1998b).

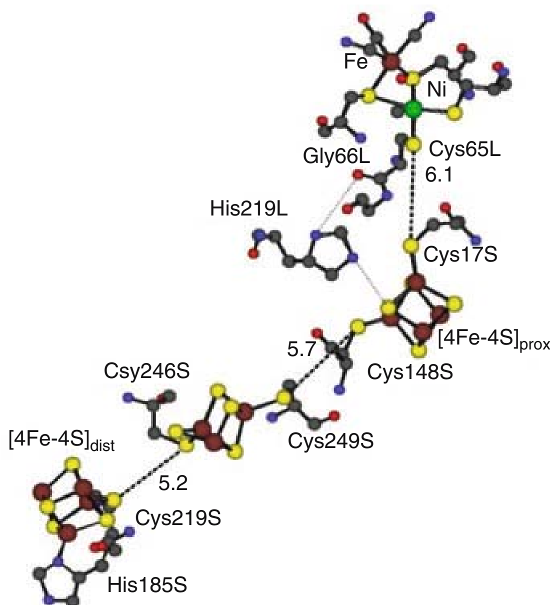


Fig. 5. A hypothetical electron transfer pathway in the [NiFe] hydrogenase of *D. gigas*. The active site is shown along with the Fe-S centers and relevant amino acids. Key distances between atoms (thick dashed lines) are given in Å. Hydrogen bonds are indicated by thin dashed lines. Residues are labeled according to their location in the large (L) and small (S) subunit (based on Volbeda et al. [1995]).

The [NiFe] hydrogenase small subunits are less conserved. In the H_2-sensing proteins, the set of amino acids responsible for coordinating the medial iron-sulfur cluster contains a cysteine at the position of the conserved proline residue, suggesting the existence of a medial [4Fe-4S] cluster in this group of proteins (Kleihues et al., 2000). A similar iron-sulfur cluster composition has been reported for the F_{420}-reducing [NiFe(Se)] hydrogenase of *Methanococcus voltae*. Reconstitution of a medial [3Fe-4S] cluster by site-directed mutagenesis yielded a protein displaying near wildtype activity with benzyl viologen but drastically decreased activity with the physiological acceptor coenzyme F_{420} (Bingemann and Klein, 2000). The conversion of the native [3Fe-4S] to a [4Fe-4S] cluster in the [NiFe] hydrogenase of *D. fructosovorans* had little effect on the enzymatic activity irrespective of whether redox dyes or cytochrome c_3 were used as electron acceptors. On the other hand, the mutation conferred pronounced oxygen sensitivity to the protein (Rousset et al., 1998b). This observation supports the notion that in some organisms, the [3Fe-4S] cluster protects the enzyme from inactivation by oxygen (Albracht, 1994).

A few [NiFe] hydrogenases, including the NAD-reducing multimeric hydrogenases and the *E. coli* type 3 hydrogenases, contain a minimal version of a functional hydrogenase module consisting of a [NiFe] active site in the large subunit and one proximal [4Fe-4S] cluster in the small subunit. It was demonstrated with *R. eutropha* mutants that this subform meets the minimal requirements for being catalytically active (Massanz et al., 1998).

THE NIFE ACTIVE SITE Comparison of over 100 deduced amino-acid sequences clearly show that [NiFe] hydrogenases share a number of common motifs. The large hydrogenase subunit contains a set of at least five related signatures in the N-terminal and C-terminal regions that are located close to the active site (Voordouw et al., 1989; Wu and Mandrand, 1993). A systematic site-directed mutagenesis was initiated with the multimeric NAD-reducing hydrogenase of *R. eutropha* and shed some light on the possible role of some of these conserved residues in catalysis (Massanz and Friedrich, 1999; Burgdorf et al., 2002). The pattern, inferred from multiple sequence alignments of two specific motifs, the so-called signatures "L1" and "L2," including the Ni-liganding cysteines, provided the basis for the recent classification of [NiFe] hydrogenases (Vignais et al., 2001). In most cases, the L2 signature ends at a histidine residue (His536 in *D.*

gigas), which marks the C-terminal endopeptidase cleavage site. In group 4 [NiFe] hydrogenases, represented by hydrogenase 3 of *E. coli*, and the related enzymes of *Rhodospirillum rubrum*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum* and *Pyrococcus furiosus*, this specific histidine is replaced by an arginine residue (reviewed by Tersteegen and Hedderich [1999]). A large peak of electron density in the vicinity of the large subunit terminal histidine was identified as an extra metal, a magnesium ion in the *D. gigas* (Volbeda et al., 1995) and the *D. vulgaris* enzymes and an iron ion in the *Dm. baculatum* hydrogenase (Garcin et al., 1999).

Hydrogen and protons have to bridge a distance of 3 nm to reach the active site or to return to the surface of the protein. Several proton pathways involving histidines, glutamates, carboxylate groups, and internal water molecules have been discussed. A possible proton pathway, proposed more recently for the *D. gigas* hydrogenase (Fig. 6), implicates two conserved glutamic acid residues (Glu 18 and Glu 46) in the large subunit and water molecules located between the [NiFe] and the Mg site (Fontecilla-Camps et al., 2001).

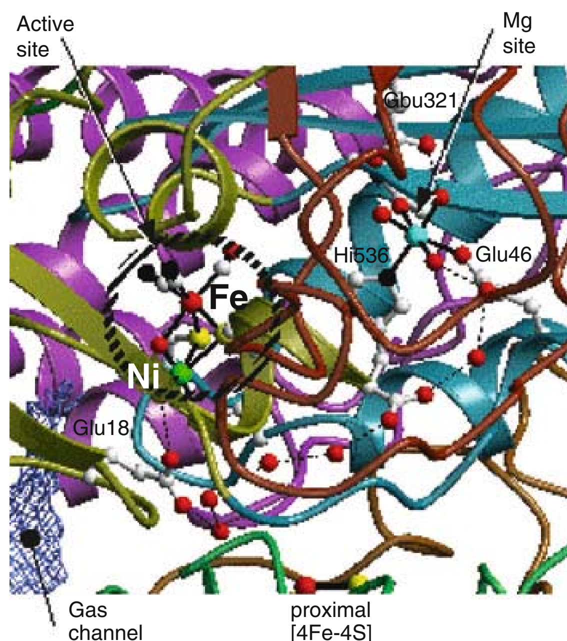


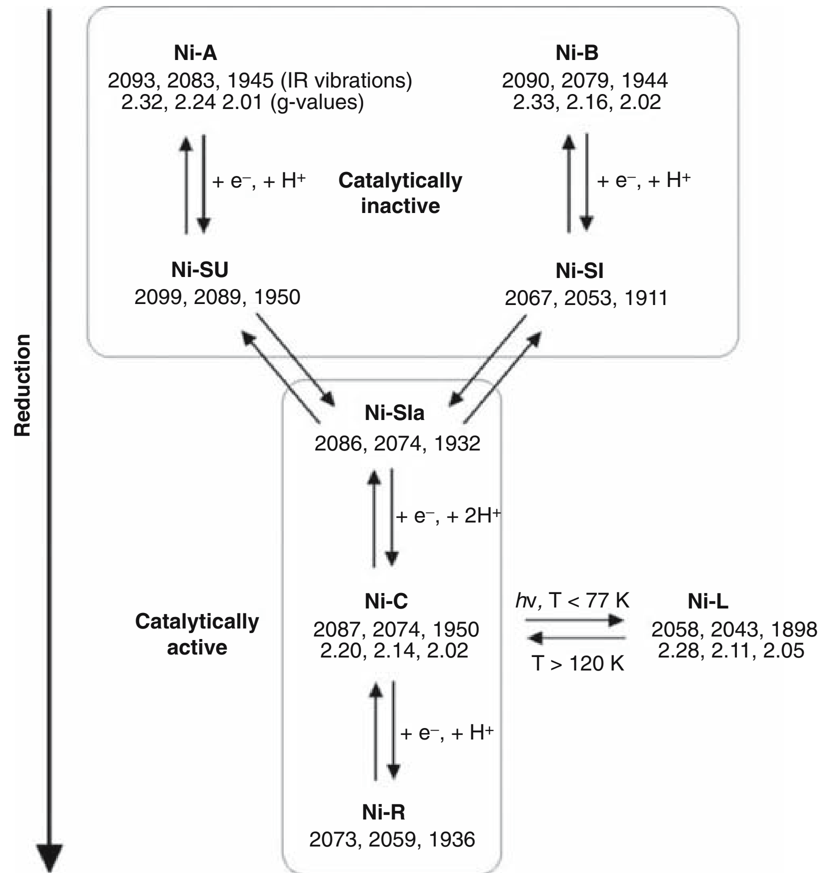
Fig. 6. Close-up view of the active site of the *Desulfovibrio gigas* [NiFe] hydrogenase. The three diatomic ligands and the bridging ligand (red) present in the oxidized form of the enzyme are shown. A putative proton pathway is indicated by the small red spheres connected by a dotted line. A blue grid (lower left) marks the inferred gas channel (Montet et al., 1997). A Mg atom is shown as blue ball. Based on Frey et al. (2000) and Fontecilla-Camps et al. (2001). Reproduced with permission.

The diffusion of molecular hydrogen, the smallest molecule in nature, through a protein matrix was for a long time considered to occur randomly. A cavity map, calculated from the electron density data for the *D. gigas* hydrogenase, showed a network of hydrophobic channels connecting the active site with the protein surface. Experiments on the diffusion of xenon in crystals of the [NiFe] hydrogenase from *D. fructosovorans* indicated that there are a few discrete hydrophobic channels for H_2 in the protein. Molecular dynamics simulations confirmed this result (Montet et al., 1997). Thus, the movement of H_2 through hydrogenase is probably not random but proceeds rather via discrete pathways.

THE CATALYTIC CYCLE Cleavage of H_2 by hydrogenases is a heterolytic process yielding H^- and H^+ as demonstrated by isotope exchange experiments a long time ago (Krasna, 1979). Although the precise reaction is still not solved, enzymatic and X-ray structure analyses in concert with primary electron paramagnetic resonance (EPR), electron nuclear double resonance (ENDOR), electron spin echo envelope modulation (ESEM), Fourier transform infrared(FTIR) spectroscopy, X-ray absorption spectroscopy (XAS), and quantum chemical calculations have shed light on the chemical mechanism of the catalytic cycle (reviewed by Albracht [1994], Fontecilla-Camps [1996], Cammack [2001], Maroney and Bryngelson [2001], and Stein and Lubitz [2002]).

Spectroscopy has shown that the unpaired electron, or the EPR-detectable unpaired spin, is located in standard [NiFe] hydrogenases close to nickel and possibly in the vicinity of one of its sulfur ligands, but not to iron. From this observation, it was inferred that the iron is retained as a low-spin Fe^{2+} during the entire catalytic cycle and that the diatomic ligands on the iron contribute to the maintainance of this redox state (Happe et al., 1997). The nickel at the active site undergoes several redox changes. Three paramagnetic, EPR-detectable Ni-states, Ni-A, Ni-B and Ni-C have been identified (Fig. 7) which differ in the infrared stretching frequencies and the position of the g_y lines of the rhombic signals. Following isolation, the hydrogenase from *D. gigas* contains a mixture of the Ni-A and Ni-B forms which display different activation kinetics. The Ni-A form, characterized as "unready" showing a Ni(III) signal with a g-tensor of 2.24 in the EPR spectrum, could be fully activated only after incubation with H_2 for several hours, whereas the Ni-B form, the "ready" state, in a Ni(III) coordination with a g-tensor of 2.16, was activated by H_2 within a few minutes (Fernandez et al., 1985). These oxidized enzyme species bear O^{-2} or OH^- molecules bridging the two metals,

Fig. 7. Redox states of nickel in standard [NiFe] hydrogenase. See text for details.



as shown by X-ray structure analysis (Volbeda et al., 1995). They are catalytically inactive and do not participate in the catalytic cycle. Sequential one-electron reductions by external reducing agents, involving several diamagnetic intermediates, finally result in the formation of the EPR-detectable catalytically active Ni-C form of the hydrogenase (van der Zwaan et al., 1990; Fig. 7). Ni-C is photosensitive and becomes converted to Ni-L upon illumination, leading to the reduction of the Ni(III) to Ni(I). Binding of CO in the Ni-C state also yields a specific paramagnetic Ni-CO form.

During reductive activation, the oxygen species dissociate from the [NiFe] active site, and the catalytic cycle is initiated. The idea that H_2 is bound to the [NiFe] center, and a hydride is formed and occupies the position of the bridging ligands, seems to be generally accepted. This process is accompanied by the release of a proton, possibly assisted by one of the terminal cysteines which might act transiently as a base, and the transfer of one electron to the oxidized proximal [4Fe-4S] cluster. To keep the cycle running, the fully reduced hydrogenase in the the diamagnetic Ni-R state (Fig. 7) has to be reconverted to the more oxidized H_2-accepting forms. This is achieved by the release of protons and the transfer of two additional electrons from the [NiFe] center via the iron-sulfur cluster(s) to an external redox partner. Once this reaction is completed, the enzyme is ready to enter a second catalytic cycle (Albracht, 1994; Cammack, 2001; Stein and Lubitz, 2002).

INTERACTIONS OF DIMERIC [NIFE] HYDROGENASES WITH REDOX PARTNERS The type of interaction with a specific redox partner can often be deduced from the structural features of the hydrogenase small subunit. In the periplasmic hydrogenases of sulfate reducers, the residues which coordinate the distal [4Fe-4S] cluster are located close to the C-terminal end of the small subunit (Fig. 8). Several species of *Desulfovibrio* contain periplasmic low-potential *c*-type cytochromes. One of these, the tetraheme cytochrome c_3, is probably one of the primary redox partners of periplasmic hydrogenases (Aubert et al., 2000; Morelli et al., 2000; Matias et al., 2001). The shuttling of electrons from the hydrogenase to membrane complexes of polyheme cytochromes of the Hmc family by *c*-type cytochromes may be a general mechanism in *Desulfovibrio* species (Rossi et al., 1993).

Membrane-bound, periplasmically oriented [NiFe] hydrogenases as found in many proteo-

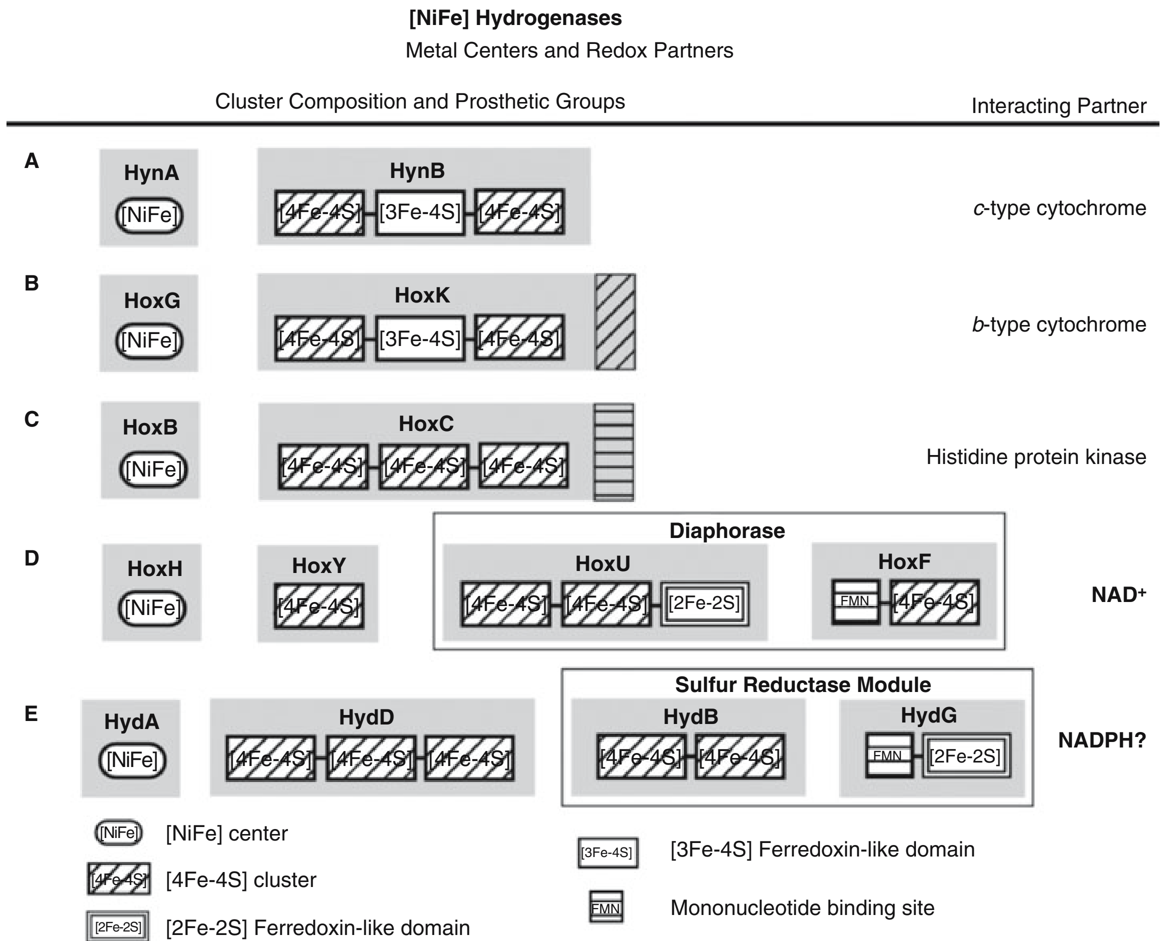


Fig. 8. Domain organization of selected [NiFe] hydrogenases. Gray boxes represent hydrogenase subunits (not to scale). Redox partners are listed at the right. A key to the symbols is given in the lower part of the figure. (A) *D. gigas* [NiFe] hydrogenase. (B) *R. eutropha* membrane-bound hydrogenase (MBH). Hatched area symbolizes the characteristic C-terminal region of the small subunit. (C) *R. eutropha* regulatory hydrogenase (RH). Hatched area symbolizes the characteristic C-terminal region of the small subunit. (D) *R. eutropha* soluble hydrogenase (SH). The diaphorase moiety is boxed. (E) *Thermococcus litoralis* sulfhydrogenase. The putative sulfur reductase module is boxed.

bacteria are characterized by a highly conserved (approx. 50 amino acid) segment at the C-terminus of the small subunit (Fig. 8). This hydrophobic region is essential for binding the hydrogenase to the membrane and coupling the electron flow to the quinone pool of the respiratory chain. A membrane-integral cytochrome *b* has been isolated as a complex with the heterodimeric [NiFe] hydrogenase from *Wolinella succinogenes* (Dross et al., 1992; Dross et al., 1993). This cytochrome, designated "HydC," binds two heme groups. Analysis of site-directed mutants revealed that substitution in HydC of three histidine residues (His25, His67 and His186), which are predicted to be heme B ligands, abolished quinone reactivity of the *W. succinogenes* hydrogenase, while benzylviologen reduction was retained. A similar phenotype was observed by mutating two conserved histidine residues in the small hydrogenase subunit HydA. One of the residues is located in the membrane-integrated C-terminal helix of HydA (His 305) and the other is supposed to be involved in the ligation of the distal [4Fe-4S] cluster. The data convincingly show that these components are necessary for electron transport from H_2 to either fumarate or polysulfide, and for quinone reactivity (Gross et al., 1998). Moreover, with cytochrome *b* deficient mutants of *R. eutropha*, it was demonstrated that the *b*-type cytochrome is bifunctional. In addition to its electron-tranferring function, it anchors the hydrogenase to the membrane (Bernhard et al., 1997). An energy-conserving system analogous to the respiration-linked proteobacterial H_2 oxidation is instrumental also in the archaeon *Methanosarcina mazei* Gö 1. The outside-oriented membrane-bound [NiFe] hydrogenase of this

strain transfers electrons from H_2 via a *b*-type cytochrome and methanophenazine to the heterodisulfide oxidoreductase. This reaction is coupled to the generation of a proton motive force (Ide et al., 1999). Thus it appears that the cytochrome *b* serves as a common primary electron acceptor for a large group of hydrogenases.

The H_2-sensing regulatory [NiFe] hydrogenases of *Bradyrhizobium japonicum* (Black et al., 1994), *Rhodobacter capsulatus* (Elsen et al., 1996), and *Ralstonia eutropha* (Lenz et al., 1997) also contain a C-terminal region of approx. 50 amino acids in the small subunit. Although the sequence of this peptide is highly conserved within this group of proteins, it is completely distinct from the C-terminal small subunit region of membrane-bound [NiFe] hydrogenases (Fig. 8). This observation points to a specific role of the C-terminal extension in partner recognition and hence signal transduction. In fact, the formation of a tight complex between the H_2-sensing hydrogenase of *R. eutropha* and its cognate signal-transmitting histidine protein kinase has been demonstrated in vitro using purified components (Bernhard et al., 2001). Unlike the standard energy-converting [NiFe] hydrogenases, which are isolated as simple heterodimers ($\alpha\beta$), the H_2-sensing hydrogenase of *R. eutropha* forms a tetramer consisting of two dimeric species ($\alpha_2\beta_2$) which accomodates an additional redox sensitive nonmetal cofactor (Bernhard et al., 2001).

Multimeric Hydrogenase Complexes Less information is available on the interaction of the hydrogenase module within multisubunit [NiFe] hydrogenase complexes. Such modules are often characterized by a truncated form of the small subunit which differs remarkably in its amino acid composition and cofactor content from the small subunit of the prototypic *D. gigas* hydrogenase (Fig. 4). Heteromultimeric [NiFe] hydrogenases generally reside in the cytoplasm or are bound to the inner surface of the cytoplasmic membrane. A typical feature of one group of multimeric hydrogenases is a tight association of the hydrogenase module with a second redox-active moiety that binds coenzymes such as F_{420} (8-hydroxy-5-deazaflavin), NAD or NADP which are reversibly reduced by H_2.

The F_{420}-reducing hydrogenases of methanogens are heterotrimeric FAD-containing enzymes which tend to form aggregates. A well-characterized example is the F_{420}-reducing hydrogenase from *Methanobacterium formicicum* (Baron and Ferry, 1989a; Baron and Ferry, 1989b), which consists of the subunits FrhA, FrhB and FrhG. The FrhA subunit contains the [NiFe] center and the FrhB subunit harbors the binding site for the cofactor F_{420}. Variants of this hydrogenase exist which contain selenium in addition to nickel and iron. Methylotrophic methanogens such as *Methanosarcina* strains possess membrane-bound hydrogenases (Fig. 3). In *Methanosarcina mazei*, a cytochrome *b* serves as the primary electron acceptor (Ide et al., 1999). The redox carrier methanophenazine shuttles electrons from the hydrogenase to heterodisulfide oxidoreductase. In hydrogenotrophic methanogens, which are devoid of cytochromes, the physiological electron acceptor of the F_{420}-nonreducing hydrogenase is not yet known.

Methanococcus voltae harbors two enzymes each of a [NiFe] and and of a [NiFe(Se)] hydrogenase. One enzyme of each pair is of the F_{420}-reducing type, the other represents the F_{420}-nonreducing type. The F_{420}-nonreducing [NiFe(Se)] hydrogenase has a peculiar structure. The large subunit lacks the C-terminal cysteinyl or selenocysteinyl ligands to Ni. This domain is contained on a separate peptide of 25 amino acids.

The soluble cytoplasmic NAD-reactive [NiFe] hydrogenases of bacterial species consist of four heterologous subunits (Fig. 2). The hydrogenase module contains a truncated form of the small electron-transferring subunit with only one Fe-S cluster as prosthetic group (Fig. 8). This moiety is associated with a heterodimeric iron flavoprotein, the so-called "diaphorase module" (Schneider and Schlegel, 1976; Schneider et al., 1984a; Tran-Betcke et al., 1990). The diaphorase consists of a large (approx. 65-kDa) polypeptide, HoxF, and a small (approx. 25-kDa) subunit, HoxU. The diaphorase moiety accomodates three to four iron-sulfur clusters and one flavin mononucleotide (Fig. 8). Sequence alignments revealed a close relationship between the diaphorase part of the NAD-linked hydrogenases and three peripheral subunits of bacterial and mitochondrial NADH ubiquinone oxidoreductases (Tran-Betcke et al., 1990; Pilkington et al., 1991; Friedrich et al., 2000). The HoxF polypeptide appears to be a fusion product of the 24- and the 52-kDa subunits of bovine complex I, and HoxU is homologous to the N-terminal part of the 75-kDa subunit. Further similarities between hydrogenases and complex I will be discussed below.

The NAD-reactive hydrogenases are found in aerobic H_2-oxidizing bacteria including the well studied *Rhodococcus opacus* (formerly *Nocardia opaca*) and *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*; Schneider and Schlegel, 1976; Schneider et al., 1984a; Tran-Betcke et al., 1990; Grzeszik et al., 1997b). A cytoplasmic, NAD-reducing hydrogenase was identified in the methanotroph *Methylococcus capsulatus* (Hanczár et al., 2002). Similar enzymes, designated "bidirectional hydrogenases" have more recently also

been found in cyanobacteria (reviewed by Tamagnini et al. [2002]). An NAD-reactive cyanobacterial hydrogenase was first characterized and sequenced from the filamentous *Anabaena variabilis* ATCC 29413 (Schmitz et al., 1995; Serebryakova et al., 1996). Subsequently this type of hydrogenase was also identified in unicellular cyanobacteria and appears to be loosely associated with cytoplasmic and thylacoid membranes (Kentemich et al., 1989; Serebryakova et al., 1994). A cytoplasmic, NAD-reducing hydrogenase was also found in the methanotroph *Methylococcus capsulatus* (Hanczár et al., 2002). Recently, a bidirectional hydrogenase was discovered in *Thiocapsa roseopersicina* (Rákhely et al., 2004). The biological function of the bidirectional hydrogenases is still obscure. A group of closely related tetrameric NADP-reactive [NiFe] hydrogenases has been found in the hyperthermophilic archaeal species of *Pyrococcus* (Ma et al., 1993; Pedroni et al., 1995; Ma et al., 2000) and *Thermococcus* (Rákhely et al., 1999). The HydA and HydD subunits of this type of enzyme constitute the hydrogenase module, whereas the HydB and HydG subunits form the flavin-containing NADP-reactive moiety of the protein (Fig. 8). It is remarkable that this group of [NiFe] hydrogenases has both H_2-oxidizing and S^0-reducing activities. During fermentation, the enzyme accepts both polysulfides or protons as electron acceptors and is therefore designated a "sulfhydrogenase" (Ma et al., 1993).

The H_2-evolving [NiFe] hydrogenases expressed under strictly anoxic conditions are constituents of multicomponent membrane-bound enzyme complexes which share the ability to couple the oxidation of carbonyl groups, derived from formate, acetate or carbon monoxide, to the reduction of protons, yielding H_2. The most extensively studied example is the formate-hydrogenlyase complex in *E. coli* comprising eight *hyc* gene products. The hydrogenase module of hydrogenase 3 consists of the active-site-containing subunit HycE and the small subunit HycG, which harbors only the proximal [4Fe-4S] cluster (Fig. 2). Both the formate dehydrogenase moiety of the complex and the two hydrogenase subunits are attached to the inner side of the cytoplasmic membrane via intrinsic membrane proteins, which serve as membrane anchors and electron mediators between the two redox proteins (Böhm et al., 1990; Sauter et al., 1992). It has so far not been possible to isolate the entire hydrogenase 3 complex.

The CO-induced [NiFe] hydrogenase of *Rhodospirillum rubrum* is a constituent of the CO-oxidizing enzyme system which permits the organism to grow in the dark with carbon monoxide as the sole energy source (Uffen, 1976). Like *E. coli* hydrogenase 3, the CooLH hydrogenase complex of *R. rubrum* is rather labile, and hence biochemical data are limited (Fox et al., 1996a; Fox et al., 1996b). Multisubunit [NiFe] hydrogenase complexes of the *E. coli* type 3 are also present in archaea such as *Methanothermobacter* species (Tersteegen and Hedderich, 1999), in *Methanosarcina barkeri* strain Fusaro (Künkel et al., 1998), and in *Carboxidothermus hydrogenoformans* (Soboh et al., 2002; Fig. 3). The intact Ech hydrogenase complex has been purified to homogeneity from cells of *M. barkeri* (Meuer et al., 1999). It consists of two membrane-spanning subunits and four hydrophilic subunits, all of which show a particularly strong resemblance to subunits of complex I (Albracht and Hedderich, 2000; Vignais et al., 2001). Ech hydrogenase catalyzes the reversible reduction of a 2[4Fe-4S] ferredoxin by H_2 (Meuer et al., 1999). With the aid of a mutant, it was elegantly shown recently that the Ech hydrogenase of *M. barkeri* has a key function in methanogenesis (Meuer et al., 2002). During acetate-dependent growth, this enzyme mediates H_2 evolution from reduced ferredoxin by the oxidation of the acetate-borne carbonyl group to CO_2. Under H_2/CO_2 growth conditions, the Ech hydrogenase catalyzes the energetically unfavorable reduction of ferredoxin, which in turn is used as a low potential electron donor for the anabolic synthesis of pyruvate. Simultanously the reduced ferredoxin serves as reducing equivalents for the first step of methanogenesis, the reduction of CO_2 to formylmethanofuran. Because of the low mid-point potential of the CO_2 + methanofuran/CHO-methanofuran couple ($E^{0\prime} = -500$ mV), the reaction is endergonic, with H_2 as electron donor ($E^{0\prime} = -414$ mV; Bertram and Thauer, 1994). It has been proposed that Ech hydrogenases function as proton or sodium pumps providing low potential reducing equivalents (Meuer et al., 1999), and on the basis of sequence comparisons, it is hypothesized that a specific ferredoxin-like subunit is the electrical driving unit of the proton pump in complex I and proton-pumping hydrogenases (Albracht and Hedderich, 2000). This subunit is absent in the dimeric [NiFe] hydrogenases and in the tetrameric NAD-reducing hydrogenases.

[FeFe] Hydrogenases

MOLECULAR CHARACTERISTICS The [FeFe] hydrogenases are found in anaerobic, primarily H_2-evolving organisms, including fermentative bacteria, sulfate-reducers, and some lower eukaryotes. They are characterized by an extremely high oxygen sensitivity, a high turnover rate, and a low affinity for the substrate

hydrogen (reviewed by Adams [1990], Peters [1999], Nicolet et al. [2000], Vignais et al. [2001], and Horner et al. [2002]). In addition to monomeric [FeFe] hydrogenases, there are dimeric, trimeric and tetrameric enzymes.

Two well characterized examples are the monomeric 61-kDa cytoplasmic [FeFe] hydrogenase of *Clostridium pasteurianum* (Adams and Stiefel, 1998) and the dimeric periplasmic enzyme of *Desulfovibrio desulfuricans* consisting of a large (43-kDa) and a small (10-kDa) subunit (Nicolet et al., 1999). The [FeFe] hydrogenase of *C. pasteurianum* uses protons as electron acceptors to rid the cell of excess reducing equivalents, thereby regenerating oxidized ferredoxin and leading to the production of H_2. The [FeFe] hydrogenase of *D. desulfuricans* plays a role in H_2 uptake. The active site consists of a unique metal-containing prosthetic group, the so-called "H-cluster" (Fig. 9). The H-cluster is composed of a single [4Fe-4S] subcluster bridged through a thioether linkage (provided by a cysteine residue) to a specific iron binuclear subcluster (Peters et al., 1998; Nicolet et al., 1999). A multiple amino acid sequence alignment revealed considerable similarity within the H-cluster-coordinating region, indicating a fairly conserved architecture of the active site. Additional domains, however, which differ remarkably in size and cofactor content, are often present in this class of hydrogenases.

Recently, the three-dimensional structure of hydrogenase isoenzyme I from *C. pasteurianum* (Peters et al., 1998) and the [FeFe] hydrogenase from *D. desulfuricans* (Nicolet et al., 1999) has been determined at a resolution of 1.8 and 1.6 Å, respectively. A comparison revealed a highly related multidomain structure divided into an active site domain, bearing the H-cluster, and accessory cluster domains (Fig. 10). Both hydrogenases share in addition to the H-cluster the so-called "F-domain," containing two [4Fe-4S] clusters in a ferredoxin-like domain (Peters, 1999; Nicolet et al., 2000). It is interesting to note that the H-cluster of the *D. desulfuricans* hydrogenase occupies a region spanning the small subunit and the N-terminal part of the large subunit.

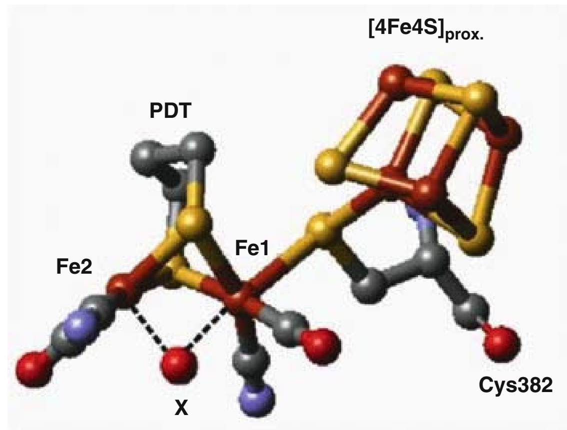


Fig. 9. The H cluster of the *D. desulfuricans* [FeFe] hydrogenase. The iron atoms are labeled "Fe1" and "Fe2." The larger bridging ligand is modeled as 1,3-propanedithiol (PDT). The smaller ligand is marked "X." Based on Nicolet et al. (1999).

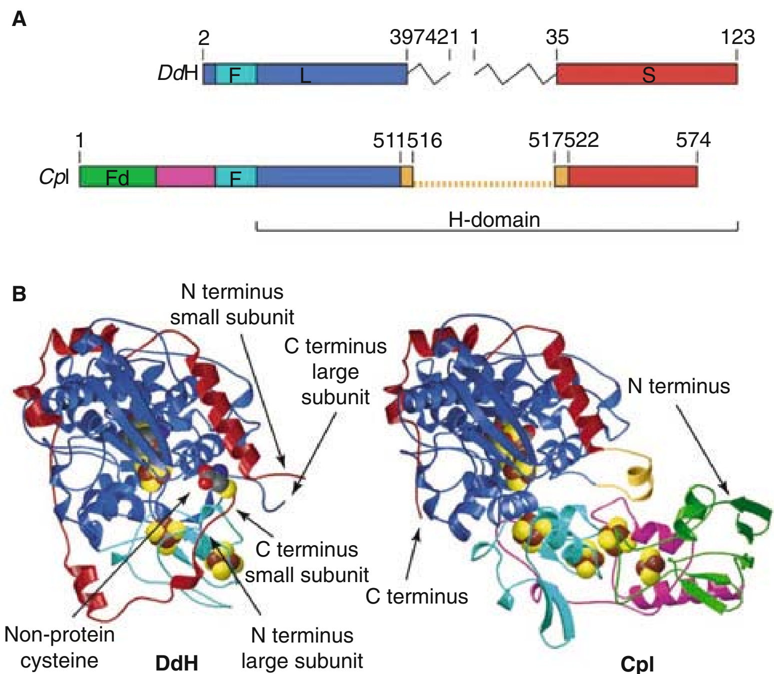


Fig. 10. Structural comparison of two [FeFe] hydrogenases. (A) Schematic representation of the *Desulfovibrio desulfuricans* [FeFe] hydrogenase (DdH) and of the *Clostridium pasteurianum* [FeFe] hydrogenase I (CpI). Structurally related domains are rendered in the same color. The small and large subunits of DdH are labeled S and L, respectively. The first 34 amino acids of the DdH small subunit and the last 24 residues of the large subunit are missing in the mature proteins. The plant-ferredoxin-like domain (Fd) is shown in green, and the F-domain, which harbors 2 [4Fe-4S] centers, in turquoise. An additional [4Fe-4S] center is colored pink. (B) The three-dimensional structures of DdH (left) and CpI (right). The color-coding is based on the diagram in (A). From Nicolet et al. (2000), with permission from Elsevier Science.

The latter provides also the binding site for the F-cluster. Thus the small subunit of the *D. desulfuricans* enzyme does not represent a single domain but rather a stretched polypeptide chain which is wrapped around the large subunit (Fig. 10). The *C. pasteurianum* hydrogenase contains two additional iron-sulfur centers not present in the *D. desulfuricans* enzyme. Both prosthetic groups are located in the N-terminal part of the protein. This implies a [2Fe-2S] cluster with structural similarity to plant-type ferredoxins and a [4Fe-4S] cluster coordinated through three cysteine ligands and a single histidine ligand (Peters et al., 1998). This coordination is equivalent to the distal [4Fe-4S] cluster coordination in the *D. gigas* [NiFe] hydrogenase (Volbeda et al., 1995).

Catalytic Reaction One of the outstanding features of the iron binuclear subcluster discovered in both the *C. pasteurianum* and the *D. desulfuricans* [Fe] hydrogenases is the existence of two terminal nonprotein ligands at each Fe atom (Fe1 and Fe2; Fig. 9). FTIR studies suggest the presence of both CN^- and CO ligands (Pierik et al., 1998) that provide a similar coordination environment as found in [NiFe] hydrogenases. Unlike the [NiFe] center, which is bound to the protein by two cysteine ligands, the Fe1-Fe2 atoms lack a direct linkage to the protein (Fig. 9). In both crystallized enzymes, the two Fe atoms are bridged through sulfur atoms, which are covalently linked to each other through a bridging molecule. In the case of *D. desulfuricans*, this bridging molecule was tentatively identified as propan dithiol (Nicolet et al., 1999). The two subgroups of [FeFe] hydrogenases obviously differ in the nature of the third bridging ligand between Fe1 and Fe2. In the enzyme from *C. pasteurianum*, this ligand was identified as a CO, and in *D. desulfuricans*, this site was modeled as an asymmetrically coordinated water molecule (Nicolet et al., 2000). A terminal water molecule was demonstrated as a fifth ligand at the bimetallic site in the *C. pasteurianum* structure (Peters et al., 1998).

The coordination environment of the two metals in [FeFe] hydrogenases favors Fe2 as a candidate for displacement and formation of a bound hydride intermediate. This assumption is consistent with the result that the competitive inhibitor CO binds to Fe2 in the enzyme from *C. pasteurianum* (Lemon and Peters, 1999). Further support comes from the observation that a hydrophobic continous channel leads from the protein surface and ends at the Fe2 coordination site of the *D. desulfuricans* structure (Hatchikian et al., 1999). This region is highly conserved in the *C. pasteurianum* enzyme and points to a specific access of H_2 to the active site as has been discussed for the [NiFe] hydrogenases (Montet et al., 1997). Finally a potential pathway for the transfer of protons was proposed involving charged and polar amino acid side chains, water molecules, and two conserved residues in the vicinity of Fe2. The latter imply a lysine residue (K358 in Cp and K237 in Dd) hydrogen bonded to a diatomic ligand of Fe2, and a cysteine residue (C299 in Cp and C178 in Dd) located in hydrogen-bonding distance to the terminally coordinated water molecule in the *C. pasteurianum* enzyme (Peters, 1999).

Diversity of [FeFe] Hydrogenases and Hydrogenase-Related Proteins The smallest [FeFe] hydrogenase unit (45–48 kDa) bearing an H-cluster subdomain only (Fig. 11) has been discovered in green algae (Happe and Naber, 1993; Happe et al., 1994). This type of enzyme, first reported for *Chlamydomonas reinhardtii*, appears to be present also in *Scenedesmus obliquus* (Florin et al., 2001; Wünschiers et al., 2001) and *Chlorella fusca* (Winkler et al., 2002). The hydrogenase is linked to the photosynthetic electron transport chain and receives its electrons for H_2 evolution from reduced [2Fe-2S] ferredoxin, which is generated during the fermentative metabolic cycle of these organisms (Florin et al., 2001). Another simple [FeFe] hydrogenase, which contains in addition to the H-cluster a clostridial F-subdomain (Fig. 9), is represented in prokaryotic species such as *Megasphera elsdenii* (Atta and Meyer, 2000; Fig. 11) as well as in lower eukaryotes such as *Trichomonas vaginalis* (Bui and Johnson, 1996; Horner et al., 2000). In spite of the absence of additional prosthetic groups, the *M. elsdenii* hydrogenase uses electron donors of the type found in clostridia including a 2[4Fe-4S] ferredoxin and a flavodoxin (Atta and Meyer, 2000). In the anaerobic eukaryotic organisms, the [FeFe] hydrogenases are localized to intracellular organelles of endosymbiotic origin (Müller, 1993; Martin and Müller, 1998; Moreira and Lopez-Garcia, 1998). In the case of chytrid fungi, anaerobic ciliates and trichomonads, these organelles are denoted as hydrogenosomes and considered as modified mitochondria which have lost the capacity for oxidative phosphorylation while gaining the ability for ferredoxin-coupled H_2 production from pyruvate.

Multisubunit [FeFe] hydrogenase complexes are present in some prokaryotic species. The large (73-kDa) subunit HydA harbors the active site of the heterotrimeric [FeFe] hydrogenase from the hyperthermophilic *Thermotoga maritima* (Fig. 11). Sequence analysis predicts that the enzyme is a multiple iron-sulfur cluster-containing flavoprotein which uses NADH as the electron donor. The latter two features still need

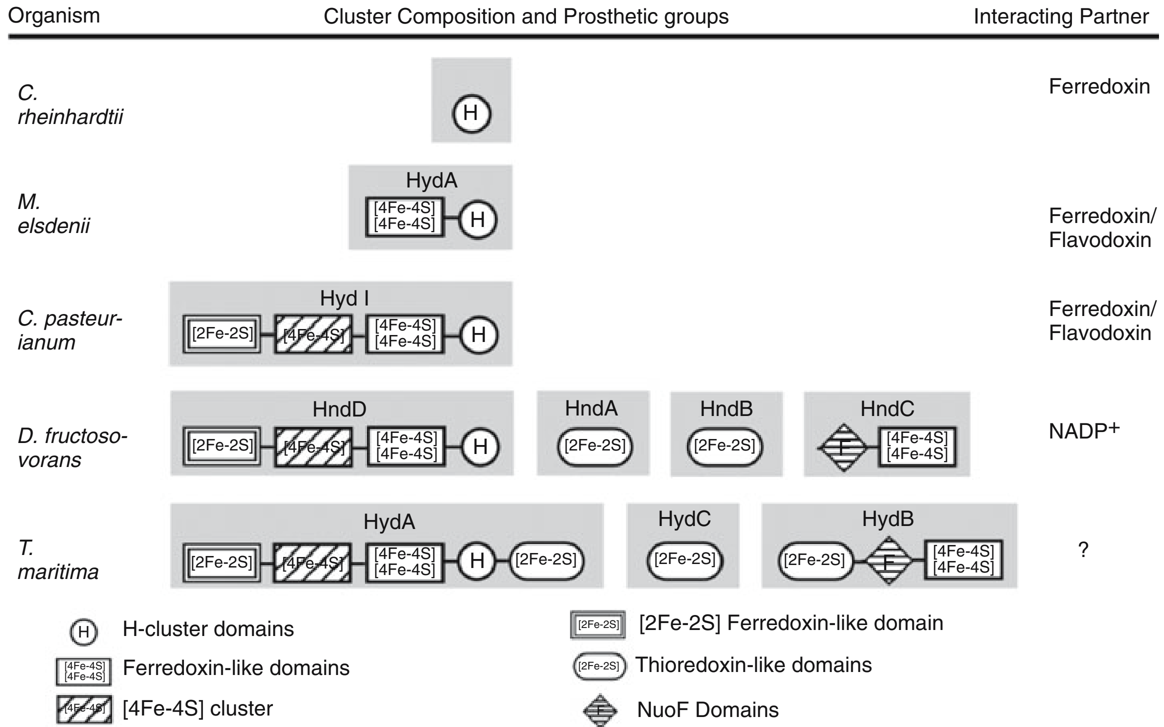


Fig. 11. Domain organization of selected [FeFe] hydrogenases. Gray boxes represent hydrogenase subunits (not to scale). Redox partners are listed at the right. A key to the symbols is given in the lower part of the figure.

to be confirmed experimentally with purified holoenzyme (Verhagen et al., 1999). Each of the three [FeFe] hydrogenase subunits contains a domain which is similar to the bacterial thioredoxin-like [2Fe-2S] ferredoxins and the NuoE polypeptide of NADH-ubiquinone oxidoreductase. An additional domain, homologous to the NuoF subunit of complex I, was uncovered in the HydB polypeptide (Fig. 11). These observations again emphasize the evolutionary relationship between complex I and hydrogenases.

The catalytic subunit of the heterotetrameric cytoplasmic NADP-reducing [FeFe] hydrogenase of *D. fructosovorans* has a similar accessory domain composition as the clostridial hydrogenases (Malki et al., 1995). Its overall structure resembles that of the *T. maritima* enzyme showing the typical complex I-related domains assigned to three separate subunits (Fig. 11). Indeed it was suggested that the HynB and HynC subunits of *D. fructosovarans* are fused to a single polypeptide (HydB) in *T. maritima* (Vignais et al., 2001).

Sequences related to [FeFe] hydrogenase genes have been identified in genomes of aerobic eukaryotes, including the human genome. Members of this novel class of nuclear proteins are termed "nuclear prelamin A recognition factors" (NARFs; Barton and Worman, 1999). A general cellular function of NARFs, such as a role in the regulation of the cell cycle, is the subject of ongoing discussion. Since H_2-converting processes have so far not been observed in higher eukaryotes, it is unlikely that NARF proteins are implicated in energy metabolism.

[Fe] Hydrogenases

The H_2-forming methylentetrahydromethanopterin dehydrogenases (Hmd's) of methanogenic archaea represent a third, phylogenetically distinct class of hydrogenases (Zirngibl et al., 1990; Thauer et al., 1996; Berkessel, 2001). These homodimeric enzymes, characterized as H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd), are present in methanogenic archaea where they catalyzes a step in met hanogenesis, namely, the reversible reduction of N^5,N^{10}-methenyltetrahydromethanopterin with H_2 to N^5,N^{10}-methylenetetrahydromethanopterin (Thauer, 1998). The product is used by the F_{420}-specific N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase for the reduction of F_{420} (Fig. 3). Both enzymes are coordinately expressed at a high level under nickel depletion (Afting et al., 1998). With the aid of the hydrogen

isotope assays and two-dimensional nuclear magnetic resonance (2D NMR), it was possible to explore the H_2-forming reaction in further detail and to demonstrate a stereoselective hydride transfer (Thauer et al., 1996). Unlike other hydrogenases, which are iron-sulfur proteins, the Hmd's contain only redox-active iron bound to a thermolabile cofactor. Exposure of the Hmd's to UV-A or blue light results in inactivation of the enzyme and release of iron (Buurman et al., 2000; Lyon et al., 2004).

Maturation of Hydrogenases

The complex architecture of the active site in [NiFe] and [FeFe] hydrogenases raises the question of how such metal centers are synthesized and assembled into the polypeptides. Auxiliary proteins which control metal insertion and coordinated folding of the protein are often involved in the biosynthesis of metalloenzymes. Inspection of the nucleotide sequences of [NiFe] hydrogenase gene complexes revealed numerous highly conserved accessory genes, which are usually closely linked to the corresponding structural genes. Mutant analysis delivered the first evidence for a complex co- or post-translational metal insertion pathway. This chapter focuses on the maturation of [NiFe] hydrogenases (Böhm et al., 1990; Friedrich and Schwartz, 1993; Maier and Böck, 1996; Casalot and Rousset, 2001; Robson, 2001; Blokesch et al., 2002). At present, scarcely anything is known about the insertion of the H-cluster into [FeFe] hydrogenases. This article does not address the acquisition of metals, the synthesis of iron-sulfur clusters, and other prosthetic groups which are shared with other metalloproteins. Depending on the cellular location and specific functional properties, [NiFe] hydrogenases undergo various levels of maturation. The maturation process will be discussed below step by step.

[NiFe] Center Insertion

At least six *hyp* gene products (HypA, HypB, HypC, HypD, HypE and HypF), present in bacterial as well as archaeal [NiFe] hydrogenase-containing species, are involved in the insertion of the heterodinuclear metal center (Table 3). The designation *hyp* stands for "genes affecting *hy*drogenases *p*leiotropically." This means that mutations in the individual *hyp* genes either reduce or abolish the activity of multiple [NiFe] hydrogenase isoenzymes, as demonstrated for *Escherichia coli* (Lutz et al., 1991) and *Ralstonia eutropha* (Dernedde et al., 1996; Wolf et al., 1998).

The sequence of events that lead to the formation of active [NiFe] hydrogenases is now emerging. A current model of the reaction cycle is predominantly based on studies on hydrogenase 3 of *E. coli*. Its [NiFe] center-containing subunit HycE is part of a protein complex attached to the inner face of the cytoplasmic membrane (Rossmann et al., 1994; Fig. 2). The working model (Fig. 12) postulates a sequential insertion of the two metals into the precursor. First, iron and its diatomic ligands are assembled. A HypC-HypD complex seems to act as a platform for this process. The insertion of at least one CN- is mediated by HypF (Paschos et al., 2001) in concert with HypE (Blokesch et al., 2002). According to the model, carbamoyl phosphate is the source of at least one of the CN- ligands. HypF, which contains several conserved functional domains (Table 3), converts carbamoyl phosphate to carbamoyl adenylate in an ATP-dependent reaction. In a transient HypE-HypF

Table 3. Characteristics of Hyp proteins.

Protein	Size[a] (kDa)	Sequence motif(s)	Putative function[b]
HypA	13.2	Cysteine cluster $CX_2CX_{12}CX_2C$	Ni insertion
HypB[c]	31.6	Cysteine cluster CX_2CGC, GTP-binding domain, His-rich region	Ni insertion and in some cases also Ni storage
HypC	9.7	Conserved N-terminal Cys residue	Chaperone which forms a complex with HypD and the large subunit
HypD	41.4	[Fe-S] cluster	Fe-dependent complex formation with HypC
HypE	33.7	ATP-binding site	Complex formation with HypF, ATPase
HypF	82.0	*o*-Carbamoyl transferase domain, two zinc finger motifs, acylphosphatase domain, and glycine-rich motif	Conversion of carbamoyl phosphate to the CN^- ligand, carbamoyl phosphate-dependent ATP cleavage
HypX[c]	65.7	N_{10}-formyltetrahydrofolate binding site, and enoyl-CoA hydratase/isomerase signature	Insertion of extra CN^-

Abbreviations: *hyp*, genes affecting *hy*drogenases *p*leiotropically; Cys, cysteine; and CN^-, cyanide ion.
[a]Sizes of *E. coli* K12 Hyp proteins are given (Lutz et al., 1991; Maier et al., 1996).
[b]References are given in the text.
[c]HypB of *E. coli* is devoid of a stretch of His residues. HypX is not present in *E. coli*.

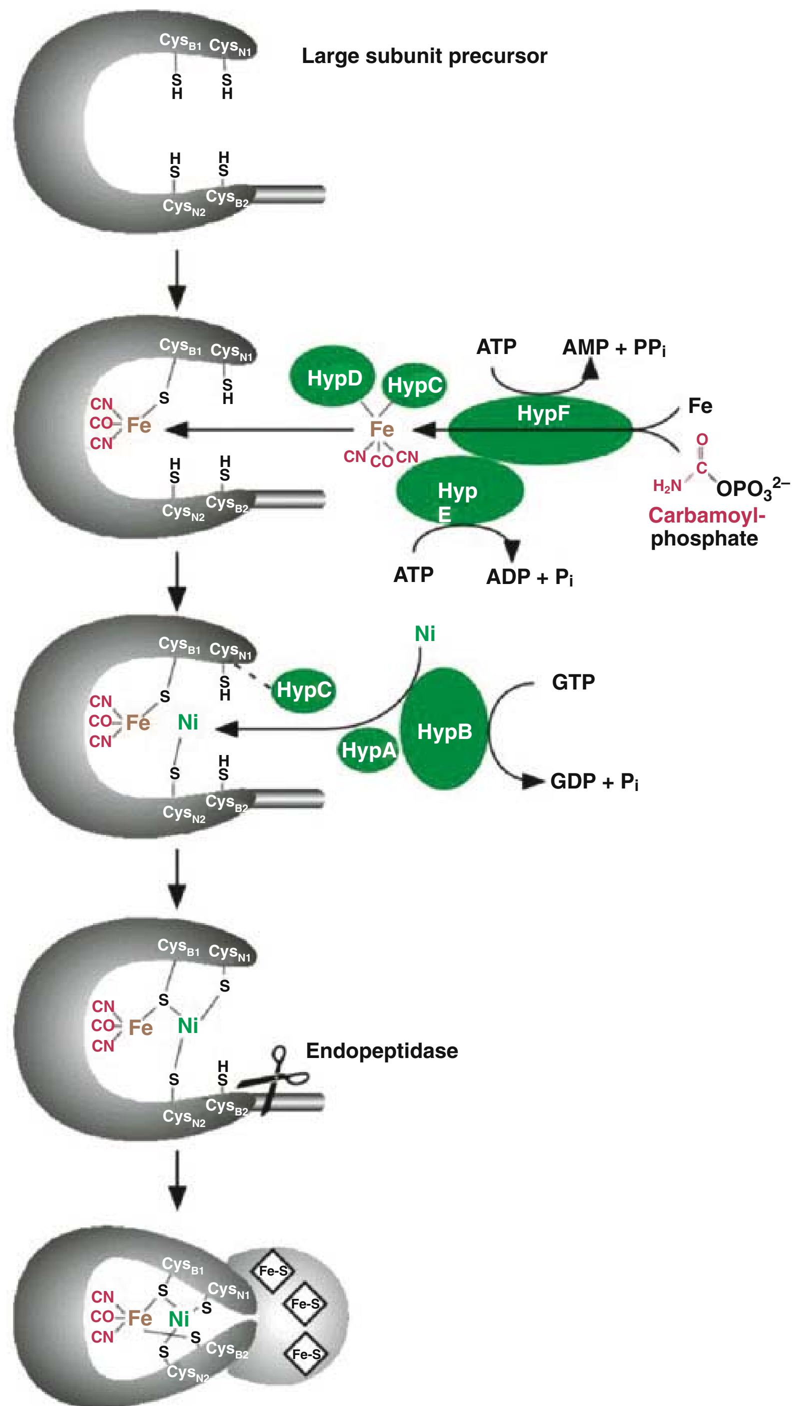


Fig. 12. Molecular model for the maturation of [NiFe] hydrogenases based mainly on studies on *E. coli* hydrogenase 3. The large and small subunits of the hydrogenase are shown in dark and light gray, respectively. The Hyp proteins are depicted in green. Iron-sulfur clusters are indicated as diamonds (Fe-S). See text for details.

complex, HypF mediates the carbamoylation of HypE at the C-terminal cysteine. Finally, HypE catalyzes the dehydration of the carbamoylate moiety to thiocyanate and then transfers this group to the iron waiting on HypC-HypD. (Paschos et al., 2001; Blokesch et al., 2002; 2004).

The steps leading to the formation of the other two ligands are as yet unknown. It is postulated that the Fe(CN-)2CO group is completed on the HypC-HypD complex and then transferred to the large subunit (Blokesch et al., 2002). A HypC-HycE complex is an intermediate in the latter step. One of the nickel-coordinating cysteine residues (CysN1; Fig. 12) is required for the formation of the complex. Although the mechanism of HycE-HypC complex formation is not yet known, disulfide bridge formation between HycE and HypC can be excluded (Magalon and

Böck, 2000). Because of the lack of data from other organisms, it would be premature to generalize the above mechanistic scheme. A global two-hybrid study an *Helicobacter pylori* indicates a HypE/HypF interaction (Rain et al., 2001). In *Thiocapsa roseopersicina* there are two distinct HypC species. Both are required for the assembly of the hydrogenases in this organism (Maróti et al., 2003). Various complexes including HypC-HypD, HypE-HypF1, HypC-HypD-HypE and HypC-HoxH have been detected in *R. eutropha* (Jones et al., 2005).

Metal center assembly proceeds by nickel incorporation catalyzed by the HypB protein in conjunction with HypA (Olson et al., 2001; Hube et al., 2002; Fig. 12). HypB proteins share a GTP-binding site (Table 3) and GTP hydrolysis is essential for HypB-mediated nickel insertion in *E. coli* (Maier et al., 1995). Some of the hydrogenase activity, however, can be rescued in a *hypB* mutant by supplying high concentrations of Ni^{+2} to the medium (Waugh and Boxer, 1986). Unlike *E. coli* HypB, the majority of HypB proteins contain stretches of His residues with potential Ni-chelating function. In fact, binding of Ni^{+2} ions has been demonstrated for the HypB proteins of *Sinorhizobium leguminosarum* (Rey et al., 1994) and *Bradyrhizobium japonicum* (Fu et al., 1995). This observation suggests that in some organisms HypB has a dual function: It not only delivers Ni^{+2} ions to the hydrogenase but acts also as a nickel storage protein.

An additional Hyp protein, HypX (formerly designated "HoxX"), has so far been found only in aerobically H_2-oxidizing bacteria, including *B. japonicum* (Van Soom et al., 1993), *R. eutropha* (Lenz et al., 1994) and *S. leguminosarum* (Rey et al., 1996). Mutations in *hypX* decreased the activity of hydrogenase under certain growth conditions in two nitrogen-fixing organisms (Rey et al., 1996; Durmowicz and Maier, 1997) as well as in a chemolithotroph (Buhrke and Friedrich, 1998). The HypX protein reveals two interesting sequence motifs: an N-terminal N_{10}-formyltetrahydrofolate binding site and a C-terminal signature that is typical for enoyl-CoA hydratases and isomerases. It was postulated that HypX plays a role in the recruitment of diatomic ligands in a tetrahydrofolate (THF)-coupled reaction (Rey et al., 1996). Recent studies with the NAD-reducing hydrogenase from *R. eutropha*, isolated from a *hypX* mutant, suggest that HypX is indeed involved in the delivery of an extra CN^- to the Ni atom (Bleijlevens, 2002; Buhrke, 2002).

Genomic sequencing has now uncovered *hypX* isologs in other prokaryotes including *Aquifex aeolicus* (Deckert et al., 1998) and *Streptomyces avermitilis* (Omura et al., 2001). The *hyp* genes are lacking in *Thermotoga maritima*, an organism which harbors [FeFe] hydrogenase only (Nelson et al., 1999). Thus Hyp proteins are obviously not involved in the synthesis of [FeFe] hydrogenases. If there is a protein-mediated metal center assembly process for this class of hydrogenases, different and as yet unknown components must be involved.

The Final Step Involves Proteolysis

Once the [NiFe] center is incorporated into the active site (Fig. 12), the chaperone HypC dissociates from the hydrogenase precursor HycE and a specific endopeptidase, HycI in the case of *E. coli* hydrogenase 3, completes the reaction cycle by cleaving 32 amino acids from the C-terminus of the active site subunit (Rossmann et al., 1994). During this process, the endopeptidase is proposed to inspect correct metal insertion and to trigger a conformational change by proteolysis, henceforth allowing the hydrogenase to enter a folded, oligomeric state. Crystal structure analysis of HybD, the endopeptidase specific for hydrogenase 2 of *E. coli*, has uncovered a metal binding site (Glu16, Asp62, His93) which is obviously implicated in nickel recognition during the proofreading process (Fritsche et al., 1999; Theodoratou et al., 2000a).

C-terminal proteolysis in multiple [NiFe] hydrogenase-containing organisms is mediated by individual endopeptidases which are functionally not interchangeable. The length of the cleaved peptides varies considerably (between 13 and 32 amino acids), whereas the cleavage sites seem to be rather conserved. A basic amino acid is separated by three amino acids from the terminal Ni-coordinating cysteine (Cys_{B2}; Fig. 12). The exact position of endoproteolytic cleavage was experimentally determined to be an arginine residue in HycE of the *E. coli* hydrogenase 3 (Rossmann et al., 1994; Theodoratou et al., 2000b) and a histidine residue in HoxH of the *R. eutropha* NAD-reducing hydrogenase (Thiemermann et al., 1996). Crystal structure analysis of the [NiFe] hydrogenases from the sulfate reducers revealed a similar situation: All primary sequences terminate at a histidine residue (Volbeda et al., 1995; Higuchi et al., 1997; Montet et al., 1997; Matias et al., 2001).

Not all hydrogenases, however, undergo C-terminal proteolysis during maturation. The H_2-sensing proteins (reviewed by Vignais et al., 2001), the CO-induced hydrogenase of *Rhodospirillum rubrum* (Fox et al., 1996a; Fox et al., 1996b) and the Ech hydrogenase of *Methanosarcina barkeri* (Künkel et al., 1998) are examples. Although experimental results have shown that metal center insertion into the H_2-sensing proteins of *B. japonicum* (Olson et al., 1997), *R. capsulatus* (Colbeau et al., 1998) and *R. eutropha*

(Buhrke et al., 2001) relies on *hyp* gene products, a C-terminal extension at the active site subunit is dispensible for metal center assembly. This contradicts data obtained with most hydrogenases, e.g., hydrogenase 3 of *E. coli* (Binder et al., 1996) and the NAD-reducing hydrogenase of *R. eutropha* (Massanz et al., 1997). In the latter cases, it was unambiguously shown that genetically designed extension-free HycE and HoxH mutants failed to express active hydrogenase and to incorporate nickel. At present, the discrepancy between the two systems cannot be resolved.

Membrane Translocation of Hydrogenases

Periplasmic and periplasmically oriented membrane-bound [NiFe] and [FeFe] hydrogenases belong to a group of redox proteins that are exported across the cytoplasmic membrane through a special translocation pathway which functions independently of the general secretion (Sec) machinery of the cell (reviewed by Berks [1996], Voordouw [2000], and Wu et al. [2000]). All proteins that undergo this mode of translocation share a signal sequence bearing a common conserved motif (S/T)-R-R-X-F-L-K referred to as the "twin arginine leader." This type of leader occurs in bimetallic hydrogenases in addition to periplasmic redox proteins containing iron-sulfur clusters, flavine adenine dinucleotide, polynuclear copper sites or molybdopterin cofactors. It directs the various redox proteins to the recently identified Mtt (membrane targeting and translocation) or synonymously termed Tat (twin arginine translocation) pathway. The salient feature of this system is that it transports proteins in partially or completely folded form (Bogsch et al., 1998; Hynds et al., 1998; Santini et al., 1998; Sargent et al., 1998; Weiner et al., 1998). The Mtt/Tat pathway resembles the DpH-driven import of proteins into the thylacoid lumen of chloroplasts (Settles et al., 1997).

In Tat deficient mutants of *Azotobacter chroococcum* (Yates et al., 1997), *E. coli* (Rodrigue et al., 1999) and *R. eutropha* (Bernhard et al., 2000), membrane targeting of [NiFe] hydrogenases is arrested and they accumulate in the cytoplasm. Although the mislocated enzymes are physiologically inactive, they nevertheless have catalytic activity, as evidenced in assays of soluble extracts using redox dyes as electron mediators. This clearly shows that metal center assembly by the Hyp proteins occurs in the cytoplasm prior to translocation of the hydrogenase.

Only the small subunit of [NiFe] hydrogenases has an unusually long N-terminal twin-arginine leader peptide of 30–50 amino acid residues, whereas the large subunit lacks an export signal. Early observations on the [FeFe] hydrogenase of *Desulfovibrio vulgaris* (Hildenborough) led to the proposal that the large subunit is translocated with the small subunit (Voordouw and Brenner, 1985; van Dongen et al., 1988). Since then, experimental evidence has accumulated which clearly points to a tandem export of the two subunits. Genetic studies conducted with [NiFe] hydrogenases from *E. coli* (Menon et al., 1991; Menon and Robson, 1994a; Rodrigue et al., 1999), *Desulfovibrio vulgaris* (Nivière et al., 1992), *R. eutropha* (Bernhard et al., 1996), *Desulfovibrio gigas* (Rousset et al., 1998a) and *Wolinella succinogenes* (Gross et al., 1999) are all in line with a cotranslocation model. Several requirements have to be met to achieve proper export of the oligomeric hydrogenases. Membrane targeting depends on an intact twin-arginine leader peptide at the C-terminus of the small subunit, on the presence of the large subunit, its nickel acquisition, and its C-terminal processing. Thus, periplasmic and periplasmically oriented membrane-bound [NiFe] hydrogenases undergo the most complex maturation process as depicted in a schematic model (Fig. 13).

It seems likely that additional proteins participate in the maturation of this group of hydrogenases. The role of chaperonins (GroES and GroEL) on the activity of the three hydrogenase isoenzymes in *E. coli* has been investigated and evidence presented for an involvement of these chaperonins in the biosynthesis of hydrogenase 3 (Rodrigue et al., 1996). Preliminary data suggest that additional hydrogenase-specific auxiliary proteins control translocation of oligomeric hydrogenass (Fig. 13). It is remarkable that membrane-bound hydrogenase operons contain sets of tightly linked accessory genes whose precise function is not understood. Inactivation of *hyaE* and *hyaF* in the hydrogenase 1 operon of *E. coli* (Menon et al., 1991) and the homologous gene sequences *hoxO* and *hoxQ* in the MBH operon of *R. eutropha* (Bernhard et al., 1996) lead to the formation of catalytically inactive hydrogenase, which accumulates in the cytosol.

Crystal structure analysis of the [FeFe] hydrogenase from *Desulfovibrio desulfuricans* (Nicolet et al., 1999) has confirmed earlier predictions concerning export of hydrogenases in sulfate reducers (Voordouw and Brenner, 1985). In the mature functional molecule, the 34-amino-acid twin arginine signal peptide, predicted from the DNA sequence of the small subunit, is missing (Hatchikian et al., 1999; Nicolet et al., 1999). This result clearly indicates that periplasmic [FeFe] hydrogenases are subject to a similar translocation process as [NiFe] hydrogenases. A second peptide of 24 amino acids, assigned to the C-terminus of the large *D. desulfuricans* subunit, is also absent in the mature protein, but whether this processing is related to translocation or

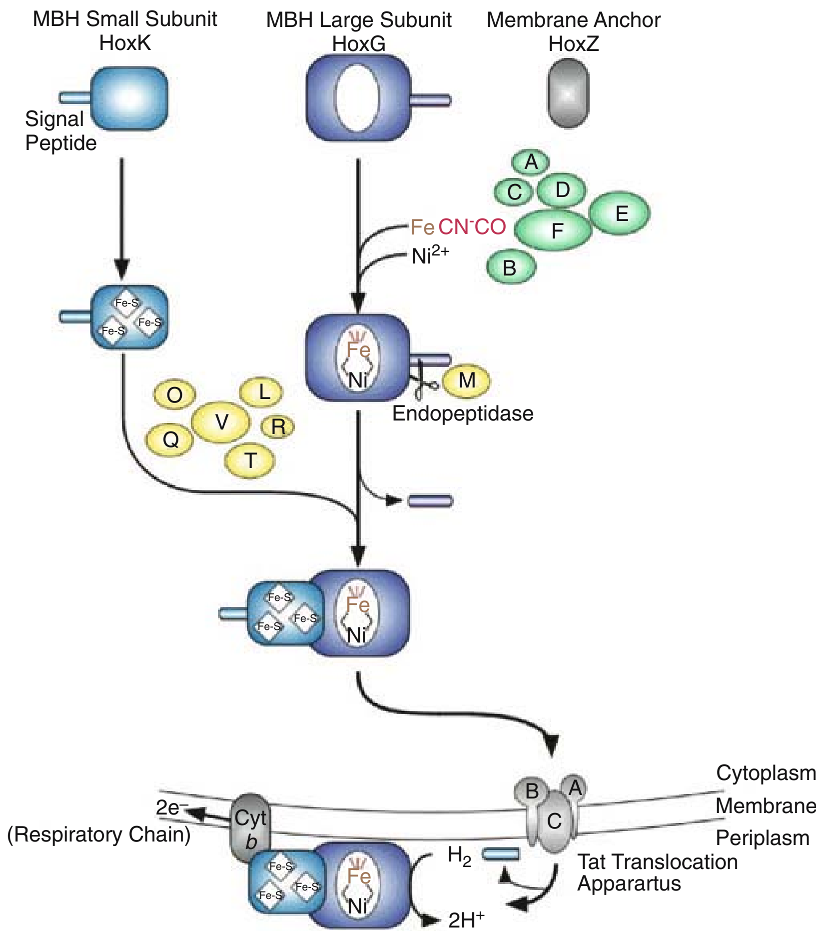


Fig. 13. Molecular model for the maturation of the *R. eutropha* membrane-bound hydrogenase. Hyp proteins (A, B, C, D, E and F) and specialized Hox accessory proteins (M, L, O, Q, R, T and V) are labeled. See text for details.

other maturation functions is still a matter of debate.

Hydrogenase Genes and Their Regulation

Genetic and molecular studies have led to the identification and characterization of the genetic determinants of numerous hydrogenase enzymes in bacteria, archaea and lower eukaryotes. More recently, genomic sequencing projects have contributed to the base of genetic data on hydrogenases. It is now possible to sketch the genetic organization of the hydrogenase genes of representatives of several important taxonomic groups. In some cases, the transcriptional units have been defined by Northern analysis. Detailed information on hydrogenase expression and the underlying regulatory mechanisms is only available for a few systems (see Friedrich et al. [2001] for a list).

It has long been known that various prokaryotes contain multiple hydrogenase isoenzymes. This raised the question regarding the total number of hydrogenases in a given organism. Genome sequence data is obviously an important source of information here and can provide a tentative answer which is, of course, subject to biochemical confirmation. The *E. coli* genome sequencing project confirmed the presence of the four previously identified hydrogenase operons (Blattner et al., 1997). The genome sequence of *Methanothermobacter thermoautotrophicus* predicts the existence of five distinct hydrogenases in this organism (Smith et al., 1997). Three of these have been characterized biochemically. Three sets of hydrogenase genes have been identified in the genome of *A. aeolicus* (Deckert et al., 1998). No hydrogenase genes were identified in the genomes of *H. influenzae* and *M. tuberculosis*.

In many prokaryotes, hydrogenase genes are carried on autochthonous plasmids, some of which are transmissible via conjugation (reviewed by Friedrich and Schwartz [1993]). Plasmid-linked hydrogenase genes are often found in organisms in which H_2 metabolism is part of a facultative lifestyle. Plasmid-borne hydrogenase genes provide a simple explanation for the combinations of hydrogenases of various biochemical types found in some prokaryotes.

Nucleotide sequences are now available for well over 100 hydrogenases. A compilation of these data presents, at first glance, a complex and confusing picture. This is, in part, due to the fact that, depending on the type of hydrogenase and its metabolic context, a more or less complex set of gene products is required. As outlined above, the three types of hydrogenases differ with respect to maturation. Furthermore, the requirement for regulatory proteins is different; conditionally expressed hydrogenases are governed by cognate regulators, which are superfluous in organisms in which the enzyme is synthesized constitutively. In general, five different classes of hydrogenase-related genes can be differentiated on the basis of function: 1) structural genes, i.e., the genes encoding peptides directly involved in the reversible activation of H_2; 2) genes coding for the additional subunits of multisubunit hydrogenases, e. g., the *b*-type cytochromes of bacterial membrane-bound hydrogenases; 3) accessory genes coding for proteins involved in hydrogenase maturation; 4) genes for redox couplers which are not components of a multisubunit hydrogenase enzyme, such as the polyferredoxins encoded by archaeal hydrogenase operons; and 5) regulatory genes. Adding to the complexity, different combinations of the above genes are grouped in transcriptional units which can be either adjacent or scattered over a replicon. Finally, the relationship of evolutionarily conserved genes is obscured by a hodgepodge of inconsistent nomenclature. The following survey summarizes data pertaining to genetic organization for a selection of representatives of the various groups of hydrogenases, pointing out some basic patterns underlying the diversity. In keeping with the premise that the three classes of hydrogenases ([NiFe], [FeFe] and [Fe]) originated independently, the discussion is divided into three parts.

Genetic Organization

[NiFe] Hydrogenases The bulk of the presently available nucleotide sequence data relates to the [NiFe] hydrogenases. Sorting these sequences solely on the basis of operon structure reveals seven groups, each of which defines a conserved gene pattern. On the one hand, these groups correlate well with the subclusters defined by clustering analysis of the deduced amino acid sequences of the hydrogenase subunits (Vignais et al., 2001). On the other hand, they reflect common biochemical and physiological properties of the respective enzymes. The seven groups are as follows: 1) the *vho* and *vht* operons encoding the methanophenazine-reducing hydrogenases of the methanogenic archaeon *Methanosarcina mazei*; 2) the operons encoding the bacterial membrane-bound hydrogenases; 3) the *mvh* operon of *Methanothermobacter* and the operons for F_{420}-nonreactive hydrogenases of *Methanococcus* strains; 4) the *fru*, *frc* and *frh* operons for the F_{420}-reactive hydrogenases of *Methanococcus voltae*, *Methanothermobacter thermoautotrophicus* and *Methanosarcina* species; 5) the operons for the cytoplasmic sulfhydrogenases of fermentative archaea, such as *Pyrococcus furiosus* and *Thermococcus litoralis*; 6) the genes for the cytoplasmic, NAD-reducing enzymes of *R. eutropha* and cyanobacteria; and 7) the operons for the multimeric, membrane-bound hydrogenases of the *E. coli* hydrogenase 3 family.

Archaeal Membrane-Bound Hydrogenases In *M. mazei*, two operons (*vhoGAC* and *vhtGACD*) encoding F_{420}-nonreactive hydrogenases have been identified and sequenced (Deppenmeier, 1995a; Deppenmeier et al., 1995b). Each operon contains genes for the hydrogenase small (*vhoG* and *vhtG*) and large subunits (*vhoA* and *vhtA*) followed by a gene for a *b*-type cytochrome (*vhoC* and *vhtC*). The *vht* operon contains a fourth gene, *vhtD*, which predicts a protein with similarity to the maturation proteases associated with bacterial membrane-bound hydrogenases, suggesting that at least one of the two *M. mazei* F_{420}-nonreactive hydrogenases undergoes a similar maturation process. The genomic sequence of *M. mazei* provides additional evidence for this assumption (Deppenmeier et al., 2002): A set of *hyp* genes (*hypC*, *hypD*, *hypA* and *hypE*) is located adjacent to the *vho* operon in the opposite orientation. Furthermore, tandem copies of *hypB* and *hypC* are found a few kilobases away from the *vhtGACD* operon and solitary copies of *hypF* and *hypE* are present at remote sites. Northern analysis confirmed that the *vho* and *vht* operons are both expressed as single transcripts approx. 4,500 nucleotides (nt) in size (Fig. 14).

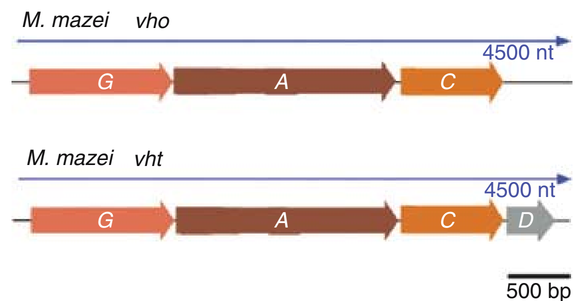


Fig. 14. Organization of the *vho* and *vht* operons of *Methanosarcina mazei*. Light brown arrows represent genes for the hydrogenase small subunits, dark brown arrows, genes for the large subunits, and orange arrows, genes for *b*-type cytochromes. A gene for a putative maturation protease, *vhtD*, is shown in gray. Thin blue arrows symbolize experimentally determined transcripts (sizes given in nucleotides).

Bacterial Membrane-Bound Hydrogenases The closest relatives of the Vho and Vht hydrogenases of *M. mazei* are the bacterial membrane-bound hydrogenases. Examples of this type of hydrogenase are found in both Gram-negative and Gram-positive bacteria. The most thoroughly studied representatives are the membrane-bound hydrogenases of various proteobacteria (Friedrich and Schwartz, 1993; Vignais and Toussaint, 1994). In contrast to the *vho* and *vht* operons, the proteobacterial genes form large, polycistronic transcriptional units. In the following, four examples of this group are discussed: The MBH locus of *R. eutropha*, the *hup*/*hyp* regions of *R. capsulatus* and *R. leguminosarum* and the *E. coli hya* operon.

The MBH locus of *R. eutropha* is a contiguous series of hydrogenase genes occupying approx. 23 kb on the megaplasmid pHG1 (Kortlüke et al., 1992; Dernedde et al., 1993; Dernedde et al., 1996; Fig. 15). The first two genes, *hoxK* and *hoxG*, which code for the small and large subunit of the hydrogenase, respectively, are followed by a gene (*hoxZ*) encoding a *b*-type cytochrome. This is the primary electron acceptor of the membrane-bound hydrogenase and also mediates attachment of the enzyme to the outer surface of the cytoplasmic membrane (Bernhard et al., 1997). The *hoxZ* gene is followed by a series of genes designated *M*, *L*, *O*, *Q*, *R*, *T* and *V*. The *hoxM* gene encodes a specific protease required for the C-terminal processing of the large subunit. The function of the other gene products is unclear. Immediately downstream of *hoxV* lies a second group of accessory genes called "*hyp* genes." The *hyp* genes are involved in the assembly of the [NiFe] center of the hydrogenase active site (see the section Maturation of Hydrogenases in this Chapter). Immediately downstream of the *R. eutropha* hyp region is a set of regulatory genes designated "*hoxA*," "*hoxB*," "*hoxC*" and "*hoxJ*" (Lenz et al., 1997). Their function will be discussed below in detail.

The same basic pattern of genes with minor variations is found in several other representatives of the proteobacteria. In *R. capsulatus*, a *hypF* gene is absent from the *hyp* operon. In its place is the regulatory gene *hupR1*. A set of regulatory genes (*hupTUV*), which corresponds to *hoxJ*/*hoxB*/*hoxC*, is encoded together with a *hypF* gene in a transcriptional unit immediately upstream of the main hydrogenase operon (Elsen et al., 1996; Fig. 15). In *R. leguminosarum*, an additional gene (*hupE*) of unknown function is located downstream of the protease gene, *hupD*. In *Methylococcus capsulatus* (Bath) hupE is adjacent to the structural genes hupSL (Csáki et al., 2001). The *E. coli hya* operon, which encodes a membrane-bound hydrogenase isoenzyme designated "hydrogenase 1," is simpler than the three examples discussed above (Menon et al., 1990; Fig. 15). This is partly due to the fact that maturation of this enzyme is mediated by the Hyp proteins encoded in an operon adjacent to the *hyc* operon. In addition to a gene for a maturation protease, *hyaD*, two other accessory genes, *hyaE* and *hyaF*, are present. It is not clear whether, aside from the Hyp proteins, accessory proteins encoded in the other hydrogenase operons are required for the function of hydrogenase 1.

An interesting variant is the *hynSL* operon of *Thiocapsa roseopersicina*. The genes *hynS* and *hynL*, which encode the small and large subunits, respectively, of a membrane-bound hydrogenase, are separated by two reading frames desig-

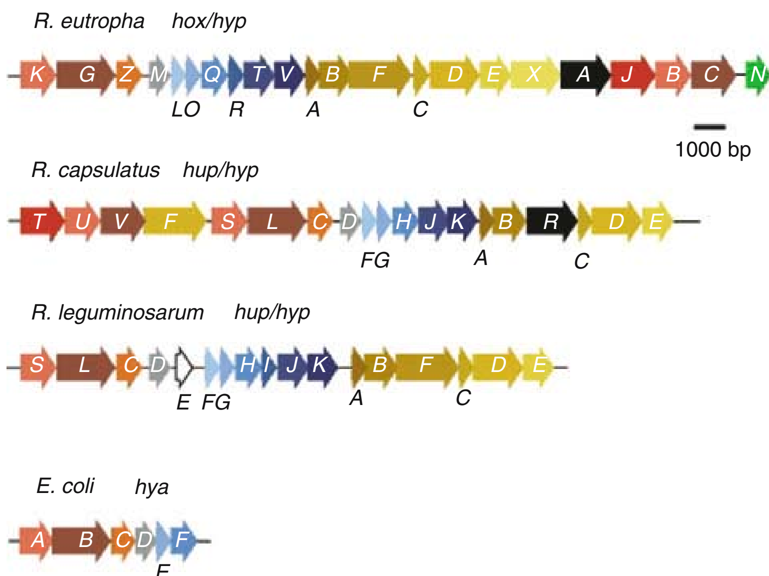


Fig. 15. Organization of hydrogenase operons of *R. eutropha*, *R. capsulatus*, *R. leguminosarum* and *E. coli*. Color-coding emphasizes similarity at the level of deduced amino-acid sequences. Light brown arrows represent genes for the small subunits, dark brown arrows, genes for the large subunits, and orange arrows, genes for *b*-type cytochromes. Genes for maturation proteases are shown in gray. The *hyp* genes are shown in shades of yellow and gold, and other accessory genes, in shades of blue. NtrC-type response regulators are black, and histidine protein kinases are red. The nickel transporter *hoxN* is colored green. Singular genes of unknown function are left uncolored.

nated *isp1* and *isp2*. These reading frames are not related to any known hydrogenase accessory genes (Rákhely et al., 1998).

The Cytoplasmic F_{420}-Nonreactive Hydrogenases The operons encoding the F_{420}-nonreactive hydrogenases of *M. thermoautotrophicus* (*mvhDGAB*) and *M. voltae* (*vhcDGAB* and *vhuDGAB*; Reeve et al., 1989; Halboth and Klein, 1992) are representatives of another conserved family (Fig. 16). The first open reading frame (ORF) of each operon predicts a protein of unknown function. This is followed by genes for the small (*mvhG*, *vhcG* and *vhuG*) and large subunits (*mvhA*, *vhcA* and *vhuA*) of the hydrogenase. A fourth gene encodes a polyferredoxin, which may be the primary electron acceptor interacting with the hydrogenase in vivo. The *vhuDGAUB* operon is an interesting variant (Halboth and Klein, 1992). In place of a gene for the large subunit, as is found in the other operons, there are two ORFs. The second, short ORF, designated *vhuU*, predicts a polypeptide related to the C-terminus of typical large subunits. This is particularly intriguing, since this part of the protein participates in coordination of the [NiFe] center.

The F_{420}-Reactive Hydrogenases Genes for F_{420}-reactive hydrogenases have also been cloned and sequenced. *Methanococcus voltae* and *M. barkeri* each contains two such operons (Halboth and Klein, 1992; Vaupel and Thauer, 1998; Fig. 17). The four operons share the same pattern: The hydrogenase large and small subunits are encoded by the first and third genes, respectively. The final gene of the set encodes the third subunit of the trimeric enzyme, which may carry the site of interaction with the cofactor F_{420}. In all four operons, the genes for the large and small subunits are separated by an additional ORF which probably encodes a specific protease responsible for the C-terminal proteolytic processing of the large subunit.

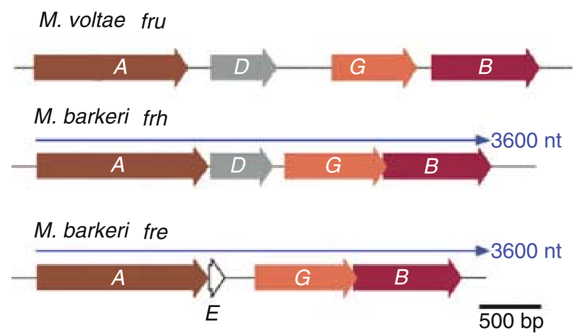


Fig. 17. Organization of the *M. voltae fru* operon and of the *M. barkeri frh* and *fre* operons. The genes for the subunit with the F_{420}-binding site are dark red. For additional details, see the legends to Figs. 14 and 15.

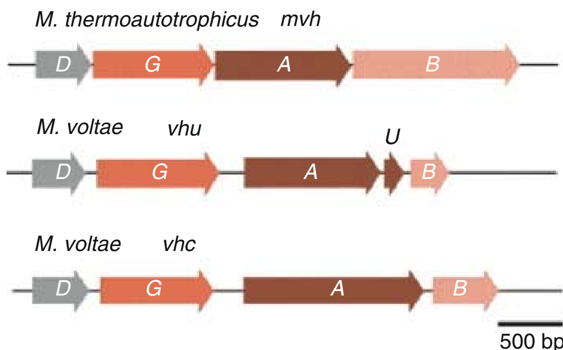


Fig. 16. Organization of the *M. thermoautotrophicus mvh* operon and of the *M. voltae vhc* and *vhu* operons. The genes for polyferredoxins are shown in pink. For additional details see the legends to Figs. 14 and 15.

Sulfhydrogenases The tetrameric, H_2-producing sulfhydrogenases of archaea are encoded by four-gene transcriptional units (Pedroni et al., 1995; Rákhely et al., 1999). In *P. furiosus* and *T. litoralis*, the small and large subunits are encoded by the third and fourth genes of each operon. The first and second genes encode proteins related to subunits of sulfite reductases. The latter gene products probably form the dimeric sulfur-reducing module of the tetrameric enzyme (Fig. 18).

The Cytoplasmic, NAD-Reducing Hydrogenases The cytoplasmic, NAD-reducing hydrogenase of *R. eutropha* is the prototype of a family of multimeric enzymes related both structurally and physiologically. The hydrogenases of this family are often called "bidirectional." The *R. eutropha* enzyme is encoded by a complex operon consisting of four structural genes and five accessory genes (Tran-Betcke et al., 1990; Thiemermann et al., 1996; Wolf et al., 1998; Fig. 19). The operon is expressed as a 7,600-nt primary transcript, which is apparently cleaved into smaller secondary mRNAs (Oelmüller et al., 1990). The first two genes of the operon (*hoxF* and *hoxU*) code for the NADH-oxidoreductase (diaphorase) moiety of the enzyme. The two genes immedi-

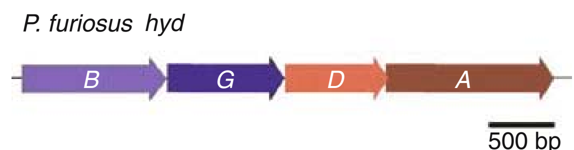


Fig. 18. Organization of the *P. furiosus hyd* operon. The genes for the sulfur reductase module are purple. For additional details, see the legends to Figs. 14 and 15.

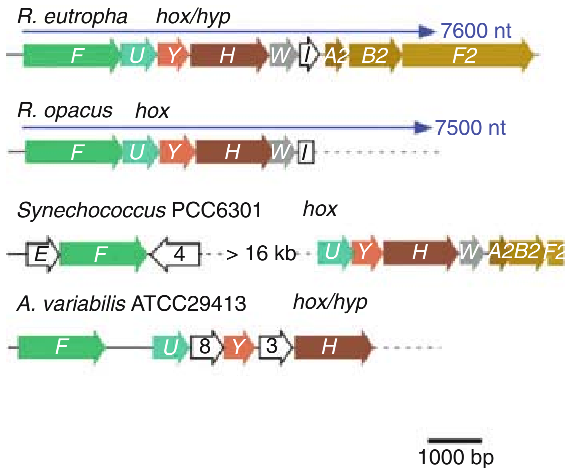


Fig. 19. Organization of the *R. eutropha* and *R. opacus* SH loci and of the *Synecchococcus* PCC6301 (*A. nidulans*) and *A. variabilis hox* regions. The genes encoding subunits of the NADH oxidoreductase module are shown in shades of green, and the *hyp* genes, in shades of yellow and gold. For additional details see the legends to Figs. 14 and 15.

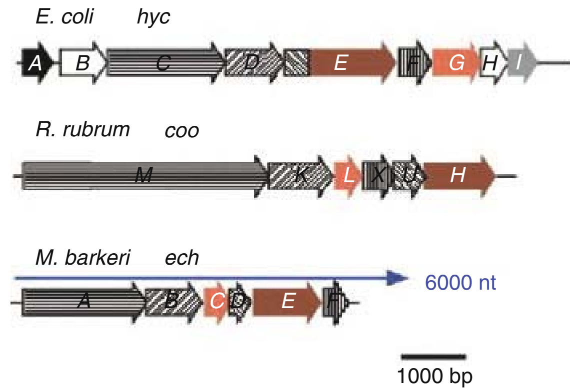


Fig. 20. Organization of the *E. coli hyc* operon, the *R. rubrum coo* operon, and the *M. barkeri ech* operon. Genes encoding proteins related to components of NADH:ubiquinone oxidoreductase I of *E. coli* are hatched. The gene products of *E. coli hycC*, *hycD*, *hycE* (partial) and *hycF* (and of the related genes) are isologous to *E. coli* proteins NuoL/NuoM/NuoN, NuoH and NuoI, respectively. For additional details see the legends to Figs. 14 and 15.

ately downstream (*hoxY* and *hoxH*) encode the small and large subunits, respectively, of the hydrogenase module. The product of *hoxW* is a specific protease which mediates C-terminal processing of the large subunit (Thiemermann et al., 1996). The product of *hoxI* is identical to the so-called "B protein," which is coexpressed with the SH (Kärst et al., 1987). Downstream of *hoxI* is a duplicated set of *hyp* genes: *hypA2*, *-B2* and *-F2* (Wolf et al., 1998). The operon encoding the soluble, tetrameric hydrogenase of the Gram-positive bacterium *Rhodococcus opacus* seems to be a carbon copy of the *R. eutropha* operon. The sequenced segment of the *R. opacus* operon revealed the genes *hoxF*, *-U*, *-Y*, *H*, *-W* and part of *hoxI* (Grzeszik et al., 1997a). A 7,500-nt transcript has been reported, suggesting that here, too, *hyp* genes are included in the hydrogenase mRNA. A similar set of genes directs the expression of a so-called "bidirectional hydrogenase" in the unicellular cyanobacterium *Synecchococcus* PCC6301 *Anacystis nidulans*; Boison et al., 1996). As in the case of *R. eutropha*, a set of structural genes (*hoxUYH*) is followed by a contiguous set of accessory genes (*hoxWhypABF*). A *hoxI*-like gene is missing in this series of genes. A *hoxF* homologon accompanied by a gene designated "*hoxE*" is encoded in a separate transcriptional unit located at a distance of 16 kb. HoxE is related to the protein NuoE of complex I and is a component of the mature hydrogenase (Schmitz et al., 2002). Another variant of the same genetic pattern is found in the filamentous cyanobacterium *Anabaena variabilis*. The bidirectional hydrogenase of this organism is encoded by an operon containing the genes *hoxF*, *-U*, *-Y* and *-H* (Schmitz et al., 1995). ORF8 and ORF3, which flank *hoxY*, code for proteins of unknown function (Fig. 19).

Multimeric H_2-Evolving Hydrogenases Both archaea and bacteria contain multimeric H_2-evolving hydrogenases encoded in complex operons (Fig. 20). The prototype of this class of enzymes is the *E. coli* hydrogenase 3, which uses electrons from formate to reduce protons, thereby generating H_2 gas. Hydrogenase 3 is encoded in a complex locus together with a set of Hyp proteins. The corresponding operons (*hycBCDEFGHI* and *hypABCDE*) are adjacent to each other and are transcribed from divergent promoters (Böhm et al., 1990; Figs. 20 and 23). The gene for the positive regulator FhlA is located downstream of the *hyp* operon and is transcribed from its own promoter. Two additional genes form a separate transcriptional unit located in the same gene cluster: *hypF*, which encodes a maturation protein, and *hydN*, whose function is unknown. The hydrogenase large and small subunits are encoded by the genes *hycE* and *hycG*, respectively. The *hycI* gene encodes a maturation endopeptidase. The function of the other gene products is largely unknown. The product of *hycF* is probably an FeS-containing protein. The *hycC* and *hycD* genes encode membrane-spanning proteins. The latter three proteins, as well as HycE and HycG, are related to components of the NADH:ubiquinone oxidoreductase complex (complex I) of *E. coli* and other organisms. Similar sets of genes have been identified in the anoxygenic phototroph *R. rubrum* (Fox et al., 1996a), the chemolithoau-

totroph *C. hydrogenoformans* (Soboh et al., 2002), and the methanogen *M. barkeri* (Künkel et al., 1998). The genomes of *P. furiosus*, *M. marburgensis*, *M. thermoautotrophicus*, *M. kandleri* and *M. janaschii* encode related hydrogenases with a more complex subunit composition (Tersteegen and Hedderich, 1999). *Escherichia coli* contains a second operon of the same type designated "*hyf*" (Andrews et al., 1997; Skibinski et al., 2002), the function of which is unknown. The operons of this family differ in gene number and order. Nevertheless, they all encode multisubunit hydrogenases containing proteins related to various components of complex I.

The above summary leads to the conclusion that typical patterns of gene organization are correlated with the major structural types of hydrogenases. Thus, conserved metabolic and structural specialization are reflected in conserved operonic structures. Basic blueprints of genetic organization persist across taxonomic boundaries.

[FeFe] Hydrogenases The genetic information for [FeFe] hydrogenases is contained, judging by the available sequence data, in simple transcriptional units consisting of 1–3 genes. The [FeFe] hydrogenase of *Desulfovibrio vulgaris* (Hildenborough), the first hydrogenase ever to be cloned and sequenced, is encoded by a pair of genes for the large and small subunits (Voordouw and Brenner, 1985). The structure of the *hydAB* operon of the closely related strain *Desulfomicrobium norvegicum* (formerly *Desulfovibrio desulfuricans* strain Norway) is the same (Hatchikian et al., 1999). Owing to the lack of transcript data on the one hand and to the paucity of sequence data on the other, it cannot be ruled out that additional genes belong to the same transcriptional unit or that hydrogenase accessory genes are present at other sites. The monomeric hydrogenase (CpI) of *Clostridium pasteurianum* is encoded by a solitary gene which appears to be a fusion of the paired genes which normally code for the two hydrogenase subunits (Meyer and Gagnon, 1991; Fig. 10). The same applies to the monomeric hydrogenase of *M. elsdenii* (Atta and Meyer, 2000). The set of genes encoding the [FeFe] hydrogenase of the hyperthermophile *Thermotoga maritima* is a singular case. Three genes, designated "*hydC*," "*-B*" and "*-A*," determine the γ, β, and α subunits of the trimeric enzyme (Verhagen et al., 1999). The *hydA* gene encodes the basic hydrogenase moiety and resembles the "fused" gene of *C. pasteurianum* (Fig. 10). The sequence of *hydB* predicts an FeS-containing flavoprotein related to HndC of *D. fructosovorans*. The deduced sequence of HydC reveals that this protein is related to NuoE of complex I.

At present the cytoplasmic, NADP-reducing hydrogenase of *D. fructosovorans* is both biochemically and genetically in a class of its own (Malki et al., 1995). The first gene of the *hndABCD* operon, *hndA*, predicts a protein similar to the large diaphorase subunit of the NAD-reducing hydrogenase of *R. eutropha*. The deduced product of *hndC* also shows marked similarity to the diaphorase moiety of the *R. eutropha* enzyme. The N-terminal part of HndC resembles HoxF, whereas the C-terminal part is similar to HoxU. Remarkably, HndD is not related to *R. eutropha* HoxH but rather contains sequence motifs typical of [FeFe] hydrogenases.

[Fe] Hydrogenases The [Fe] hydrogenases of *M. marburgensis* and other methanogenic archaea are N^5,N^{10}-methylene-H_2MPT dehydrogenases (Afting et al., 1998). The monocistronic gene for the homodimeric enzyme of *M. thermoautotrophicus* has been identified in the genomic sequence (Smith et al., 1997).

Regulation of Hydrogenase Genes

[NiFe] Hydrogenases The *R. eutropha hox* regulon is composed of two operons located on the 450-kb pHG1 megaplasmid and separated by ca. 70 kb (Eberz et al., 1986; Figs. 15 and 21). The MBH operon encodes the dimeric, membrane-bound hydrogenase and is transcribed as a >17-kb mRNA (Schwartz et al., 1999). The genes for the cytoplasmic, NAD-reducing hydrogenase belong to the SH operon. A 7,600-nt transcript has been mapped to this locus (Oelmüller et al., 1990). Expression of the two operons is coordinate and responds to two environmental conditions (Fig. 21): 1) the availability of H_2 and 2) the quality of the carbon and energy sources present in addition to H_2 (Friedrich et al., 1981a; Friedrich, 1982). The expression of the hydrogenase regulon is controlled at the level of transcription. The -24 and -12 promoters have been mapped upstream of the two operons and their activity is strictly dependent on the minor transcription factor σ^N (Schwartz et al., 1998). The activity of P_{MBH} and P_{SH} is governed by the NtrC-type activator protein HoxA (Eberz and Friedrich, 1991; Schwartz et al., 1998). This positive transcriptional regulator is encoded by the gene *hoxA* located downstream of the *hyp* genes in the MBH operon (Eberz and Friedrich, 1991). A moderate, σ^D-dependent promoter (P_{hoxA}) drives low-level, constitutive transcription of *hoxA* and perhaps of the downstream genes *hoxB*, *hoxC* and *hoxJ* as well. Constitutive transcription of *hoxA* guarantees that at least a basal level of HoxA is present in the cell at all times, making sure that the organism is poised to respond to the environmental cue for hydroge-

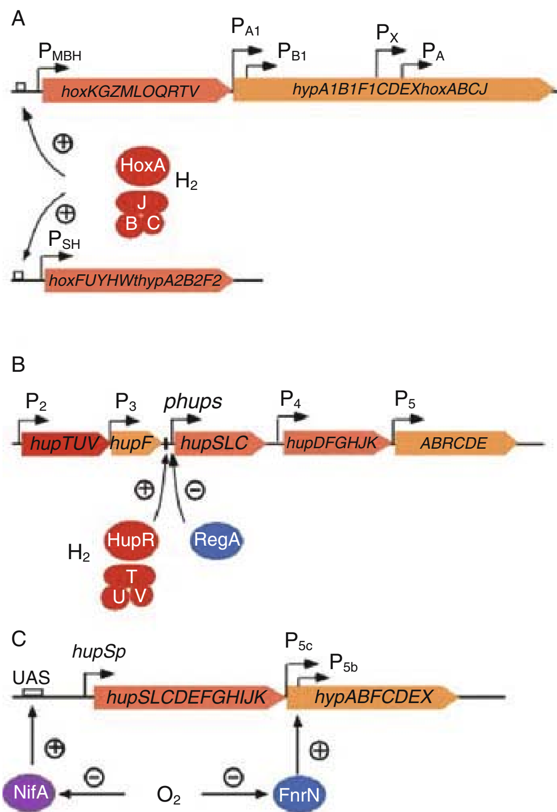


Fig. 21. Molecular models for the regulation of hydrogenase expression in *Ralstonia eutropha* (A), *Rhodobacter capsulatus* (B) and *Rhizobium leguminosarum* (C). Colored arrows represent genes or operons. Colored ovals symbolize regulatory proteins. Binding sites of regulatory proteins are indicated by open boxes.

nase expression, i.e., H_2 (Schwartz et al., 1999). At least some of the transcripts initiated at P_{MBH} extend a full 17,000 nt, encompassing *hoxA*. As a result, P_{MBH} and *hoxA* form a positive feedback system. Induction of the hydrogenase regulon results in the amplification of both MBH and HoxA. The amplified levels of HoxA also drive up the production of SH. HoxA is, like other members of the NtrC family, a DNA-binding protein (Zimmer et al., 1995). Deletion analysis of the SH upstream region pointed out a tandem palindrome 5′ of the -24 and -12 promoters. A similar motif is also present in the MBH upstream region. The distance between the palindromic element and promoter in the two upstream regions is compatible with the standard model for -24/-12 promoter activation, involving binding of the activator protein and protein-protein contact between activator and σ^N -RNA polymerase (RNAP) holoenzyme. In vitro studies using HoxA-containing extracts indicate that HoxA binds to DNA-fragments from the SH upstream region harboring the tandem palindrome, suggesting an NtrC-like mechanism of transciptional activation (Zimmer et al., 1995).

Detailed investigation of H_2-dependent hydrogenase expression led to the identification of an H_2-responsive signalling pathway (Friedrich et al., 1996; Lenz and Friedrich, 1998). This pathway is mediated by three components: 1) the response regulator HoxA, 2) a histidine protein kinase designated "HoxJ" and 3) a cytoplasmic hydrogenase-like protein, called a "regulatory hydrogenase" (RH), encoded by the genes *hoxB* and *hoxC*. HoxA and HoxJ constitute a two-component system. As in other two-component systems, HoxJ phosphorylates itself in an autocatalytic reaction. Subsequently, transfer of the phosphate group to the cognate response regulator HoxA can take place. Unlike most other two-component systems, however, phosphorylation of the response regulator has a negative effect on transcription of the subordinate genes. This conclusion is based on various experimental findings. First, deletion of gene *hoxJ* results in a deregulation of the system manifest as a drastic increase in hydrogenase gene expression in the absence of H_2. Furthermore, replacement of the conserved Asp-55 residue, the putative phosphorylation target in HoxA, has a similar effect, as does alteration of the Gly-422 in the kinase module of HoxJ (Lenz and Friedrich, 1998). The phosphotransfer reaction from HoxJ to HoxA has been demonstrated in vitro (M. Forgber, O. Lenz and B. Friedrich, unpublished observation). HoxB and HoxC comprise a nickel-containing tetramer with the structure $\alpha_2\beta_2$ (Kleihues et al., 2000; Bernhard et al., 2001). Comparisons of deduced amino-acid sequences revealed that HoxBC is a relative of the dimeric [NiFe] hydrogenases. The protein does, indeed, catalyze low but significant rates of H_2-dependent methylene blue reduction in vitro (Bernhard et al., 2001). The RH forms a complex with HoxJ (Bernhard et al., 2001). This complex can be reconstituted in vitro by mixing purified components. A stretch of amino acids at the C-terminus of HoxB is essential for both the formation of the $\alpha_2\beta_2$ tetramer and the complex formed between this tetramer and HoxJ (Buhrke, 2002). Notably, this C-terminal peptide is different from the C-termini of the energy-generating membrane-bound hydrogenases. In RH null mutants, hydrogenase gene expression is totally abolished, indicating that the RH is a positive regulator (Lenz and Friedrich, 1998). The available data support the following molecular model for H_2 sensing (Lenz et al., 2002; Fig. 22): A cytoplasmic H_2-sensing complex, consisting of RH and HoxJ, governs the phosphorylation status of HoxA. In the absence of H_2, HoxJ mediates phosphotransfer to HoxA, rendering it inactive. When molecules of H_2 enter the cell, they engage

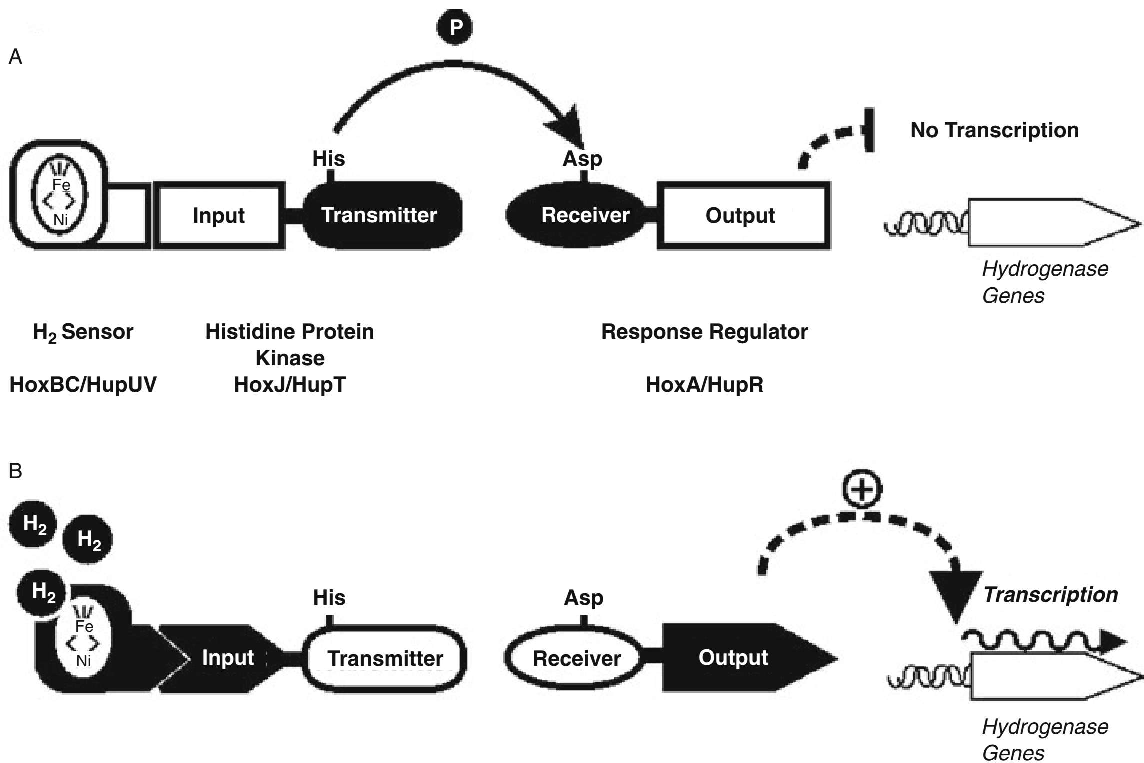


Fig. 22. Molecular model for H_2 sensing in *R. eutropha* and *R. capsulatus*. The upper part of the diagram (A) illustrates the interactions between the components of the H_2-sensing apparatus in the absence of H_2. The lower part (B) represents the protein-protein interactions in the presence of H_2. Transmitter and receiver domains of the histidine kinases and response regulators, respectively, are shown in red. The solid arrow symbolizes the phosphotransfer reaction. Dashed arrows indicate positive (+) or negative (–) control.

the RH, unleashing a specific interaction between it and HoxJ. The latter interaction may involve the transfer of electrons between the additional cofactor in the RH and the PAS domain in the input module of HoxJ. Thus, the RH blocks the net phosphoryl transfer from HoxJ to HoxA and ultimately is a positive control on H_2-dependent transcription.

Interestingly, the signal transduction pathway is cryptic in some natural isolates of *R. eutropha* (Lenz et al., 1997). In *R. eutropha* H16, a single-nucleotide exchange in the region of *hoxJ* corresponding to the transmitter domain reactivates signal transduction.

A second layer of regulation is superimposed on H_2-responsive signalling. This involves control of hydrogenase expression exerted by additional carbon and energy sources. Catabolite control is probably very important for *R. eutropha*, since in the natural habitat, it will very likely be confronted with both H_2 and organic substrates at the same time. Monitoring hydrogenase gene expression in the presence of both H_2 and organic substrates reveals a clear correlation between substrate quality and expression levels: Expression is high when poor substrates are available in addition to H_2 and vice versa (O. Lenz and B. Friedrich, unpublished results). In this context, substrate quality is judged on the basis of the corresponding growth rate. The underlying regulatory mechanisms are not yet known.

Despite major differences in the organization of the hydrogenase determinants in the phototroph *R. capsulatus* and the chemolithotroph *R. eutropha*, the H_2-oxidizing systems of these two organisms share a common regulatory mechanism. The dimeric [NiFe] hydrogenase of *R. capsulatus* is encoded in the *hupSLC* operon under the control of the p*hupS* promoter (Toussaint et al., 1997; Fig. 21). The *hupDFGHJK*, *hypAB-hupR-hypCDE* and *hupTUVF* operons are transcribed from separate promoters. An additional promoter is located upstream of *hypF*. Promoter p*hupS* is under the control of an NtrC-like regulator, HupR, but is σ^D-dependent as evidenced by mutagenesis of promoter sequences (Dischert et al., 1999) and by experiments with *rpoN* mutants (Colbeau and Vignais, 1992). HupR binds to the palindromic sequence TTG-N_5-CAA upstream of p*hupS* (Toussaint et al., 1997; Dischert et al., 1999). An integration host

factor (IHF)-binding motif located between the upstream activation sequence (UAS) and p*hupS* is another feature typical of promoters controlled by NtrC-like regulators. As in other systems, the architectural protein IHF is not essential for hydrogenase expression but has a pronounced stimulatory effect (Toussaint et al., 1991). The presence of H_2 triggers a 10-fold induction of hydrogenase activity (Toussaint et al., 1997). This effect depends on an H_2-sensing system similar to that found in *R. eutropha*. A histidine protein kinase, HupT, and an H_2-sensing hydrogenase, HupUV, cooperate with HupR in mediating signal transduction. HupT is a negative regulator (Elsen et al., 1993; Dischert et al., 1999). According to the model, HupT mediates phosphorylation of HupR in the absence of H_2 (Fig. 22). As in the case of *R. eutropha* HoxA, the phosphorylated form of HupR is inactive, preventing transcription of the hydrogenase genes under noninducing conditions (Dischert et al., 1999). HupUV is a cytoplasmically localized dimeric [NiFe] hydrogenase with a low but significant level of hydrogenase activity (Vignais et al., 1997; Vignais et al., 2000). HupUV presumably interacts with HupT governing the HupT/HupR phosphotransfer reaction. This interaction is, however, diametrically different from that of its *R. eutropha* counterpart. Since HupUV$^-$ mutants express hydrogenase constitutively (Elsen et al., 1996), HupUV must have a stimulatory effect on the net phosphoryl transfer to HupR. Apart from the specific, H_2-dependent control, hydrogenase expression is also regulated by the global, redox-responsive regulator pair RegA/RegB (Elsen et al., 2000). RegA exerts a negative effect on p*hupS* by binding to a site located between the promoter and the HupR UAS.

In contrast to *R. eutropha* and *R. capsulatus*, hydrogenase expression in the symbiotic N_2-fixer *R. leguminosarum* is not controlled by H_2 availability. In the latter organism, hydrogenase is expressed in bacteroids but not in vegetative cells (Palacios et al., 1990). The hydrogenase genes, which belong to three transcriptional units, are under the control of O_2-sensitive global regulators (Fig. 21). The first operon, containing the hydrogenase structural genes, is regulated by NifA (Brito et al., 1997). Transcription is driven by the -24/-12-type promoter *hupS*$_P$ upstream of *hupS* (Hidalgo et al., 1992). Deletion analysis of the region 5′ of *hupS*$_P$ indicated the existence of sequence elements essential for *hup* gene transcription. Experiments in a heterologous system indicated that transcription from *hupS*$_P$ is dependent on σ^N and is under the control of NifA (Brito et al., 1997). Similar experiments also indicated the involvement of IHF in the activation of *hupS*$_P$. Thus, the *R. leguminosarum hup* genes belong to the *nif* regulon. The coexpression of hydrogenase and nitrogenase in *R. leguminosarum* is not surprising, since the physiological role of hydrogenase in this organism is the utilization of H_2 generated as a byproduct of N_2 fixation. A second operon contains *hyp* genes: *hypBFCDEX*. Expression of this operon is induced under microaerobic conditions both in bacteroids and in vegetative cells (Palacios et al., 1990). Two transcriptional start sites have been mapped upstream of *hypB*, suggesting the existence of two promoters designated "P_{5a}" and "P_{5b}" (Hernando et al., 1995). While the significance of P_{5a} is unclear, P_{5b} appears to be a typical Fnr-dependent promoter with a characteristic anaerobox. *Rhizobium leguminosarum* contains two copies of the gene *fnrN* (Gutierrez et al., 1997). The expression of the *hypBFCDEX* operon is reduced in single *fnrN* mutants and abolished in the double mutant. A third transcriptional unit consists of the gene *hypA* (Hernando et al., 1998). Unlike the other *hyp* genes, *hypA* is expressed only under symbiotic conditions. The regulation of the *hypA* promoter, P_{5c}, is not yet understood. An intriguing finding is the discovery of a defective copy of a gene for a response regulator in the *Rhizobium leguminosarum* hydrogenase gene cluster (Brito et al., 1997). This remnant could be a relic of a defunct H_2-dependent regulatory system.

A very different regulatory mechanism controls expression of the *hyc* genes which encode the hydrogenase isoenzyme 3 of *E. coli*. The divergently transcribed *hycBCDEFGHI* and *hypABCDE* operons belong to the formate regulon (Böhm et al., 1990; Lutz et al., 1990; Lutz et al., 1991). A third transcriptional unit, separated from the *hycBCDEFGHI* operon by the functionally unrelated genes *ascB*, *-F* and *-G*, consists of the genes *hydN* and *hypF* (Maier et al., 1996). These operons are transcribed from three σ^N-dependent promoters designated "P_C," "P_P" and "P," respectively (Fig. 23). The activator protein FhlA governs the expression of the regulon in response to intracellular formate accumulation (Schlensog and Böck, 1990; Schlensog et al., 1994). The FhlA protein forms a complex with formate, binds to sites upstream of the subordinate promoters, and is, itself, transcribed by the weak constitutive, σ^D-dependent promoter P_{fhlA}. However, transcripts originating at P_P encompass *fhlA*, leading to an amplification of intracellular FhlA levels under inducing conditions. A second regulator, HycA, counteracts FhlA and thus mediates negative control of the regulon.

Hydrogenases 1 and 2 of *E. coli* are encoded by the operons *hyaABCDEF* and *hybOABCDEFG*, respectively (Menon et al., 1990; Menon et al., 1994b). Both biochemical and genetic studies have led to a coherent picture of the regula-

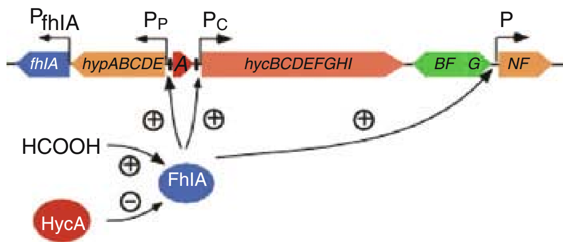


Fig. 23. Molecular model for the regulation of the *E. coli hyc* and *hyp* operons. See Fig. 21 for details.

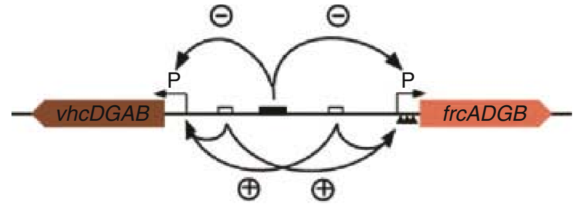


Fig. 24. Molecular model for the regulation of the selenocysteine-containing hydrogenases in *Methanococcus voltae*. The colored arrows represent the *vhc* and *frc* operons. Promoters are symbolized by arrows marked "P." A solid box denotes a negatively acting sequence element. Two positively acting sequence elements are represented by open boxes. Solid triangles denote a repetitive sequence motif.

tion of these operons (Ballantine and Boxer, 1985; Ballantine and Boxer, 1986; Sawers et al., 1985; Sawers and Boxer, 1986a; Brøndsted and Atlung, 1994; Brøndsted and Atlung, 1996; Wu et al., 1989; Richard et al., 1999). Like the *hyc* operon, both the *hya* and *hyb* operons are induced under anaerobic conditions. The ArcA/ArcB system is involved in the regulation of both operons albeit differently. Under anaerobic conditions ArcA suppresses *hyb* expression and activates *hya* expression (Richard et al., 1999). The fumarate and nitrate reduction regulator (FNR) is also involved in the expression of the active holoenzymes but the effect is evidently indirect. Nitrate, acting via the NarX/NarL and NarQ/NarP systems, represses the synthesis of both hydrogenases. Another two-component system, DpiA/DpiB stimulates expression of the *hya* operon. The latter effect is mediated by the positive regulator AppY.

An unusual regulatory mechanism has been reported for the *Anabaena* sp. strain PCC 7120 *hup* operon (Carrasco et al., 1995). This filamentous cyanobacterium is capable both of oxygenic photosynthesis and nitrogen fixation. Since nitrogenase is an O_2-labile enzyme, the processes of nitrogen fixation and photosynthesis demand a spatial separation. In *Anabaena* PCC7120, nitrogen fixation takes place only in heterocysts. In the vegetative cells, which do not require uptake hydrogenase, the *hupL* gene is inactive owing to the presence of a 10.5-kb intervening sequence element. In the course of differentiation to a heterocyst, the intervening element is excised resulting in a continuous *hupL* reading frame. Another recent discovery is the observation that expression of hydrogenase genes in *Rhodospirillum rubrum* and *Synechococcus* sp. PCC7942 oscillates in a circadian rhythm (Van Praag et al., 2000; Schmitz et al., 2001). In the case of *Synechococcus*, both the *hoxEF* and *hoxUYHWhypAB* operons are controlled at the level of transcription by a circadian clock. A photoreceptor in the form of bacteriophytochrome is probably responsible for entrainment of the oscillation (Schmitz et al., 2000).

Experimental data on the regulation of hydrogenase genes in archaea are scant. The best studied system is the methanogen *Methanococcus voltae* (Sorgenfrei et al., 1997). In *M. voltae*, four operons encode two selenium-containing and two selenium-free hydrogenase isoenzymes (Halboth and Klein, 1992). It is, therefore, not surprising that selenium is a key factor governing expression of the hydrogenase genes (Berghöfer et al., 1994).

The *fruADGB* and *vhuDGAUB* operons, which code for the selenium-containing enzymes, are expressed both in the presence and absence of selenium. In contrast, transcripts of the corresponding operons for the selenium-free enzymes are detectable only under selenium limitation. The latter operons, *frcADGB* and *vhcDGAB*, are arranged in a head-to-head orientation and are transcribed from divergent promoters located in the common 453-bp upstream region (Fig. 24). Transcription from the *frc* and *vhc* promoters is coordinately regulated (Noll et al., 1999). Deletion analysis of the upstream region suggests the presence of both positively and negatively acting sequence elements. One of the negatively acting sequence elements is located between the *frc* promoter and the start codon of *frcA*, and consists of a tandem repetition of a heptamer (Noll et al., 1999). An undecamer (5′-TCTATATAAAC-3′) located upstream of each of the promoters was shown to mediate positive control. Interestingly, mutations in either sequence element affect both promoters. A 55-kDa protein which binds specifically to this sequence element has been purified by DNA-affinity chromatography (Müller and Klein, 2001).

A study on the expression of the *M. barkeri ech* operon revealed that *ech* transcript was present under all growth conditions tested, suggesting that *ech* transcription is constitutive (Künkel et al., 1998). In *M. mazei*, the *vho* and *vht* operons are differentially expressed

(Deppenmeier, 1995a): *vht* is expressed during growth on H_2 and CO_2 but not on acetate. The *vho* operon, on the contrary, seems to be expressed constitutively. This points to a degree of physiological specialization of the two isoenzymes.

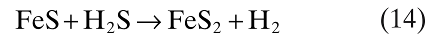
The examples discussed above reveal that hydrogenase enzymes are integrated into specific physiological functions by diverse regulatory mechanisms. It is evident that the regulatory scheme is dictated more by the physiological context than by the phylogenetic origin of the given hydrogenase.

[FeFe] Hydrogenases Not much is known about the regulation of genes for [FeFe] hydrogenases. *Clostridium acetobutylicum* produces high levels of hydrogenase activity during acidogenic fermentation and very low levels after switching to solventogenesis (Gorwa et al., 1996). The monocistronic *hydA* operon, which encodes a [FeFe] hydrogenase, is expressed as a 1900-nt transcript (Gorwa et al., 1996). The intracellular levels of *hydA* mRNA show the same pattern of substrate dependence as the enzyme activity levels, suggesting that *hydA* is regulated at the level of transcription.

[Fe] Hydrogenases *Methanothermobacter marburgensis* forms an [Fe] hydogenase called "H_2-forming methylenetetrahydromethanopterin" or "Hmd." This enzyme is synthesized regardless of the availability of nickel. However, under nickel-limited conditions the cytoplasmic level of the enzyme is raised sixfold. Under the latter conditions, Hmd substitutes for the F_{420}-reducing hydrogenase, a [NiFe] hydrogenase, in a reducing step of methanogenesis (Fig. 3). The expression of Hmd is regulated at the transcriptional level and responds to Ni concentration but not to the availability of H_2 (Afting et al., 2000).

Evolutionary Aspects

Since the pioneering work of Miller (1953) showing that the synthesis of the simpler organic building blocks of living organisms could occur spontaneously under conditions assumed to be similar to those on the primeval earth, biologists have envisaged an evolution of prebiotic systems in an ocean rich in organic compounds (Miller and Orgel, 1974). Hence, it was widely assumed that the first organisms were heterotrophs which fed on the organic compounds in the primeval broth. More recently, discrepancies in this hypothesis have prompted a reconsideration (Maden, 1995). One of the incongruities confronting the assumption of a heterotrophic origin of life is the fact that many of the deepest branching lineages of the phylogenetic tree contain autotrophic organisms. This indicates that autotrophy is not of recent origin but rather very ancient. Taking this into account, various hypotheses postulating an autotrophic origin of life have been put forward. The most comprehensive and rigorous of these was proposed by Wächtershäuser (Wächtershäuser, 1988; Wächtershäuser, 1990; Wächtershäuser, 1992). According to his theory of "pyrite-pulled surface metabolism," the primordial, energy-yielding process for prebiotic evolution is the reaction of ferrous sulfide and H_2S yielding pyrite and H_2:



$$FeS + H_2S \rightarrow FeS_2 + H_2 \quad (14)$$

The free energy for this reaction under standard conditions is -38 kJ/mol. This is sufficient to drive an archaic CO_2-fixing cycle similar to the reductive citric-acid cycle of contemporary organisms. Wächtershäuser suggests that pyrite formed in this process could serve as a matrix for the growing pool of organic reactants. Anabolic metabolites bind tightly enough to the surface of pyrite to prevent their loss to the solution but are still capable of two-dimensional diffusion.

The theory outlined above is valuable because of its explanatory power in reconstructing events of prebiotic evolution. Is also attractive because it offers a perspective for later evolutionary phases. Once the formation of phospholipid micelles permitted the liberation of cells from the pyrite surface, the above reaction may have been harnessed by early phosphorylation-based energy metabolism. One scenario envisages a primitive system consisting of three enzymes: a sulfur reductase, a hydrogenase, and an ATPase (the latter two being integral membrane proteins). The H_2S produced inside the cell by reduction of sulfur could diffuse out and react with FeS yielding pyrite and H_2. Thereupon the H_2 could be oxidized by the hydrogenase, providing reductant for the cytoplasmic sulfur reductase. The ensuing H^+ gradient could drive ATP synthesis.

A milestone in the evolution of living systems was the appearance of compartmental organization characteristic of contemporary eukaryotic cells. Most biologists are convinced that the organelles of eukaryotic cells developed out of an endosymbiosis of a bacterium within an archaeal host (Margulis, 1970). One of the weak points of this theory is its vagueness regarding the selective pressure responsible for the original endosymbiotic association. Moreover, the classical theory does not explain the origin of hydrogenosomes, the specialized, H_2-producing organelles found in many anaerobic lower eukaryotes. Recently, a novel hypothesis has

been put forward by Martin and Müller (1998) to remedy these deficits. Their "hydrogen hypothesis" proposes that H_2 metabolism was the basis of the archaic, endosymbiotic association according to the following scenario: A strictly autotrophic, strictly H_2-dependent archaeon such as a primitive methanogen is assumed to be the host cell, and a bacterium with respiratory and fermentative (i.e., H_2-producing) capabilities is assumed to be the symbiont. The initial liason between the two must have originated in an anoxic environment with sufficient H_2 and CO_2 for growth of the host. The association of symbiont and host would render the host cell independent of environmental sources of H_2. On the other hand, in the absence of environmental H_2 the host cell would be totally dependent on the symbiont and be subject to selective pressure to optimize gas exchange between the symbiont and itself. This could lead to progressive engulfment of the symbiont. This would, in turn, necessitate that the host cell acquire transport systems (e.g., via gene transfer from the symbiont) to supply the symbiont with organic substrates for its heterotrophic metabolism. At this stage, the heterotrophic metabolism of the symbiont and the autotrophic metabolism of the host coexist. In the final stage, a progressive transfer of genetic determinants from symbiont to host could eventually replace its autotrophic metabolism by heterotrophic pathways of the symbiont. This would turn the symbiont into a mitochondrion or a hydrogenosome, depending on the remaining set of enzymes. One of the strengths of the hydrogen hypothesis is the fact that associations like the one postulated as the starting point of the archaeal/bacterial partnership are widespread in present-day microbial communities. The syntrophic associations of H_2-producers and H_2-consumers in anaerobic habitats are well-known. Another advantage of the hydrogen hypothesis compared with the original endosymbiotic hypothesis is that the former accounts for both mitochondriate and amitochondriate cells.

Another aspect of H_2 metabolism with a profound impact on the course of evolution is the production of H_2 by cyanobacterial mats. With the advent of photosynthesis, the concentration of O_2 in the atmosphere began increasing. The gradual transition to an oxidizing atmosphere over a period of about 0.5 billion years set the stage for the evolution of respiratory aerobes. Prerequisite for this development was the production of O_2 on a global scale and the concomitant removal of reduced chemical compounds. It was long believed that burial of reduced carbon species was the only process of sufficient magnitude to account for the global change in the atmospheric redox status. Recent studies on subtidal and intertidal mats suggest that H_2 production in these mats could have had a major geochemical impact (Hoehler et al., 2001; Jørgensen, 2001). The mats, which harbor varied microbial populations dominated by the cyanobacterial species *Microcoleus chthonoplastes* and *Lyngbia* spp., were the predominant terrestrial life form for 2 billion years. Measurements of gas production in the modern cyanobacterial mats revealed a dual cycle of H_2 production due in part to the activity of nitrogenase. In contrast to other microbial communities, a major fraction of this H_2 is not recycled but liberated into the atmosphere and, hence, eventually escapes into space. Extrapolation of the H_2 production by modern mats suggests that, on a geological time scale, they could have contributed significantly to the removal of reductant from the earth's biogeochemical cycles.

Acknowledgments. The authors are indebted to their colleagues R. Conrad, T. Eitinger, R. Hedderich, K. Kovács, O. Lenz and J. M. Palacios for their helpful comments on the manuscript. Thanks to A. Volbeda for providing illustrations.

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Prokaryotes (2006) 2:564–577
DOI: 10.1007/0-387-30742-7_18

CHAPTER 1.18

Hydrocarbon-Oxidizing Bacteria

EUGENE ROSENBERG

Introduction

Periodic ecological disasters caused by large oil spills call attention, in a dramatic manner, to the toxicity of petroleum. The fact that hydrocarbons persist for months and even years following major oil spills indicates that hydrocarbon biodegradation is slow in most natural environments. To the microbiologist, the fundamental questions are: What are the biochemical mechanisms of hydrocarbon degradation? Which microorganisms are involved? What are their special properties? What limits the rate of hydrocarbon degradation in the environment? And from an applied point of view, what (if anything) can be done to accelerate this rate? Several decades of research on hydrocarbon-oxidizing bacteria have provided considerable data relevant to these questions. This chapter will discuss the distribution, nutritional requirements, enumeration, isolation identification, special physiologic characteristics, and potential applications of hydrocarbon-degrading bacteria. The specific class of methane oxidizers will be presented in separate chapters.

Habitats

Hydrocarbons are a ubiquitous class of natural compounds. Not only are they found in petroleum-polluted areas, but chemical analyses have revealed the presence of significant quantities of aliphatic and aromatic hydrocarbons in most soils and sediments (Giger and Blumer, 1974; Stevenson, 1966). The most probable origin of the low concentrations of widely distributed hydrocarbons is ongoing biosynthesis by certain plants and microorganism (Fehler and Light, 1970; Hardwood and Russel, 1984; Hunt et al., 1980; Juttner, 1976; Kolattukudy et al., 1972; Mikkelsen and Von Wettstein-Knowles, 1978; Winters et al., 1969). Hydrocarbons are produced by reduction of fatty acyl-CoA by enzymes which utilize NADH or NADPH. Other sources of hydrocarbons are natural seeps on the ocean floor and unburned fuel from oil-burning engines (Floodgate, 1984). Since hydrocarbons are natural products as well as pollutants, it is not surprising that hydrocarbon-oxidizing bacteria are widely distributed in nature. A sample of ecological studies on hydrocarbon-degrading bacteria is shown in Table 1.

It can be seen that hydrocarbon oxidizers are located in virtually all natural areas, although with large variations in cell concentration. As would be expected, the ratio of hydrocarbon-oxidizing bacteria to the total population of heterotrophic bacteria, as well as the variety of hydrocarbon-degrading microorganisms found in a particular ecosystem, may change according to the time of sampling or the extent of oil pollution (Geiselbrecht et al., 1996). Atlas (1981) has discussed many of the factors that limit the growth of hydrocarbon-oxidizing bacteria in nature. These include physical constraints, such as temperature, availability of oxygen, salinity, pH, and the extent to which the particular habitat is an open or closed ecosystem. Nutritional factors are also important and include the availability of utilizable sources of nitrogen, phosphorus and other elements, the nature of the hydrocarbon substrate and its effective concentration, and the possible presence of toxic substances either in the petroleum product or in the environment itself.

Effect of Oil Pollution

The localization of hydrocarbon-oxidizing bacteria in natural environments has received considerable attention because of the possibility of utilizing their biodegradation potential in the treatment of oil spills. Because of the enormous quantities of crude and refined oils that are transported over long distances and consumed in large amounts, the hydrocarbons have now become a very important class of potential substrates for microbial oxidation. It is not surprising, therefore, that hydrocarbon-oxidizing microorganisms have recently been isolated in large numbers from a wide variety of natural aquatic and terrestrial environments. Several

Table 1. Sample habitats and characteristics of hydrocarbon-utilizing microorganisms.

Location	Source	Carbon source	Cell concentration	Reference
Prince William Sound	Surface	Hexadecane	$2–12 \times 10^3$ per g	Lindstrom et al. (1991)
Alaska, beach gravel	Subsurface	Hexadecane	$1–12 \times 10^3$ per g	
Tyrolean Alps	Subsoil	Diesel	$0.2–3 \times 10^4$ per g	Margesin and Schinner (1997)
Bayway Refinery, New Jersey	Surface	Jet fuel	$1–4 \times 10^3$ per g*	Song and Bartha (1990)
	Surface	Jet fuel	$1–6 \times 10^3$ per g*	
Prince William Sound	Surface	Fuel oil	$10^3–10^4$ per g	Haines et al. (1996)
Alaska, Duck Island	Sand	—		
Pudget Sound, Washington	Contaminated sediment	Phenanthrene	$10^4–10^3$ per g	Geiselbrecht et al. (1996)
	Uncontaminated sediment	Phenanthrene	$10^3–10^4$ per g	
Barataria Bay (Louisiana Coast)	Over 200 stations along the coast	Lightweight paraffin oil	$10^3–10^4$ per g mud	ZoBell and Prokop (1966)
Chesapeake Bay	Eastern Bay (water)	Nondetergent motor oil	$0.5–6 \times 10^3$ per ml	Walker and Colwell (1976b)
	Eastern Bay (sediment)	Nondetergent motor oil	$8–99 \times 10^3$ per g	Walker and Colwell (1976b)
	Colgate Creek (water)	Nondetergent motor oil	$90–4.4 \times 10^3$ per ml	Walker and Colwell (1976b)
	Colgate Creek (sediment)	Nondetergent motor oil	$10–9.0 \times 10^3$ per g	Walker and Colwell (1976b)
Atlantic Ocean sediment off North Carolina coast	250 meters off shore (depth 9 meter)	Model petroleum substrate	$1.5–1.2 \times 10^2$ per ml	Walker and Colwell (1976)
	50 km from shore, Continental Shelf (depth, 62 meters)	Model petroleum substrate	$3 \times 10^2–3 \times 10^3$	Walker et al. (1976)
	375 km from shore (depth, 5,000 meters)	Model petroleum substrate	4×10^4 per ml	Walker et al. (1976)
Alaskan waters	Chuchi Sea	Crude oil	$10^3–10^4$ per ml	Horowitz and Atlas (1977)
	Port Valdez	Crude oil	3×10^2 per liter	Robertson et al. (1973)
	Prudhoe Bay	Crude oil	7×10^2 per liter	Atlas and Schofield (1975)
	Cape Simpson oil	Crude oil	3×10^6 per g soil	Atlas and Schofield (1975)
Southern Louisiana marsh sediments	Airplane Lake	Crude oil	$10^3–10^2$ per g sediment	Crow et al. (1975)
	Martigan Point	Crude oil	$10^3–10^2$ per g sediment	Crow et al. (1975)
Field plots	Marcus Hook, PA	Hexadecane	$4.4–11 \times 10^4$ per g	Raymond et al. (1976)
	Tulsa, OK	Hexadecane	$1–5 \times 10^2$ per g	Raymond et al. (1976)
	Corpus Christi, TX	Hexadecane	$3–66 \times 10^4$ per g	Raymond et al. (1976)
Lake Mendota, WI	Surface water	Hexadecane	$10^2–8 \times 10^3$ per ml	Ward and Brock (1976)
Athabasca oil sands	River sediment	Hexadecane	7×10^4 per ml	Wyndham and Costerton (1981)
		Naphthalene	1×10^3 per ml	

*Determined by FDA (fluorescein diacetate) epifluorescence.

investigators have demonstrated an increase in the number of hydrocarbon-oxidizing bacteria in areas that suffer from oil pollution (Table 1). Walker and Colwell (1976a, b) observed a positive correlation between the percentage of petroleum-degrading bacteria in the total population of heterotrophic microorganisms and the amount of heptane-extractable material in sediments of Colgate Creek, a polluted area of Chesapeake Bay. In contrast, no correlation was found when total numbers of hydrocarbon oxidizers (rather than percentages) were compared to hydrocarbon levels. Horowitz and Atlas (1977b) observed shifts in microbial populations in an Arctic freshwater lake after the accidental spillage of 55,000 gallons of leaded gasoline. The ratio of hydrocarbon-utilizing to total heterotrophic bacteria was reported to be an indicator of the gasoline contamination. These investigators also studied shifts in microbial populations in Arctic coastal water using a continuous flow-through system, following the introduction of an artificial oil slick (Horowitz and Atlas, 1977a). The addition of the oil

appeared to cause a shift to a greater percentage of petroleum-degrading bacteria. Atlas and Bartha (1973b) found similar results in an oil-polluted area in Raritan Bay off the coast of New Jersey. Hood et al. (1975) compared microbial populations in sediments of a pristine salt marsh with those of an oil-rich marsh in southeastern Louisiana. These investigators also found a high correlation between the percentage of hydrocarbon oxidizers and the level of hydrocarbons in the sediments. Significant increases in the number of hydrocarbon-utilizing microorganisms were found in field soils following the addition of several different oil samples (Raymond et al., 1976). No estimate of the ratio of hydrocarbon oxidizers to the total heterotrophic population was presented.

From the studies discussed above, it is clear that the presence of hydrocarbons in the environment frequently brings about a selective enrichment in situ for hydrocarbon-utilizing microorganisms. Evidence also has been presented suggesting that the supplementation of certain ecosystems, particularly oil-polluted marine environments with nitrogen and phosphorus may increase the relative number of hydrocarbon oxidizers (Atlas and Bartha, 1973a; Gutnick and Rosenberg, 1977; Reisfeld et al., 1972; Song and Bartha, 1990).

Isolation and Enumeration

The use of hydrocarbons as substrates for bacterial growth presents special problems to both the microorganism using them as a source of carbon and energy and to the investigators in the field of hydrocarbon microbiology. Depending on the solubility of the particular hydrocarbon in water, its physical state (solid, liquid, or gas), and toxicity, different isolation methods must be employed. In all cases, the heterogeneity of the system complicates sampling, enumeration, and growth measurement procedures. After a discussion of general nutritional requirements for hydrocarbon-degrading bacteria, several specific procedures for the selective enrichment and isolation of the different hydrocarbon degraders will be presented.

General Nutritional Requirements

In addition to the requirements for suitable cell-hydrocarbon interactions and the specific genetic potential of the organism for hydrocarbon oxidation, a number of general nutritional conditions must be fulfilled for bacteria to utilize hydrocarbons. These nutritional requirements depend on the fact that hydrocarbons, as the name denotes, are compounds composed solely of carbon and hydrogen atoms. Thus, all other elements essential for cell growth must be available in the growth medium. These include molecular oxygen, utilizable forms of nitrogen, phosphorus, sulfur, metals, and trace components. The requirement for molecular oxygen has been given much attention, particularly with respect to maximum production of single-cell protein by hydrocarbon-degrading microorganisms (Mimura et al., 1973; Schocken and Gibson, 1984). The limitation for oxygen is easily overcome in small-scale laboratory studies, or in open aqueous systems where the oil-water interface is in direct contact with air at all times. The possibility of anaerobic decomposition of hydrocarbons has received considerable attention (Hollinger and Zehnder, 1996). Although hydrocarbon utilization by strictly anaerobic sulfate-reducing bacteria (e.g., Rosenfeld, 1947) has been reported, evidence that pure cultures of sulfate-reducing bacteria can attack hydrocarbon in the absence of additional sources of organic carbon is not definite. However, a few microbial species appear to be able to grow on pure alkane in the absence of molecular oxygen, if provided with nitrate as an electron acceptor (Senez and Azoulay, 1961; Mihelcic and Luthy, 1988) or sulfate (Rueter et al., 1994; Rabus et al., 1996).

The nitrogen and phosphorus requirements for maximum growth of hydrocarbon oxidizers can generally be satisfied by ammonium phosphate. Alternatively, these requirements can be met with a mixture of other salts, such as ammonium sulfate, ammonium nitrate, ammonium chloride, potassium phosphate, sodium phosphate, and calcium phosphate. When ammonium salts of strong acids are used, the pH of the medium generally decreases with growth. This problem can often be overcome by using urea as the nitrogen source. In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1 g of hydrocarbon to cell material. In open systems, the high water solubility of most utilizable sources of nitrogen and phosphorus reduces their effectiveness because of rapid dilution. In principle, this problem can be solved by using oleophilic nitrogen and phosphorus compounds with low C:N and C:P ratios. It was found that a combination of paraffinized urea and octyl phosphate was able to replace nitrate and inorganic phosphate, respectively (Atlas and Bartha, 1973a). A more economical way may be to add water insoluble controlled-release nitrogen and phosphorus fertilizers. This technology has been successfully demonstrated in laboratory and field experiments (Rosenberg et al., 1996). One intriguing possibility to obviate the need for addition of nitrogen compounds to the medium is to use a

bacterium that is capable of both hydrocarbon degradation and nitrogen fixation. Such microorganisms were reported following enrichment on hydrocarbon media lacking nitrogen salts (Coty, 1967).

In addition to utilizable sources of nitrogen and phosphorus, the mineral requirements of hydrocarbon-degrading bacteria can be met by the addition of K^+, Mg^{2+}, Fe^{2+}, and SO_4^{2-} to purified media. All other inorganic ions required by bacteria to obtain optimum growth are commonly present in sufficient concentration as contaminants in these salts. For most marine hydrocarbon degraders, artificial sea water (or filtered sea water), supplemented simply with phosphate, a nitrogen source, and the hydrocarbon serves as an adequate medium for enrichment culture studies. In certain aquatic environments under conditions in which the water was supplemented with nitrogen and phosphorus, a high concentration of iron may limit oil biodegradation (Dibble and Bartha, 1976). Under these conditions, an encapsulated oleophilic iron compound, ferric octoate, was found to be as effective in stimulating biodegradation as various water-soluble iron derivatives, such as ferric ammonium citrate.

Enumeration of Hydrocarbon-Degrading Bacteria

The determination of the concentration of hydrocarbon-degrading bacteria is one of the methods commonly used for monitoring oil pollution in the environment. Theoretical difficulties associated with the interpretation of these data have been discussed elsewhere (Floodgate, 1973). The enumeration of hydrocarbon-degrading bacteria presents two special technical problems, sampling and choice of carbon source. Petroleum-degrading bacteria tend to adhere to hydrophobic materials (Fig. 1). Thus, unless the bacteria are removed from the material and dispersed prior to enumeration, only minimum cell numbers can be obtained. The choice of a carbon source is an even more serious problem. Petroleum is an extremely complex-mixture of hydrocarbons. Because certain bacteria may grow only on minor components in the oil, it would be necessary to incorporate large quantities of petroleum into the growth medium to ensure sufficient substrate for these bacteria to grow well. However, high concentrations of petroleum and mixtures of hydrocarbons cannot be used, because they are toxic to bacteria (Vestal et al., 1984). Thus, the enumeration of hydrocarbon-degrading bacteria using petroleum as the carbon source selects primarily for bacteria that can degrade major components of the oil mixture. Often, pure hydrocarbons and mixtures of pure hydrocarbons and fractions of crude oil can be used to advantage in replacing petroleum as the carbon source in the isolation medium. The following four methods have been used to enumerate hydrocarbon-degrading bacteria in the marine, estuarine, and freshwater environments:

Fig. 1. Phrase contrast photomicrograph of bacterial strain UP-2 (Horowitz et al., 1975) growing on supplemented 0.1% hexadecance-seawater medium. During expontial growth most of the cells appear to be in the form of microcolonies tightly bound to oil droplets. Diameter of oil droplets approximately 150 μm.

Enumeration of Hydrocarbon-Degrading Bacteria in Marine Material Not Miscible with Water (Gunkel and Trekel, 1967)

1. Approximately 2 g of the material to be examined is placed in a sterile bottle containing 100 ml sterile sea water or salts medium.
2. After 1 ml of a sterile. nontoxic, nonionic emulsifier and 1 drop of an antifoam agent are added to the sample, the mixture is homogenized to disperse and break up the bacterial aggregates (e.g., an Ultra Turrax homogenizer run at 24,000 rpm for 30 s).
3. The homogenized sample is then diluted serially in steps of 1 : 10 in sterile sea water or salts medium.
4. One-ml samples of the appropriate dilutions are then inoculated into bottles or tubes containing the following sterile medium:

Aged sea water	750 g
Distilled water	250 ml
NH_4Cl	0.5 g
K_2HPO_4	0.5 g
NaH_2PO_4	1.0 g

5. After addition of 1 drop of sterile hydrocarbon. the samples are incubated for 2–6 weeks, depending on the temperature of incubation.
6. Bottles remaining turbid after addition of 1 ml HCl to dissolve inorganic salts are scored, and the most-probable-number calculated from tables published in Standard Methods (American Public Health Association, 1995).

Enumeration of Hydrocarbon-Utilizing Bacteria by Direct Plating of Estuarine Water and Sediment Samples (Colwell et al., 1973)

Estuarine salts solution:

Distilled water	l liter
NaCl	10 g
$MgCl_2$	2.3 g
KCl	0.3 g

Oil powder: 10 g of hydrocarbon dissolved in 30 ml of diethyl ether is mixed with 10 g of silica gel, allowed to evaporate, and then added to the following basal medium prior to autoclaving.

Oil agar medium:

Distilled water	1 liter
NaCl	10 g
$MgSO_4$	0.5 g
NH_4NO_3	1.0 g
$FeCl_3$ (25 g ·ml $^{-1}$)	1 drop
Purified agar (Difco)	20 g
Oil powder	10 g
KH_2PO_4 (10 g ·100 ml $^{-1}$)*	3.0 ml
K_2HPO_4 (10 g ·100 ml $^{-1}$)*	7.0 ml
Fungizone*	10 mg

*added after autoclaving

Enumeration of Hydrocarbon-Degrading Bacteria in Fresh Water (Ward and Brock, 1976)

Basal medium:

Distilled water	1 liter
NaCl	0.4 g
NH_4Cl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$NaHPO_4 \cdot 7H_2O$*	0.05 g
KH_2PO_4*	0.05 g

* Added after autoclaving

Serial dilutions are made in the basal medium. One drop of sterile hydrocarbon is added to 10 ml of basal medium. After incubation at the appropriate temperature, growth is detected by pellicle formation at the surface of the oil droplet. Most-probable-number is determined from the tables published in Standard Methods (American Public Health Association, 1995).

Enumeration of Hydrocarbon-degrading Bacteria by a 96-well Plate Procedure (Haines et al., 1996)

The 96-well plates were processed with a Beckman Biomek 1000 laboratory robot (Beckman Instruments, Fullerton, CA, USA) which filled the wells with medium, performed ten-fold serial dilutions of the sample, and added oil to the inoculated wells. The robot added 180 ml of BH (Bushnell-Haas medium, Difco) to each well in 11 of the 12 rows, leaving the first row empty. It transferred 200 ml of undiluted sample to the wells in the first row, mixed their contents, and then transferred 20 ml to each well in the second row. The contents of the second row were mixed, and 20 ml was transferred to each well in the third row. This procedure of mixing and transfer was carried out for all except the last row, which served as a sterile control. Sterile pipet tips were used for each transfer. After the dilutions were completed, 2 ml of oil was added to each well as the growth substrate. The plates were sealed in plastic bags and incubated for 14 days at 20°C. Positive wells were scored in one of two ways. When F2 (number 2 fuel oil) was the carbon source, 50 ml of a sterile solution (3 g ·liter $^{-1}$) of INT (iodonitrotetrozolium violet; Research Organics, Cleveland, OH, USA) was added to each well. INT competes with O_2 for electrons from the respiratory electron transport chain, and it is reduced to an insoluble formazan that deposits as a red precipitate in the presence of active respiring microorganisms. Red or pink wells were scored as positive. When a crude oil was used as the carbon source, a smooth oil slick developed in each well. Positive wells were scored by emulsification or dispersion of this oil slick. INT cannot be used effectively with crude-oil substrates, because their dark color interferes with detection of formazan deposition.

Enrichment Culture for Hydrocarbon-Degrading Bacteria

Since hydrocarbons are natural products that are widely distributed in nature, it is not surprising that bacteria able to degrade hydrocarbons can easily be isolated by standard enrichment culture procedures. By varying parameters, such as temperature, pH, hydrocarbon concentration, and basal medium, a wide variety of different hydrocarbon-degrading and emulsifying bacteria can be obtained from either aquatic or terrestrial environments. In most studies, crude oi1 or a petroleum distillate was used as the sole carbon and energy source in the enrichment culture procedure. Under those conditions, bacteria that specialize in the oxidation of low-molecular-weight n-alkanes are generally obtained. Bacteria that grow more slowly or oxidize minor components of crude oil never increase much in batch enrichments, although the activity of these microorganism may be of special significance in natural environments. To overcome this difficulty, enrichment culture procedures have to be employed using different carbon sources. The following examples represent only a few of the possible variations.

Enrichment of Crude Oil-Degrading Bacteria in Supplemented Sea Water (Reisfeld et al., 1972)

To 20 ml of unsterilized sea water in a 125-ml flask were added: 155 mg unsterilized crude oil, 0.056 mM KH_2PO_4, and 7.6 mM $(NH_4)_2SO_4$. After inoculation with about 1 g beach tar or oily sand, the flask was incubated at 30°C with shaking. After about 1 week, the oil became evenly dispersed throughout the liquid. One ml of this culture was then transferred to 20 ml sterile sea water supplemented with 0.056 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, and 1 drop of sterile crude oil. (The crude oil was sterilized by filtration through a Millipore 0.45 mm membrane filter.) After one passage, the oil became emulsified in 2–4 days. Such mixed cultures were maintained by serial transfers to fresh media at 3 to 4-day intervals. Pure cultures were obtained by streaking the enrichment culture either onto the above medium solidified with 1.5% agar (Difco) or nutrient agar (Difco) prepared with filtered sea water. Isolated colony types were found to grow both on nutrient and oil-containing media.

Enrichment culture procedures used to isolate crude oil-degrading bacteria, such as that described in the preceding paragraph, yield a mixture of several different strains even after several transfers. One reason for this is the heterogeneity of the carbon source. Low-molecular-weight paraffin oxidizers (C_{10} to C_{25}) generally dominate the cultures because of their more rapid growth rate. To isolate bacteria that could utilize other fractions of crude oil, the following sequential enrichment culture can be employed:

Sequential Enrichment of Hydrocarbon-Degrading Bacteria on Crude Oil in Supplemented Sea Water (Horowitz et al., 1975)

Inoculate the following sterile medium with a pure culture of an n-paraffin-oxidizing bacterium (which can be obtained by standard enrichment culture procedures): One liter filtered water, 10 mg $K_2HPO_4 \cdot 3H_2O$, 450 mg urea, and 0.7 ml crude oil. After 3 days incubation with shaking at 30°C, the residual oil is extracted with l liter of benzene-pentane-ether (3:l:1, v/v/v). The oil remaining after evaporation of the organic solvent in vacuo is referred to as "bacteria-depleted oil." An enrichment culture is now carried out using the "bacteria-depleted oil" in place of crude oil as the sole source of carbon and energy.

The general failure of investigators to isolate microorganism on highly water-insoluble, solid hydrocarbons, such as anthracene, may be due to the fact that the cells remain firmly bound to the substrate. Thus, a standard enrichment culture procedure in which a portion of the bulk water phase is transferred would select against rather than for these specific microorganism. It may be that for successful enrichments the solid phase should be used as the inoculum during the sequential transfers.

Enrichment of Hydrocarbon-Degrading Bacteria on Bunker C Fuel Oil in Minimal Salts medium (Mulkins-Phillips and Stewart, 1974a)

One gram of beach sand sample or 1 ml of a water sample was added to the following minimal medium containing 0.125% Bunker C oil (steam-sterilized at 121°C and 15 psi for 15 min in tightly capped flasks to prevent evaporation):

Minimal salts medium:

Distilled water	l liter
NaCl	28.4 g
K_2HPO_4	4.74 g
KH_2PO_4	0.56 g
$MgSO_4$	0.50 g
$CaCl_2$	0.1 g
NH_4NO_3	2.5 g
Trace element stock (pH 7.1)	1 ml

Flasks were incubated at 20°C for 14 days and 120 rpm on a refrigerated gyratory shaker bath. Pure cultures of hydrocarbon-utilizing bacteria were isolated from the enrichment culture by streaking onto minimal salts medium to which 2% washed Ionagar No. 2 (Oxoid) was added. The carbon source consisted of 0.5 ml of the following hydrocarbon mixture added to sterile filter paper secured in the lids of the Petri dishes. The dishes were then inverted and incubated at the appropriate temperature for 1–3 weeks.

Hydrocarbon mixture:

Naphthalene	0.1 g
Anthracene	0.1 g
Dibenzothiophene	0.1 g
Decalin	5 ml
Hexadecene-1	5 ml
Hexadecane	5 ml
Octadecane	0.1 g
Dodecane	5 ml
Iso-octane	5 ml

Enrichment of Polyaromatic Hydrocarbon-Degrading Bacteria (PAHs) (Churchill et al., 1999)

Small amounts of fresh sediment known to be contaminated with PAHs were inoculated into the following mineral salts medium (g/liter):

$(NH_4)2SO_4$	10
KH_2PO_4	5.0
$MgSO_4 \cdot 7H_2O$	0.1
$Fe(NH)_2(SO_4)_2$	0.005
Pyrene	40

Trace metals (Beauchop and Elsden, 1960)

After adjusting the pH to 7.0 with NaOH, the flasks were shaken for 1 week. Pyrene-degrading bacteria were detected on pyrene-coated mineral medium (as above) agar plates. Zones of clearing around colonies indicated pyrene degradation. The same procedure can be used with other PAHs replacing the pyrene.

Enrichment on Liquid Aromatic Hydrocarbons (Gibson, 1971)

Liquid aromatic hydrocarbons, such as benzene, toluene, and ethylbenzene, are toxic to bacteria when present in the liquid phase. However, if these carbon sources are introduced in the vapor phase, good growth can be obtained. Figure 2 illustrates two methods that can be used for growing bacteria on volatile toxic hydrocarbons. Since the liquid hydrocarbons do not come in direct contact with the salts medium, they need not be sterilized. When the reservoir of volatile hydrocarbons is exhausted, it can easily be refilled with a Pasteur pipette.

Enrichment for Nitrogen-Fixing Hydrocarbon Oxidizers (Coty, 1967)

Hydrocarbon-oxidizing bacteria able to grow in the absence of added nitrogen compounds were isolated by addition of 0.1 g soil to 25 ml of mineral salts medium of the following composition (g/liter):

Na_2HPO_4	0.3
KH_2PO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.1
$FeSO_4 \cdot 7H_2O$	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.002

The containers were incubated in an atmosphere of air and hydrocarbon vapors. After turbidity developed, the cultures were streaked and reincubated on the above mineral salts medium containing 1.5% washed agar. Purification was achieved after several restreakings and culturing on nitrogen-free mineral salts agar medium. Bacteria able to utilize atmospheric nitrogen on addition of naphthenic acid, n-butane, n-tetradecane, or sodium cyclohexane carboxylate were reported to be isolated by this procedure.

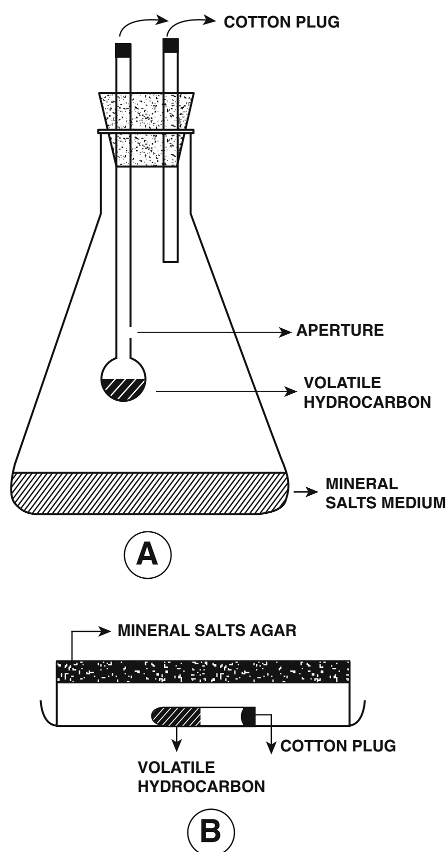

Fig. 2. Two methods for the growth of bacteria on volatile hydrocarbons using (A) liquid media and (B) solid media.

Enrichment for Solid Hydrocarbon-Degrading Bacteria (Miller and Bartha, 1989)

A slurry of soil in 0.1 M phosphate buffer, pH 7.0, with 0.1% octadecane was incubated for 1 week, with shaking. This enrichment culture was transferred (1:100 ratio) to the following medium (g/liter):

Na_2HPO_4	0.4
KH_2PO_4	0.15
NH_4Cl	0.1
$MgSO_4 \cdot 7H_2O$	0.02
Iron ammonium citrate	0.005
$CaCl_2$	0.001
Octadecane	0.1

To obtain pure cultures, the enrichment was streaked on the above medium solidified with 2% agar. The *Pseudomonas* sp. that was isolated grew on solid alkanes such as hexatriacontane (C_{36}).

Identification

The variation in bacterial populations isolated by enrichment culture depend largely on the hydrocarbon substrate used in the enrichment, the culture conditions, and the source of the inoculum. Many species capable of hydrocarbon degradation have been isolated (Table 2). The most frequently isolated bacterial genera are *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Corynebacterium*, and *Arthrobacter*. Most of the investigations on the degradation of aromatic hydrocarbons have been carried out using *Pseudomonas putida*, and species of *Beijerinckia* and *Nocardia* (Gibson, 1971). Westlake et al. (1974) studied the effect of oil quality and incubation temperature on the genetic composition of hydrocarbon-decomposing populations isolated from an area in British Columbia that had been exposed to chronic pollution with diesel fuel. All of the populations consisted predominantly of Gram-negative rods, including species of *Pseudomonas*, *Acinetobacter*, *Xanthomonas*, *Arthrobacter* and *Alcaligenes*.

An extensive study of petroleum-degrading bacteria isolated from Chesapeake Bay waters and sediments was carried out by Austin et al. (1977a, 1977b). A total of 99 strains were examined for 48 biochemical, cultural, morphological, and physiological characteristics. A statistical analysis revealed 14 phonetic groups, comprising about 85% of the hydrocarbon-degrading bacteria. These groups were characterized as actinomycetes, coryneforms, *Enterobacteriaceae*, *Klebsiella aerogenes*, species of *Micrococcus*, *Nocardia* and *Pseudomonas* and *Sphaerotilus* natans.

Physiological Properties

There are two essential characteristics that define hydrocarbon-oxidizing bacteria: 1) hydrocarbon-group-specific oxygenases and 2) mechanisms for optimizing contact between the bacterium and the hydrocarbon.

Group-Specific Oxygenases

Several reviews have appeared on the microbial metabolism of straight-chain and branched alkanes (Asperger and Kleber, 1991; Singer and Finnerty, 1984), cyclic alkanes (Perry, 1984), and aromatic hydrocarbons (Gibson, 1977; Cerniglia, 1984). It has been established that the first step in the degradation of hydrocarbons by bacteria is the introduction of both atoms of molecular oxygen into the hydrocarbon. In the case of aromatic hydrocarbons, ring fission requires a dihydroxylation reaction, the introduction of two atoms of oxygen, and the subsequent formation of a *cis*-dihydrodiol (Gibson, 1968). This reaction is catalyzed by a dioxygenase which is a multicomponent, membrane-bound enzyme system (Cerniglia, 1992). Further oxidation of the *cis*-dihydrodiol leads to the formation of catechols that are substrates for another dioxygenase that catalyzes ring fission.

Table 2. Genera of hydrocarbon-degrading bacteria.

Genus	Reference[a]	
	From soil	From aquatic environment
Achromobacter	9, 12, 27	5, 8, 10, 20, 21, 25, 28
Acinetobacter	9, 23, 27, 34	5, 8, 17, 24, 26
Actinomyces	12	4, 28, 31
Aeromonas		7, 26
Alcaligenes	9, 16, 23, 27	6, 8, 9
Arthrobacter	9–12, 14, 15, 18	5, 7, 10, 25
Bacillus	14, 23	4, 7, 10, 20, 28
Beneckea		10
Brevibacterium	10	5
Corynebacterium	12, 14, 15, 22, 27	4, 5, 7, 10, 25, 26, 28
Cycloclasticus	30	
Cytophaga	9, 27	
Erwinia	1	4
Flavobacterium	1, 13, 15, 22, 27	2, 7, 9, 10, 20, 21
Klebsiella		2
Lactobacillus		2
Leucothrix	1	2
Micrococcus	13, 14, 22	
Moraxella		2
Mycobacterium		29
Myxobacterium	3, 14, 23, 32	28
Nocardia	14, 15, 23	2, 4, 5, 10, 21, 26
Peptococcus		7
Pseudomonas	1, 9, 11–14, 16, 18, 19, 23, 27, 33	2, 4, 5, 10, 20, 21, 26, 28
Rhodococcus	35	
Sarcina	14, 22	20
Serratia	1	
Spherotilus		4
Spirillum	10	20
Vibrio	1, 30	4, 5, 10, 20, 21, 26, 28
Xanthomonas	9, 10	4

[a]Key to references: 1, Atlas et al. (1978); 2, Atlas and Bartha (1972); 3, Antoniewski and Schaefer (1972); 4, Austin et al. (1977a, b); 5, Bartha and Atlas (1977); 6, Bertrand et al. (1976); 7, Buckley et al. (1976); 8, Byrom et al. (1970); 9, Cook et al. (1973); 10, Cundell and Traxler (1973a, 1973b, 1976); 11, Jensen (1975a); 12, Jensen (1975b); 13, Jobson et al. (1972); 14, Jones and Edington (1968); 15, Kincannon (1972); 16, Kiyohara et al. (1982); 17, Makula et al. (1975); 18, McKee et al. (1972); 19, Miller and Bartha (1989); 20, Mironov (1970), Mironov and Lobed (1972); 21, Mulkins-Philips and Stewart (1974b); 22, Odu (1978); 23, Perry (1977); 24, Reisfeld et al. (1972); 25, Soli (1973); 26, Walker and Colwell (1974), Walker and Colwell (1975), Walker et al. (1976b); 27, Westlake et al. (1974); 28, ZoBell (1964). Adapted from Floodgate (1985) and Bossert and Bartha (1985). 29, Churchill et al. (1999); 30, Gieselbrecht et al. (1996); 31, Barabas et al. (1995); 32, Burback and Perry (1993); 33, Grifoll et al. (1994); 34, Ratajczak et al. (1998); 35, Whyte et al. (1998).

It is important to emphasize that the biochemical mechanism of aromatic hydrocarbon oxidation in prokaryotes is fundamentally different from that of eukaryotes. Fungi and mammalian cells metabolize aromatics using the cytochrome P-450 monooxygenase system, which leads to the formation of arene oxides. These active epoxides can form covalent bonds with nucleophilic sites in DNA, leading to mutations and carcinogenesis. Aromatic hydrocarbons that have been shown to serve as substrates for bacterial oxygenases include benzene, toluene, xylene, naphthalene, phenanthrene, anthracene, benz(a)-anthracene, biphenyl, and several of their methylated derivatives. The enzymes necessary for aromatic hydrocarbon degradation are specified, in part, by degradative catabolic plasmids.

In general, alkanes are terminally oxidized to the corresponding alcohol, aldehyde, and fatty acid (Asperger and Kleber, 1991). The hydroperoxides may serve as unstable intermediates in the formation of the alcohol (Singer and Finnerty, 1984). Fatty acids.derived from alkanes are then further oxidized to acetate and propionate (odd-chain alkanes) by inducible β-oxidation systems. The group specificity of the alkane oxygenase system is different in various bacterial species. For example, *Pseudomonas putida* PpG6 (oct) grows on alkanes of 6 to 10 carbons in chain length (Nieder and Shapiro, 1975), whereas *Acinetobacter* sp. HOI-N is capable of growth on long-chain alkanes (Singer and Finnerty, 1984). The ability of *P. putida* to grow on C^6–C^{10} alkanes was shown to be plasmid

encoded (Chakrabarty, 1973). In contrast, all activities necessary for growth of *Acinetobacter* sp. HOI-N and *A. calcoaceticus* BD413 appear to be coded by chromosomal genes (Singer and Finnerty, 1984).

Subterminal alkane oxidation apparently occurs in some bacterial species (Markovetz, 1971). This type of oxidation is probably responsible for the formation of long-chain secondary alcohols and ketones. Pirnik (1977) and Perry (1984) have reviewed the microbial oxidation of branched and cyclic alkanes, respectively.

Physical Interactions Between Bacteria and Hydrocarbons: Adhesion, Desorption, and Emulsification

The low solubility of hydrocarbons in water, coupled to the fact that the first step in hydrocarbon degradation involves a membrane-bound oxygenase, makes it essential for bacteria to come into direct contact with their hydrocarbon substrates. Two general biological strategies have been suggested for enhancing contact between bacteria and water-insoluble hydrocarbons: 1) specific adhesion mechanisms and 2) emulsification of the hydrocarbon.

To understand the special cell surface properties of bacteria that allow them to grow on hydrocarbons, it is necessary to consider the dynamics of petroleum degradation in natural environments. Following an oil spill in the sea, the hydrocarbons rise to the surface and come into contact with air. Some of the low molecular weight hydrocarbons volatilize; the remainder are metabolized relatively rapidly by microorganisms, such as *Pseudomonas* sp., which take up soluble hydrocarbons. These bacteria do not adhere to oil and do not have a high cell-surface hydrophobicity (Rosenberg and Rosenberg, 1985). The next stage of degradation involves microorganisms with high-cell-surface hydrophobicity, which can adhere to the residual high-molecular-weight hydrocarbons. In the case of *A. calcoaceticus* RAG-1, this adherence is due to thin hydrophobic fimbriae (Rosenberg et al., 1982). Mutants lacking these fimbriae failed to adhere to hydrocarbons and were unable to grow on hexadecane. Other bacteria exhibit high-cell-surface hydrophobicity as a result of a variety of fimbriae and fibrils, outer-membrane and other surface proteins and lipids, and certain small cell-surface molecules, such as gramicidin S (Rosenberg et al., 1985) and prodigiosin (Rosenberg et al., 1989). Bacterial capsules and other anionic exopolysaccharides appear to inhibit adhesion to hydrocarbons (Rosenberg et al., 1983).

Desorption from the hydrocarbon is a critical part of the growth cycle of petroleum-degrading bacteria. Petroleum is a mixture of thousands of different hydrocarbon molecules. Any particular bacterium is only able to use a part of the petroleum. As the bacteria multiply at the hydrocarbon/water interface of a droplet, the relative amount of nonutilizable hydrocarbon within the droplet continually increases until the cells can no longer grow. For bacteria to continue to multiply, they must be able to move from the depleted droplet to a fresh oil droplet. *A. calcoaceticus* RAG-1 has an interesting mechanism for desorption and for ensuring that it only reattaches to a droplet of fresh oil. When cells become starved on the "used" hydrocarbon drop or tar ball, they release their capsule. The capsule is composed of an anionic heteropolysaccharide, with fatty acid side-chains, referred to as emulsan (Rosenberg, 1986). The extracellular, amphipathic emulsan attaches avidly to the hydrocarbon/water interface, thereby displacing the cells to the aqueous phase. Each "used" oil droplet or tar ball is then covered with a monomolecular film of emulsan. The hydrophilic outer surface of the emulsan-coated hydrocarbon prevents reattachment of the RAG-1 cells. The released capsule-deficient bacteria are hydrophobic and readily adhere to fresh hydrocarbon substrate.

Many hydrocarbon-degrading microorganisms produce extracellular emulsifying agents (Desai and Banat, 1997; Rosenberg and Ron, 1997). In some cases, emulsifier production is induced by growth on hydrocarbons (Hisatsuka et al., 1971). Mutants that do not produce the emulsifier grow poorly on hydrocarbons (Itoh and Suzuki, 1972). Pretreatment of oil with emulsifying agents can both inhibit and stimulate oil biodegradation (e.g., Foght et al., 1989; Nakahara et al., 1981; Tiehm, 1994; Thibault et al., 1996; Liu et al., 1995; Zhang and Miller, 1994). As discussed above, emulsification may be a by-product of a cell/hydrocarbon detachment process. An entire chapter of this book is devoted to bioemulsifiers (Rosenberg and Ron, 1997).

Acinetobacter sp. HOI-N accumulates extracellular membrane vesicles of 20-50 nm in diameter when grown on hexadecane (Kappeli and Finnerty, 1980). The isolated vesicles partition exogenously supplied hydrocarbons in the form of a microemulsion. These vesicles appear to play a role in the uptake of alkanes. Miller and Bartha (1989) have been able to overcome the difficulties involved in the transport of water-insoluble, solid hydrocarbons by using unilamellar vesicles. A *Pseudonomas* isolate grew on octadecane (C_{18}) and hexatri-acontane (C_{36}) with K_s values of 2,450 and 2,700 mgliter $^{-1}$, compared to 60 and 41 mgliter $^{-1}$, respectively, when the hydrocarbon was presented in the form of liposomes. The data clearly demonstrate the importance of transport in the microbial metabolism of recalcitrant hydrocarbons.

Applications

Petroleum microbiology began as an applied subject, and the applied aspects continue to provide the primary impetus for research in this field. Current areas of applied interest are:

1. Microbial spoilage of petroleum products.
2. Treatment of oil spills and disposal of petroleum wastes.
3. Enhanced oil recovery.
4. Production of surface-active agents.
5. Hydrocarbons as substrates in industrial fermentation processes.

Biodeterioration of petroleum products, such as fuels, lubricating oils, and oil emulsions, has obvious economic implications. Genner and Hill (1981) have reviewed the data on the microbial spoilage of petroleum products and emphasized that spoilage only occurs when the petroleum products come in contact with water. In addition to avoiding water, spoilage can sometimes be retarded by the use of biocides (Rogers and Kaplan, 1968) or membrane filtration.

In considering the microbial treatment of oil spills, it is essential to distinguish between open systems (e.g., the ocean) and closed ones (e.g., oil storage tanks). In the latter case, it is possible to supplement the system with appropriate sources of nitrogen, phosphones, oxygen, and seed bacteria to enhance microbial growth and petroleum degradation, emulsification, or both. Two early published accounts of the use of these fundamental microbiological principles to enhance oil conversion in a restricted area are the treatment of oily ballast water from an oil tanker (Gutnick and Rosenberg, 1977) and of contaminated soil (Raymond et al., 1976). More recently, petroleum pollution has been treated by composting (Kirchmann and Ewnetu, 1998), thermophilic bacteria (Mueller and Nielsen, 1996), in soil-water slurries (Zhang and Bouwer, 1997), and the use of water-insoluble fertilizers (Rosenberg et al., 1996). In an open system, such as the sea, the ability of resident bacteria to extensively degrade a large oil slick is limited primarily by the concentration of nitrogen and phosphorus. Since there is no economical technology for overcoming these nutrient limitations in an open system, there is at present no practical microbial solution for oil spills at sea.

The use of microorganism in tertiary oil recovery has been the subject of several international conferences and literature reviews (e.g., Westlake, 1984; Moses and Springham, 1982). After primary and secondary recovery (water-flooding) processes, approximately 70% of the reservoir oil remains underground, trapped in pore spaces and bound to inorganic minerals. The potential use of microorganisms in situ to release this oil depends on the anaerobic production of organic solvents, such as ethanol and butanol, gases such as methane and carbon dioxide, and organic acids. These materials can help overcome the physical forces holding the oil in the reservoir. Also, acid production can dissolve carbonates thus increasing the permeability of the reservoir. In addition, microbial products could enhance oil recovery by producing surface-active material and viscosity-altering polymers. Although the evidence for the positive role of microorganism in enhanced oil recovery is limited to a few poorly controlled experiments (Hitzman, 1983), the enormous potential of this technology warrants further investigation. In recent years, interest in bioemulsifiers and other microbial surface active agents has been growing. Many of these compounds are produced by hydrocarbon-degrading microorganisms (Rosenberg, 1986; Rosenberg and Ron, 1997; Desai and Banat, 1997). The advantages of microbially produced surfactants include: 1) biodegradability and controlled inactivation; 2) diversity of structure and function for different applications; 3) selectivity for specific hydrocarbon/water interfaces; and 4) characteristic surface modifications.

The use of hydrocarbons as inexpensive raw materials for the production of single cell protein (SCP) was stimulated by the publications of Champagnat and Llewelyn (1962) and Champagnat et al. (1963). During the 1960s, many large oil and fermentation companies were involved in large-scale research and development projects for the conversion of petroleum fractions into SCP. Although the anticipated market for SCP in human and animal nutrition was not realized, these technological developments have provided a rich source of information about how bacteria grow on petroleum, how a continuous process can be scaled-up, and how bulk products can be recovered economically. In the 1970s, several fermentation plants were operating with capacities of 100,000 tons of SCP per year. These were the largest biotechnology plants ever built. Because of the increased cost of hydrocarbon feedstock and more stringent governmental regulations governing its use in fermentation industry, there are presently no large-scale commercial fermentation processes based on hydrocarbon substrates. There are, however, a number of excellent microbial processes that have already been developed; these could be activated under the right set of economic conditions. These include processes for producing alcohols, organic acids, and ketones from specific alkanes, single-cell (food) oil from mixed n-paraffins, and large numbers of microbiological metabolites, including vitamins, amino

acids, pigments, polysaccharides, enzymes and alkanes.

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Prokaryotes (2006) 2:578–617
DOI: 10.1007/0-387-30742-7_19

CHAPTER 1.19

Cellulose-Decomposing Bacteria and Their Enzyme Systems

EDWARD A. BAYER, YUVAL SHOHAM AND RAPHAEL LAMED

Introduction

From an anthropocentric point of view, for millennia, human culture has been intricately involved with cellulose, the major component of the plant cell wall. The development of the wood, paper and textile industries has served to incorporate cellulosic materials into the fabric of our society. Within the past century, however, cellulosic wastes, derived mainly from the same industries, have also become a major source of environmental pollution. This chapter will concentrate mainly on cellulose and the cellulolytic bacteria, in view of their importance to mankind and world ecology. Nevertheless, the true substrate of these bacteria—i.e., the complement of plant cell wall polysaccharides in general—is much more complex than cellulose alone. Likewise, the complement of enzymes—both the cellulolytic and the non-cellulolytic glycosyl hydrolases—are produced concurrently in these bacteria for the purpose of efficient synergistic degradation of the complete substrate composite as it appears in nature. Consequently, when we discuss the cellulose-decomposing bacteria and their enzyme systems, we cannot ignore the related noncellulolytic enzymes, and these will also be treated, albeit secondarily, in the present chapter.

It should also be noted that this chapter of the *The Prokaryotes* is a sequel to the previous chapter of the same title (authored by M.P. Coughlan and F. Mayer) from the second edition of this treatise (Coughlan and Mayer, 1992). The reader is cordially invited to consult the earlier chapter (to be considered as Part A) as an excellent complement to our own (Part B).

The plant cell wall consists of an intricate mixture of polysaccharides (Carpita and Gibeaut, 1993); cellulose, hemicellulose and lignin are its major constituents. These polymers are of a very robust nature. They both equip the plant with a stable structural framework and protect the plant cell from the perils of its environment. Despite its recalcitrant nature, in the guise of dead or dying plant matter, the polysaccharides of the plant cell wall provide an exceptional source of carbon and energy, and a multitude of different microorganisms has evolved which are capable of degrading plant cell wall polysaccharides.

In any given ecosystem, the polysaccharide-degrading microbes are not alone, but rely on the complementary contribution of other bacterial and/or fungal species (Bayer and Lamed, 1992; Bayer et al., 1994; Ljungdahl and Eriksson, 1985). The polymer-degrading strains play a primary and crucial role in the ecosystem by converting the plant cell wall polysaccharides to the respective simple sugars and other degradation products (Fig. 1). They are assisted by satellite microbes, which cleanse the microenvironment from the breakdown products, producing, in the final analysis methane and carbon dioxide.

In a given polysaccharide-degrading microorganism, the enzymes that catalyze the degradation may occur either in the free state and/or in discrete complexes with other similar types of enzymes. The latter are called "cellulosomes." Both the free enzymes and cellulosomal components are usually modular proteins, which contain a multiplicity of functional domains. The "free" enzymes comprise a single polypeptide chain, which contains a catalytic domain usually connected to a cellulose-binding domain or CBD. Cellulosomes are exocellular macromolecular machines, designed for efficient degradation of cellulose and associated plant cell wall polysaccharides (Bayer et al., 1998). In contrast to the free enzymes, the cellulosome complex is composed of a collection of subunits, each of which comprises a set of interacting functional modules. Thus, one type of cellulosomal module, the CBD, is selective for binding to the substrate. Another family of modules, the catalytic domains, is specialized for the hydrolysis of the cellulose chains. Yet another complementary pair of domains—the cohesins and dockerins—serves to integrate the enzymatic subunits into the complex and the complex, in turn, into the cell surface. Multiple copies of the cohesins form an integrating subunit called "scaffoldin" to which the dockerin-containing

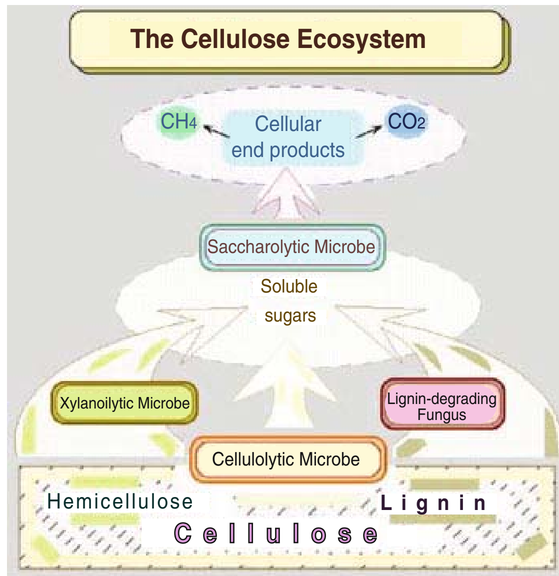

Fig. 1. Simplified schematic description of a typical ecosystem comprising degrading plant matter. Cellulolytic, xylanolytic and ligninolytic microbes combine to decompose the major polysaccharide components to soluble sugars. "Satellite" microorganisms assimilate the excess sugars and other cellular end products, which are ultimately converted to methane and carbon dioxide.

enzymes are attached. This "Lego™"-like arrangement of the modular subunits generates an intricate multicomponent complex, the enzymes of which are bound en bloc to the insoluble substrate and act synergistically towards its complete digestion.

Inherent to the study of cellulases and related enzymes is their potential industrial application—particularly towards conversion of cellulosic biomass. For reviews on the potential uses of these enzymes, the reader is referred to appropriate reviews on the subject (Bhat, 2000; Himmel et al., 1999; Lynd et al., 1991).

Plant Cell Wall Polysaccharides

Plant cells produce a composite matrix of hardy and durable polysaccharides on the outer surface of the plasma membranes, called "the cell wall" (Carpita and Gibeaut, 1993). The cell wall confers a protective coating to the plant cell, providing structure, turgidity and durability, which renders the cell resistant to the outer elements, including mechanical, chemical and microbial assault. Different types of plant cell tissues exhibit different ratios of the three major types of cell wall component; on the average, the cell wall contains roughly 40% cellulose, 30% hemicellulose and 20% lignin, but the exact composition of an individual type of plant varies greatly. The first two polymers are indeed polysaccharides. On the other hand, lignin is a heterogeneous, high-molecular-weight hydrophobic polymer, which consists of nonrepeating aromatic monomers connected via phenoxy linkages (Higuchi, 1990; Lewis and Yamamoto, 1990). Unlike cellulose and hemicellulose, which are degraded aerobically or anerobically, lignin degradation requires oxygen and is limited to filamentous prokaryotes (e.g., the Actinomycetes Streptomyces viridans) and fungi (e.g., *Phanerochaete chrysosporium*, *Bejerkendera adusta* and *Pleurotus ostreatus*), which produce a complicated set of enzymes that hydrolyze the polymer. In fact, the recalcitrant lignin interferes severely with the access of enzymes to the cellulose component, and is rate limiting for anaerobic degradation of cellulose. In any case, the lignin component must be degraded or removed, before efficient degradation of cellulose can take place. Nevertheless, considering lignin is not a polysaccharide, it will not be discussed further in this chapter.

Cellulose

Cellulose is the major constituent of plant matter and thus represents the most abundant organic polymer on Earth. Cellulose is a remarkably stable homopolymer, consisting of a linear (unbranched) polymer of β-1,4-linked glucose units. Chemically, the repeating unit is simply glucose, but structurally, the repeating unit is the disaccharide cellobiose, i.e., 4-*O*-(β-D-glucopyranosyl)-D-glucopyranose, inasmuch as each glucose residue is rotated 180° relative to its neighbor (Fig. 2). The individual cellulose chains contain from about 100 to more than 10,000 glucose units, packed tightly in parallel fashion into microfibrils by extensive inter- and intrachain hydrogen bonding interactions, which account for the rigid structural stability of cellulose. The microfibrils exhibit variable amounts of crystalline and amorphous components, again depending on the degree of polymerization, the extent of hydrogen bonding and, ultimately on the source of the cellulose. The microfibrils themselves are further assembled into plant cell walls, the tunic of some sea animals, pellicles from bacterial origin, etc. Highly crystalline forms of cellulose include cotton, bacterial cellulose (from *Acetobacter xylinum*) and the cellulose from the algae, *Valonia ventricosa*, which exhibit crystallinity levels of about 45%, 75% and 95%, respectively. The following reviews are available for more information on the structure of cellulose (Atalla, 1999; Atalla and VanderHart, 1984; Chanzy, 1990; O'Sullivan, 1997).

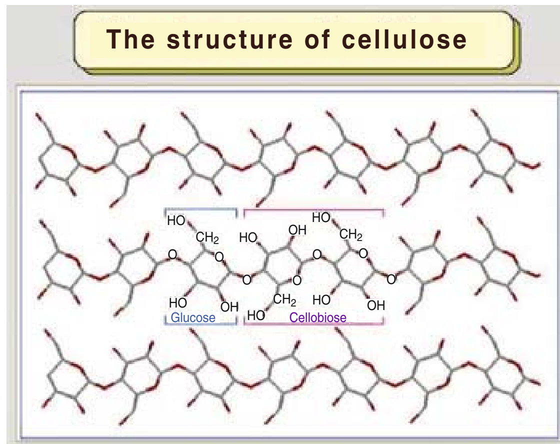

Fig. 2. Structure of cellulose. Three parallel chains that form the 0, 1, 0 face are shown, and a glucose moiety and repeating cellobiose unit are indicated. The model was built by Dr. José Tormo, based on early crystallographic data. The diagram was drawn using RasMol 2.6.

Hemicellulose

Hemicelluloses are relatively low-molecular-weight, branched heteropolysaccharides associated with both cellulose and lignin and together build the plant cell wall material (Puls and Schuseil, 1993; Timell, 1967). The main backbone of hemicellulose is usually made of one or two sugars, which determines their classification. For example, the main backbone of xylan is composed of 1,4-linked-β-D-xylopyranose units. Similarly, the backbone of galactoglucomannans is made of linear 1,4-linked β-D-glucopyranose and β-D-mannopyranose units with a-1,6-linked galactose residues. Other common hemicelluloses include arabinogalactan, lichenins (mixed 1,3-1,4-linked β-D-glucans) and glucomannan. Most hemicellulases are based on a 1,4-β-linkage and the main backbone is branched, whereas the individual sugars may be acetylated or methylated. For example, the linear xylan backbone is highly substituted with a variety of saccharide and nonsaccharide components (Fig. 3). In the

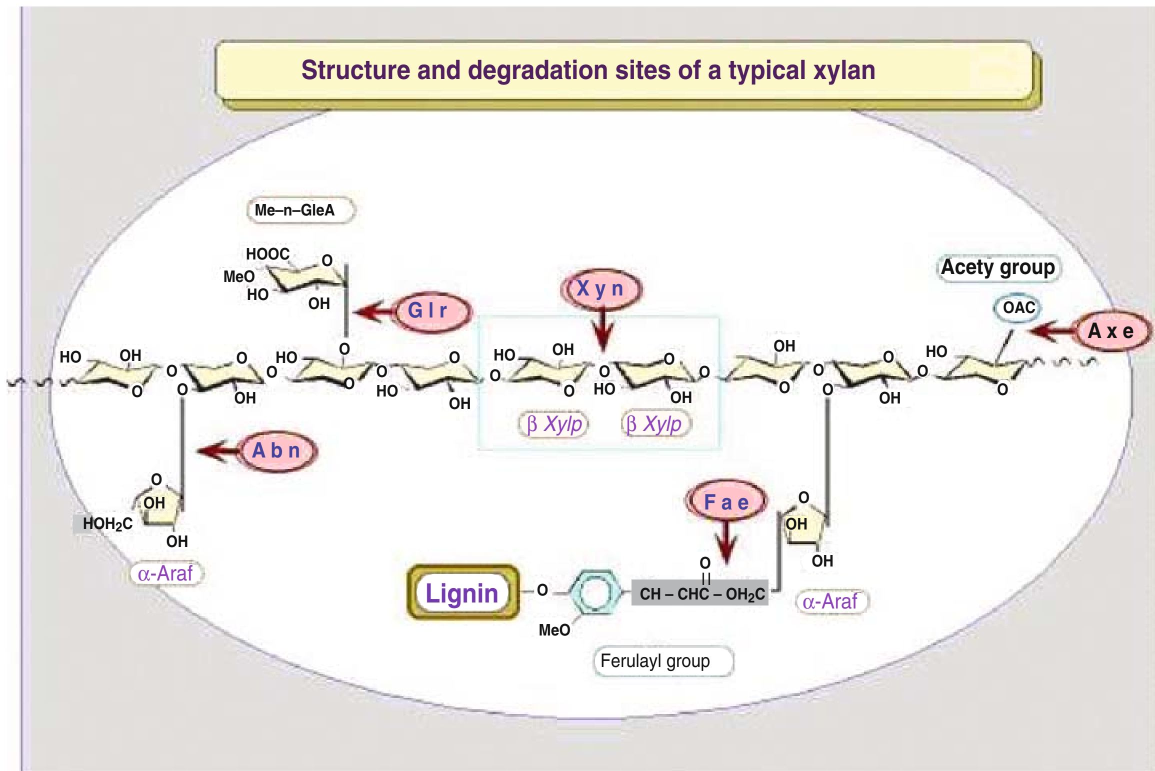

Fig. 3. Composition of a typical xylan component of hemicellulose. The xylobiose unit (β-Xylp-β-Xylp) is indicated by the blue-sided box, as are major substituents: MeaGlcA, methylglucuronic acid; αAraf, arabinofuranosyl; OAc, acetyl group. A presumed lignin attachment site to a feruloyl substituent of xylan is also illustrated. Sites of cleavage by selected hemicellulases and carbohydrate esterases are also shown: Xyn, xylanase; Abn, arabinofuranosidase; Glr, glucuronidase; Axe, acetyl xylan esterase; Fae, ferulic acid esterase.

plant cell wall, xylan is closely associated with other wall components. The 4-*O*-methyl-α-D-glucuronic acid residues can be ester-linked to the hydroxyl groups of lignin, providing cross-links between the cell walls and lignin (Das et al., 1984). Similarly, feruloyl substituents serve as crosslinking sites to either lignin or other xylan molecules. Thus, the chemical complexity of xylan is in direct contrast to the chemical simplicity of cellulose. Likewise, the structural diversity of the xylans is in contrast to the structural integrity of the cellulose microfibril. Consequently, unlike the crystalline-like character of cellulose, the hemicellulose component adopts a gel-like consistency, providing an amorphous matrix in which the rigid crystalline cellulose microfibrils are embedded.

Cellulose-Degrading Bacteria

The cellulolytic microbes occupy a broad range of habitats. Some are free living and rid the environment of plant polysaccharides by converting them to the simple sugars, which they assimilate. Others are linked closely with cellulolytic animals, residing in the digestive tracts of ruminants and other grazers or in the guts of wood-degrading termites and worms (Haigler and Weimer, 1991). Cellulose-based ecosystems include soils, swamps, marshes, rivers, lakes and seawater sediments, rotting grasses, leaves and wood, cotton bales, sewage sludge, silage, compost heaps, muds and decaying vegetable matter in hot and volcanic springs, acid springs, and alkaline springs (Ljungdahl and Eriksson, 1985; Stutzenberger, 1990).

The cellulolytic microorganisms include protozoa, fungi and bacteria and are ubiquitous in nature. The cellulose-decomposing bacteria include aerobic, anaerobic, mesophilic and thermophilic strains, inhabiting a great variety of environments, including the most extreme vis-à vis temperature, pressure and pH. Cellulolytic bacteria also have been found in the gut of wood-eating worms, termites and vertebrate herbivores, all of which exploit anaerobic symbionts for the digestion of wood and fodder.

In nature, many cellulolytic species exist in symbiotic relationships with secondary microorganisms (Ljungdahl and Eriksson, 1985). The primary microorganisms degrade cellulose directly to cellobiose and glucose. Only part of the breakdown products is assimilated by the polymer degrading strain(s), and the rest is utilized by the satellite microorganisms. Removal of the excess of sugars promotes further cellulose degradation by the primary species because cellobiose-induced inhibition of cellulase action and repression of cellulase synthesis are precluded.

Modern interest in cellulolytic microorganisms was spawned by the decay of cotton fabric in army tents and military clothing in the South Pacific jungles during World War II. The basic research program that resulted from this military problem led to the establishment of the United States Army Natick Laboratories (Reese, 1976). The resultant research led to the discovery that the causative agent for the costly problem was a cellulolytic fungi, *Trichoderma viride* (subsequently renamed *Trichoderma reesei*). Subsequent research, originally from the Natick Laboratories and later spreading to other research institutes and universities, led to the identification and classification of thousands of different strains of cellulolytic fungi and bacteria. Many of the major types of cellulolytic bacteria have been listed in Part A of the second edition of *The Prokaryotes* (Coughlan and Mayer, 1992). Since the latter publication, the major emphasis in the area has not concentrated on the discovery or description of new cellulolytic strains. Rather, research in the area during the past decade has centered on characterizing the enzymes and enzyme systems from selected bacteria that degrade cellulose in particular and plant cell wall polysaccharides in general.

Enzymes That Degrade Plant Cell Wall Polysaccharides

The chemical and structural intricacy of plant cell wall polysaccharides is matched by the diversity and complexity of the enzymes that degrade them. The cellulases and hemicellulases are family members of the broad group of glycosyl hydrolases, which catalyze the hydrolysis of oligosaccharides and polysaccharides in general (Gilbert and Hazlewood, 1993; Kuhad et al., 1997; Ohmiya et al., 1997; Schülein, 1997; Tomme et al., 1995a; Viikari and Teeri, 1997; Warren, 1996; Wilson and Irwin, 1999).

Historically, the type of substrate and manner in which a given enzyme interacted with its substrate were decisive in the classification of the glycosidases, as established first by the Enzyme Commission (EC) and later by the Nomenclature Committee of the International Union of Biochemistry (IUB). Enzymes were usually named and grouped according to the reactions they catalyzed. Thus, cellulases, xylanases, mannanases and chitinases were grouped a priori in different categories. Moreover, enzymes that cleave polysaccharide substrates in the middle of the chain ("endo"-acting enzymes) versus those which clip at the chain ends ("exo"-acting enzymes) were also placed in different groups.

For example, in the case of cellulases, the endoglucanases were grouped in EC 3.2.1.4, whereas the exoglucanases (i.e., cellobiohydrolases) were classified as EC 3.2.1.91.

The historical division of enzymes is inappropriate for classification of the cellulases and other glycosyl hydrolases. Like other enzymes (e.g., proteases, etc.), previous classification systems of the glycosyl hydrolases centered on the types of substrates and the bonds cleaved by a given enzyme. The problem with the glycosyl hydrolases is that the polysaccharide substrates and particularly the bonds they cleave are all quite similar, and classification of the different types of enzymes according to conventional criteria often misses the mark. Consequently, alternative approaches were pursued. The recent trend is to classify the different glycosyl hydrolases into groups based on common structural fold and mechanistic themes (Davies and Henrissat, 1995; Henrissat, 1991; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Henrissat et al., 1998). A comprehensive website that provides a catalog of the different glycosyl hydrolase families is now available (Coutinho and Henrissat, 1999a; Coutinho and Henrissat, 1999c; [Carbohydrate-Active Enzymes server (afmb.cnrs-mrs.frl)]. The website also provides excellent introductory explanatory material, and the interested reader is encouraged to use this site extensively.

It is interesting that the distinction between endo- and exo-acting enzymes is also reflected by the architecture of the respective class of active site, even within the same family of enzymes (Fig. 4). The endoglucanases, for example, are commonly characterized by a groove or cleft, into which any part of a linear cellulose chain can fit. On the other hand, the exoglucanases bear tunnel-like active sites, which can only accept a substrate chain via its terminus. The exo-acting enzyme apparently threads the cellulose chain through the tunnel, wherein successive units (e.g., cellobiose) would be cleaved in a sequential manner. The sequential hydrolysis of a cellulose chain is a relatively new notion of growing importance, which has earned the term “processivity” (Davies and Henrissat, 1995), and processive enzymes are considered to be key components which contribute to the overall efficiency of a given cellulase system.

Though instructive, there is growing dissatisfaction with the endo/exo terminology. As our understanding of the nature of catalysis by these enzymes progresses, it has become clear that some enzymes are capable of both endo- and exo-action (Johnson et al., 1996; Morag et al.,

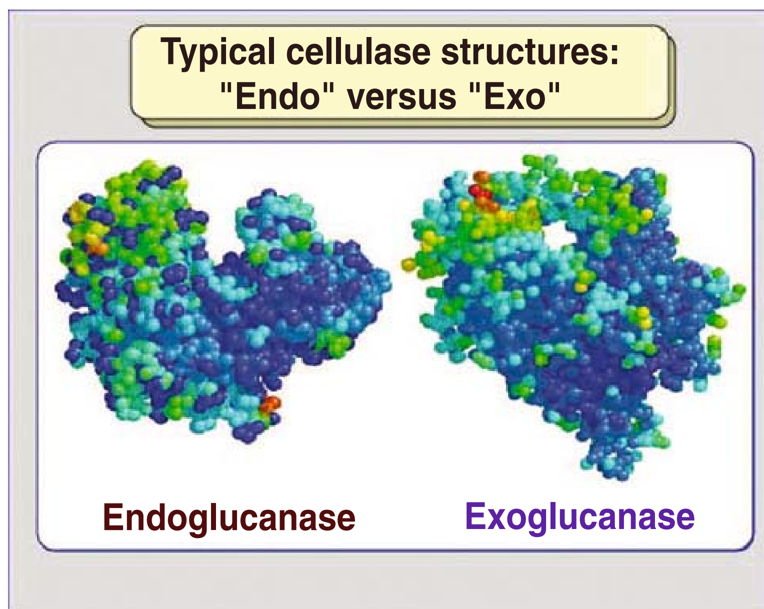

Fig. 4. Structures of a typical endoglucanase and exoglucanase. In each case, the structure is viewed from a perspective, which demonstrates the comparative architecture of the respective active site. Despite the sequence similarity of both enzymes and their classification as family-6 glycosyl hydrolases, their respective active-site architecture is different. The endoglucanase (endoglucanase E2 from the bacterium, *Thermomonospora fusca*, PDB code 1TML) is characterized by a deep cleft to accommodate the cellulose chain at any point along its length, whereas the active site of the exoglucanase (cellobiohydrolase CBHI from the cellulolytic fungus, *Trichoderma reesei*, PDB code 1CEL) bears an extended loop that forms a tunnel, through which one of the termini of a cellulose chain can be threaded. The ribbon diagrams, showing the secondary structures (α-helices and β-strands) of the two enzymes, were drawn using RasMol 2.6.

1991; Reverbel-Leroy et al., 1997; Sakon et al., 1997). Moreover, some glycosyl hydrolase families include both endo- and exoenzymes, again indicating that the mode of cleavage can be independent of sequence homology and structural fold. In this context, relatively minor changes in the lengths of relevant loops in the general proximity of the active site, may dictate the endo- or exo-mode of action without significant differences in the overall fold.

Owing to subtle but diverse chemical and structural aspects of the substrates involved, plant cell wall degrading enzymes do not follow the same rules as common enzyme standards, such as simple proteases, DNAse, RNAse and lysozyme. In fact, the cellulases and hemicellulases are usually very large enzymes, whose molecular masses often exceed those of proteases by factors of 2–5 and more. Their polypeptide chains partition into a series of functional modules and linker segments (frequently glycosylated), which together determine their overall activity characteristics and interaction with their substrates and/or with other components of the cellulolytic and hemicellulolytic system.

Cellulases

The cellulases include the large number of endo- and exoglucanases which hydrolyze β-1,4-glucosidic bonds within the chains that comprise the cellulose polymer (Béguin and Aubert, 1994; Haigler and Weimer, 1991; Tomme et al., 1995b). Thus, in principle, the degradation of cellulose requires the cleavage of a single type of bond. Nevertheless, in practice, we find that cellulolytic microorganisms produce a variety of complementary cellulases of different specificities from many different families.

It may seem somewhat surprising that the combined effect of so many different enzymes are required to degrade such a chemically simplistic substrate. This complexity reflects the difficulties an enzyme system encounters upon degrading such a highly crystalline substrate as cellulose. As described in the previous section, cellulases that degrade the cellulose chain can be either "endo-acting" or "exo-acting." Moreover, the degradation of crystalline cellulose should be viewed three-dimensionally and in situ, where the cellulose chains are packed within the microcrystal, thus generating the remarkably stable physical properties of the crystalline substrate. The enzymes have to bind to the cellulose surface, localize and isolate suitable chains, destined for degradation. It would seem logical that amorphous regions or defects in the crystalline portions of the substrate would be favorable sites for initiation of the process. The structural as opposed to chemical heterogeneity of the substrate dictates the synergistic action of a complex set of complementary enzymes towards its complete digestion.

Various models have been suggested to account for the observed synergy between and among two or more different types of cellulases. For example, an endo-acting enzyme can produce new chain ends in the internal portion of a polysaccharide backbone, and the two newly exposed chains would then be available for action of exo-acting enzymes. In addition, two different types of exoglucanases may exhibit different specificities by acting on a cellulose chain from opposite ends (i.e., the reducing versus the nonreducing end of the polymer). Likewise, an endoglucanase may be selective for only one of the two sterically distinct glucosidic bonds on the cellulosic surface. In addition, some cellulases may display high levels of activity at the beginning of the degradative process, i.e., on the highly crystalline material, whereas others would be selective for newly exposed, partially degraded chains, otherwise embedded within the crystal. Still others would show very high levels of activity after the degradative process has advanced, and cellulose chains that have been freed of the crystalline setting would then be hydrolyzed quite rapidly. A collection of various enzymes, which exhibit complementary specificities and modes of action, would account for the observed synergistic action of the complete cellulase "system" in digesting the cellulosic substrate.

In addition to endo- and exoglucanases, included in the overall group of cellulases are the β-glucosidases (EC 3.2.1.21), which hydrolyzes terminal, nonreducing β-D-glucose residues from cello-oligodextrins. In particular, this type of enzyme cleaves cellobiose—the major end product of cellulase digestion—to generate two molecules of glucose. Some β-glucosidases are specific for cellobiose whereas others show broad specificity for other β-D-glycosides, e.g., xylobiose. Often, the β-glucosidases are associated with the microbial cell surface and hydrolyze cellobiose to glucose before, during or after the transport process.

Hemicellulases

Strictly speaking, hemicellulases are not the precise subject of this chapter, since they do not directly sever the β-1,4-glucosidic bond of cellulose. Nevertheless, in nature, they are essential to the bacterial degradation of insoluble cellulose because the natural bacterial substrate—the plant cell wall—comprises an architecturally

cogent composite of cellulose and hemicellulose. In natural systems, the two types of polysaccharides cannot be easily separated, and microbial systems have to deal simultaneously with both. The xylan component is particularly of interest for several reasons: 1) xylan is a major hemicellulosic component of the plant cell wall, 2) the xylanases are well defined enzymes, closely associated with the cellulase and 3) the repeating units (both xylose and xylobiose) bear striking structural resemblance to their cellulosic counterparts (i.e., glucose and cellobiose).

In contrast to cellulose degradation, the degradation of the hemicelluloses imposes a somewhat different challenge, since this group of polysaccharides includes widely different types of sugars or non-sugar constituents with different types of bonds. Thus, the complete degradation of hemicellulose requires the action of different types of enzymes. These enzymes, the hemicellulases, can differ in the chemical bond they cleave, or, as in the case of the cellulases, they may cleave a similar type of bond but with different substrate- or product specificity (Biely, 1985; Coughlan and Hazlewood, 1993; Eriksson et al., 1990; Gilbert and Hazlewood, 1993).

Hemicellulases can be divided into two main types, those that cleave the mainchain backbone, i.e., xylanases or mannanases, and those that degrade sidechain substituents or short end products, such as arabinofuranosidase, glucuronidase, acetyl esterases and xylosidase. Like the cellulases, hemicellulases can be of the endo- or exo-types. A schematic view of the types of bonds that would be hydrolyzed by different types of hemicellulases is presented in Fig. 3.

XYLAN-DEGRADING ENZYMES. The xylanases are by far the most characterized and studied of the hemicellulases and involve the cleavage of a major mainchain backbone. Endoxylanases (1,4-β-D-xylan xylanhydrolase, EC 3.2.1.8) hydrolyze the 1,4-β-D-xylopyranosyl linkage of xylans, such as D-glucurono-D-xylans and L-arabino-D-xylan. These single-subunit enzymes from both fungi and bacteria exhibit a broad range of physiochemical properties, whereby two main classes have been described: alkaline proteins of low M_r (<30,000) and acidic proteins of high M_r. This general classification scheme correlates with their assignment into glycosyl hydrolase families 10 and 11, whereby the former represents the high M_r xylanases and the latter coincides with the low M_r enzymes. The two families also differ in their catalytic properties, such that the family 10 enzymes seem to display a greater versatility towards the substrate than that observed for those of family 11, and are thus typically able to hydrolyze highly substituted xylan more efficiently. The family 10 xylanases exhibit a (β/α)8 topology whereas those from family 11 form a β-jelly roll fold. Both families show a retaining catalytic mechanism of hydrolysis.

MANNAN-DEGRADING ENZYMES. Glucomannans and galactoglucomannans are branched heteropolysaccharides found in hardwood and softwood. The degradation of these polymers again involve many hydrolytic enzymes, including endo-1,4-β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), β-glucosidase (EC 3.2.1.21), and α-galactosidase (EC 3.2.1.22). 1,4-β-D-Mannanases hydrolyze mainchain linkages of D-mannans and D-galacto-D-mannans. These enzymes, both of the endo- or exo-types, are produced in various microorganisms, including *Bacillus subtilis*, *Aspergillus niger* and intestinal and rumen bacteria and commonly occur in families 5 and 26.

LICHENIN-DEGRADING ENZYMES. Lichenase (1,3-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.73) is a mixed linkage β-glucanase, which cleaves the β-1,4 linkages adjacent to the β-1,3 bonds of the lichenin substrate. According to [{afmb.cnrs-mrs.fr/~pedro/CAZY/db.html}{modern structure-based classification, lichenases can be members of families 8, 16 or 17.

β-D-XYLOSIDASES. The 1,4-β-D-xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) hydrolyze xylo-oligosaccharides (i.e., xylan breakdown products and mainly xylobiose) to xylose. These enzymes are either intracellular or extracellular components and are closely associated with hemicellulolytic activities. Monomeric, dimeric and tetrameric xylosidases have been found with Mrs of 26,000 to 360,000. Many of the xylosidases act on a variety of substrates. For example, *Aspergillus niger* produces an enzyme classified as a β-xylosidase that can hydrolyze β-galactosides, β-glucosides and α-arabinosides, in addition to β-xylosides.

SIDECHAIN-DEGRADING ENZYMES. α-D-Glucuronidases (EC 3.2.1.39) catalyze the cleavage of the α-1,2 glucosidic bond of 4-*O*-methyl-α-D-glucuronic acid side chain. This bond has a stabilizing effect on the neighboring xylosidic bonds of the main chain. Several α-glucuronidase genes have recently been cloned and sequenced and usually occupy family 67.

α-L-Arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) is another important enzyme that cleaves non-reducing terminal α-L-arabinofuranosidic linkages in arabinoxylan, L-arabinan, and other

L-arabinose containing polysaccharides. These enzymes are found either in the cell-associated or extracellular form and can be members of families 43, 51 or 62.

1,4-β-Mannosidases hydrolyze 1,4-linked β-D-mannosyl groups from the nonreducing end. These enzymes (similar to β-xylosidases) hydrolyze mainly the end products of the mannanases, i.e. mannobiose and mannotriose.

Carbohydrate Esterases

The side chain substituents of xylan are composed not only of sugars but also of acidic residues, such as acetic, ferulic (4-hydroxy-3-methoxycinnamic) or *p*-coumaric (4-hydroxycinnamic) acids. Carbohydrate esterases that cleave these residues (see Fig. 3) are found in enzyme preparations from both hemicellulolytic and cellulolytic cultures (Borneman et al., 1993). Such enzymes sometimes represent separate modules, separated by linker segments from other cellulolytic or hemicellulolytic catalytic modules in the same polypeptide chain. Like the glycosyl hydrolases, the carbohydrate esters are currently classified according to sequence homology and common structural fold.

Cellulases and Hemicellulases are Modular Enzymes

The initial contribution of biochemical methods for determining the characteristics of a given cellulase was extended immeasurably by the contribution of molecular biology and bioinformatics. By comparing the sequences of the cellulases and related enzymes, an entirely new view of these enzymes emerged.

Cellulases and hemicellulases are composed of a series of separate modules. This fact explains the very large size of some of these enzymes and gives us some insight into their complex mode of action. Each module or domain comprises a consecutive portion of the polypeptide chain and forms an independently folding, structurally and functionally distinct unit (Coutinho and Henrissat, 1999; Gilkes et al., 1991; Teeri et al., 1992). Each enzyme contains at least one catalytic module, which catalyzes the actual hydrolysis of the glycosidic bond and provides the basis for classification of the simple enzymes (i.e., those containing a single catalytic module). Other accessory or "helper" domains assist or modify the primary hydrolytic action of the enzyme, thus modulating the overall properties of the enzyme. Some of the different themes illustrating the modular compositions of the cellulases and related enzymes are illustrated in Fig. 5.

The Catalytic Modules—Families of Enzymes

The definitive component of a given enzyme is the catalytic domain. Former EC-based classification schemes according to substrate specificity are now considered somewhat obsolete because they fail to take into account the structural features of the enzymes themselves. The catalytic domains of glycosyl hydrolases are presently categorized into families according to amino acid sequence homology (Coutinho and Henrissat, 1999; Henrissat, 1991; Henrissat and Bairoch, 1996; Henrissat and Davies, 1997; Henrissat et al., 1998). For more information, see the Carbohydrate-Active Enzymes (CAZy), designed and maintained by Pedro Coutinho and Bernard Henrissat.

The enzymes of a given glycosyl hydrolase family display the same topology, and the positions of the catalytic residues are conserved with respect to the common fold. In recent years, X-ray crystallography has provided a general overview of the structural themes of the glycosyl hydrolases and their interaction with their intriguing set of substrates (Bayer et al., 1998; Davies and Henrissat, 1995; Henrissat and Davies, 1997).

The mechanism of cellulose and hemicellulose hydrolysis occurs via general acid catalysis and is accompanied by either an overall retention or an inversion of the configuration of the anomeric carbon (Davies and Henrissat, 1995; McCarter and Withers, 1994; White and Rose, 1997; Withers, 2001). In both cases, cleavage is catalyzed primarily by two active-site carboxyl groups. One of these acts as a proton donor and the other as a nucleophile or base. Retaining enzymes function via a double-displacement mechanism, by which a transient covalent enzyme-substrate intermediate is formed (Fig. 6A). In contrast, inverting enzymes employ a single-step mechanism as shown schematically in Fig. 6B. The distance between the acid catalyst and the base represents the major structural difference between the two mechanisms. In retaining enzymes, the distance between the two catalytic residues is about 5.5 Å, whereas in inverting enzymes the distance is about 10 Å. In the inverting enzymes, additional space is provided for a water molecule, involved directly in the hydrolysis, and the resultant product exhibits a stereochemistry opposite to that of the substrate. In all cases, the mechanism of hydrolysis is conserved within a given glycosyl hydrolase

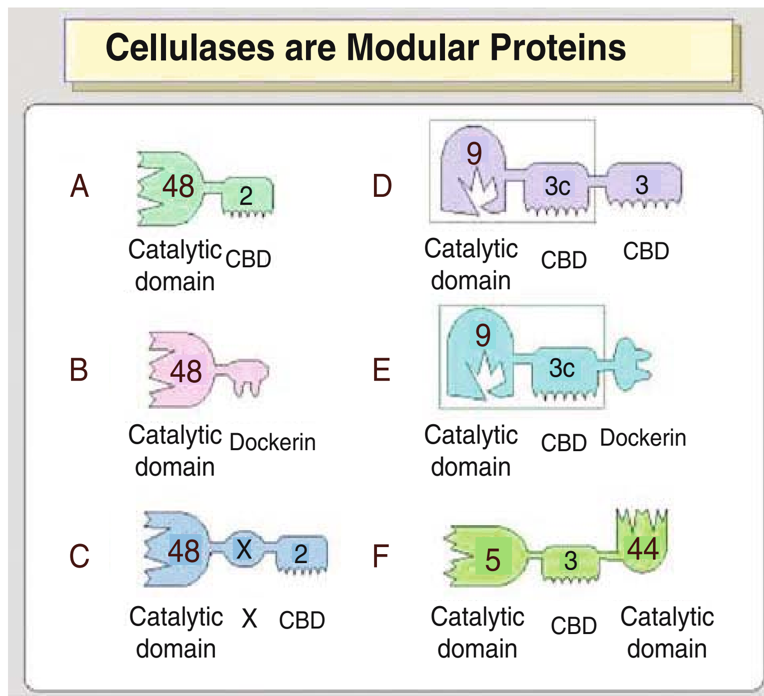

Fig. 5. Scheme illustrating the diversity of the modular architecture of cellulases and other glycosyl hydrolases. The different modules are grouped into families according to conserved sequences as shown here symbolically. A. One of the most common types of cellulases consists of a catalytic module or domain, flanked by a cellulose-binding domain (CBD) at its *N*- or *C*-terminus. This particular enzyme shown in "A" comprises a catalytic domain from family 48 and a family-2 CBD. B. Cellulosomal enzymes are characterized by a "dockerin domain" attached to a catalytic domain. In this case, the same type of enzyme as in "A," carrying a family-48 catalytic module, harbors a dockerin domain instead of a CBD. C. Many cellulases contain "X domains," i.e., domains of unknown (as yet undefined) function. D. Some enzymes have more than one CBD or other type of carbohydrate-binding module (CBM). Often, one CBD, such as the family-3 CBD shown here, serves to bind the cellulase strongly to the flat surface of the insoluble substrate, whereas the other one (the family-3c CBD) acts in concert with the catalytic module by binding transiently to a single cellulose or hemicellulose chain. E. Some cellulosomal cellulases have a CBD or CBM together with a dockerin in the same polypeptide chain. F. Some cellulases have more than one type of catalytic module, such as the family-5 and family-44 modules shown here, and the two probably work in concerted fashion to degrade the substrate efficiently.

family (Coutinho and Henrissat, 1999; Davies and Henrissat, 1995; Henrissat and Davies, 1997).

Cellulose-Binding Domains Versus Carbohydrate-Binding Modules

In addition to the catalytic module, free cellulases and hemicellulases usually contain at least one cellulose-binding domain (CBD) as an integral part of the polypeptide chain (Linder and Teeri, 1997; Tomme et al., 1995a). The CBD serves predominantly as a targeting agent to direct and attach the catalytic domain to the insoluble crystalline substrate. Like the catalytic domains, the CBDs are categorized into a series of families according to sequence homology and consequent structural fold.

In some cases, the term "CBD" is deceptive because not all of the CBDs bind to crystalline cellulose. Some families (or subfamilies or family members) bind either preferentially or additionally to other insoluble polysaccharides, e.g., xylan or chitin. For example, the family-5 CBD and some of the members of the family-3 CBDs bind to chitin as well as cellulose (Brun et al., 1997; Morag et al., 1995). Moreover, the family-2 CBDs can be divided into two subfamilies, one of which indeed binds preferentially to insoluble cellulose, but the other binds to xylan (Boraston et al., 1999). The molecular basis for this was proposed to reflect the fact that in the first subfamily, 3 surface-exposed tryptophans contribute to cellulose binding (Simpson et al., 1999b; Williamson et al., 1999). However, in the case of the xylan-binding members, one of these tryptophans is missing, whereas the other two assume a different conformation, thereby allowing them to stack against the hydrophobic surfaces of two xylose rings of a xylan substrate. Other types of CBD prefer less crystalline substrates (e.g., acid-swollen cellulose), single

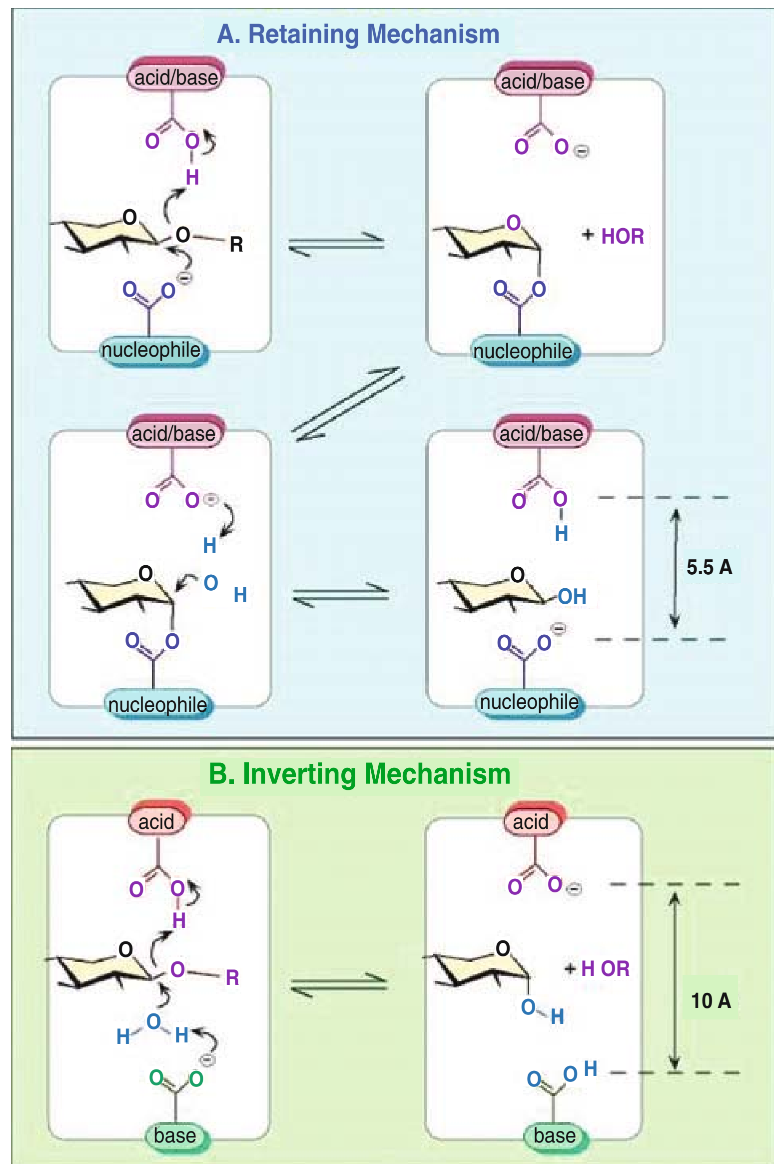

Fig. 6. The two major catalytic mechanisms of glycosidic bond hydrolysis. A. The retaining mechanism involves initial protonation of the glycosidic oxygen via the acid/base catalyst with concomitant formation of a glycosyl-enzyme intermediate through the nucleophile. Hydrolysis of the intermediate is then accomplished via attack by a water molecule, resulting in a product that exhibits the same stereochemistry as that of the substrate. B. The inverting mechanism involves the single-step protonation of the glycosidic oxygen via the acid/base catalyst and concomitant attack of a water molecule, activated by the nucleophile. The resultant product exhibits a stereochemistry opposite to that of the substrate. The type of mechanism is conserved within a given glycosyl hydrolase family and dictated by the active-site architecture and atomic distance between the acid/base and nucleophilic residues (aspartic and/or glutamic acids).

cellulose chains and/or soluble oligosaccharides, e.g, laminarin (1,3-β-glucan) and barley 1,3/1,4-β-glucan (Tomme et al., 1996; Zverlov et al., 2001). Still others exhibit alternative accessory function(s), a topic to be described below in more detail. Moreover, the CBDs responsible for the primary binding event may further disrupt hydrogen bonding interactions between adjacent cellulose chains of the microfibril (Din et al., 1994), thereby increasing their accessibility to subsequent attack by the hydrolytic domain.

Consequently, the concept of CBD has been broadened and redefined as "CBM" i.e., carbohydrate-binding module (Boraston et al., 1999; Coutinho and Henrissat, 1999). To date (March 2001), 26 different CBM families have been described. The structures of CBDs from a number of families and subfamilies have been determined, and an understanding of their structures has provided interesting information regarding the mode of binding to cellulose. Those that bind to crystalline substrates, appear to do so via a similar type of mechanism. One of the surfaces of such CBDs is characteristically flat and appears to complement the flat surface of crystalline cellulose. A series of aromatic amino acid residues on this flat surface form a planar strip (Mattinen et al., 1997; Simpson and Barras, 1999a; Tormo et al., 1996) that stack opposite the glucose rings of a single cellulose chain. In addition, to the planar aromatic strip, several polar amino acid residues on the same surface appear to anchor the CBD to two adja-

cent cellulose chains. The binding of the CBD to crystalline cellulose would thus involve precisely oriented, contrasting hydrophobic and hydrophilic interactions between the reciprocally flat surfaces of the protein and the carbohydrate substrate. Together they provide a selective biological interaction, which contributes to the specificity that a CBD exhibits towards its structure.

In contrast to the interaction with the crystalline cellulose surface, other CBMs seem to interact with single cellulose chains. The family-3c and family-4 CBDs preferentially bind to noncrystalline forms of cellulose and clearly have a different function in nature (Johnson et al., 1996; Sakon et al., 1997; Tomme et al., 1996). For example, the role of family-4 CBD may be to recognize, bind to and deliver an appropriate catalytic module to a cellulose chain, which has been loosened or liberated from a more ordered arrangement within the cellulose microfibril. The binding of the family-3c CBD to single cellulose chains and its remarkable role in cellulose hydrolysis will be discussed later (Fig. 9).

The Family-9 Cellulases: An Example

This section pertains to enzyme diversity and how a single type of catalytic module can be modified by the class of helper module(s) that flank its *C*- or *N*-terminus. We are only at the beginning in our understanding of how the modular arrangement affects the overall activity and function of a given enzyme.

In its simplest form, an enzyme would presumably consist of a single catalytic domain, usually with a standard CBM, which would target the enzyme to the crystalline substrate. Indeed, this is the norm for many individual glycosyl hydrolase families. However, in others, e.g., the family-9 cellulases, the catalytic domains commonly occur in tandem with a number of accessory modules. Although the story is still rather incomplete, we can discuss the currently available information regarding family 9 and draw several interesting conclusions from the few publications on this currently developing subject.

Family-9 Theme and Variations. The crystal structure of the family-9 catalytic module is known and displays an $(\alpha/\alpha)6$-barrel fold and inverting catalytic machinery. However, few of the prokaryotic family-9 enzymes consist of a solitary catalytic module (Fig. 7A). Actually, there are numerous family-9 cellulases of plant origin, the great majority of which are such lone catalytic modules that lack accessory modules. Another type of eukaryotic family-9 cellulase that lacks helper modules is produced by the termite. The prokaryotic family-9 enzymes, however, are almost invariably decorated with a variety of subsidiary modules that modulate the activity of the catalytic module.

Microbial family-9 cellulases commonly conform to one of the themes shown in Fig. 7. In one of these, the catalytic module is followed immediately downstream by a fused family-3c CBM (Fig. 7B). This particular type of CBM imparts special characteristics to the enzyme (see below). A second theme consists of an immunoglobulin-like (Ig) domain (of unknown function) immediately upstream to the catalytic domain (Fig. 7C). A variation of the latter theme includes a family-4 CBM at the *N*-terminus of the enzyme, followed by an Ig domain and family-9 catalytic domain (Fig. 7D). In addition to the above-described modular arrangement, each of the free prokaryotic enzyme systems includes a standard CBD that binds strongly to crystalline cellulose.

Until very recently, there has been but one example in the prokaryotic world of a family-9 enzyme that contains no helper domain. This is the family-9 glycosyl hydrolase of the cellulosomal scaffoldin from the cellulolytic anaerobic bacterium, *Acetivibrio cellulolyticus* (Ding et al., 1999). The *A. cellulolyticus* enzyme forms part of a multimodular scaffoldin, but the catalytic module appears to be a functionally distinct entity that lacks adjoining helper modules. The other modules are conventional scaffoldin-associated modules, e.g., cohesins and a true cellulose-binding CBD. More recently, a dockerin-containing cellulosomal family-9 enzyme from *Clostridium cellulovorans* has been sequenced and also seems to lack adjoining helper modules (Tamaru et al., 2000b).

This thematic arrangement of the family-9 cellulases is mirrored in the respective sequences of the catalytic modules. The divergent sequences are reflected by the phylogenetic relationship of the parent cellulases (Fig. 8). Thus, the simplest cellulases (the group A eukaryotic cellulases from plants) that lack adjacent helper modules are all phylogenetically related (theme A). Interestingly, the catalytic module of CipV from *A. cellulolyticus* is distinct from the other groups designated in Fig. 8, but closest to the plant enzymes, as might be anticipated from its lack of a helper module. In a similar manner, catalytic modules from cellulases that are fused to a family-3c CBD (group B), all map within the same branch (theme B). On the other hand, the catalytic modules that bear an adjacent Ig-like domain all fall into a cluster on the opposite side of the tree. Cellulases which have the Ig-like domain only (theme C) occupy a small separate branch and those that also include a family-4 CBD (theme D) that develop distally to form a separate subcluster.

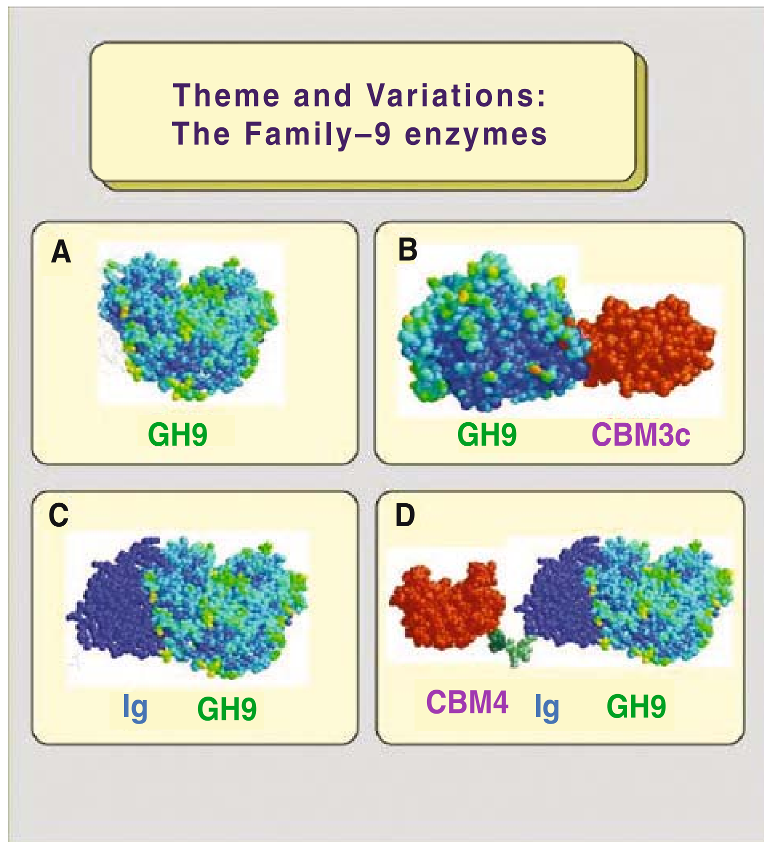

Fig. 7. Theme and variations: schematic view of the modular arrangement of the family-9 glycosyl hydrolases. A. The solitary catalytic domain. B. The catalytic domain and fused family-3c cellulose-binding domain (CBD). *C. Immunoglobulin*-like (Ig) domain, fused to the catalytic domain. D. Successive family-4 CBD, Ig and catalytic domains. The representations of the different modules are based on their known structures and are presented sequentially, left-to-right, from the *N*- to *C*-terminus. Structures (Ribbon diagrams produced by RasMol 2.6) in "A" and "B" are derived from cellulase E4 from *Thermomonospora fusca* (PDB code, 1TF4), those in "C" and "D" are from the CelD endoglucanase of *C. thermocellum* (PDB code, 1CLC). The figure used for the family-4 CBD in "D" is derived from the nuclear magnetic resonance (NMR) structure of the *N*-terminal CBD of *Cellulomonas fimi* β-1,4-glucanase CenC (PDB code, 1ULO). The structures in "B" and "C" are authentic views of the respective crystallized bi-domain protein components. The CBD in "D" has been placed manually to indicate its *N*-terminal position in the protein sequence, but its spatial position in the quaternary structure and the structure of the linker segment remains unknown.

Theme A enzymes: CipV Acece, CipV scaffoldin from the cellulolytic bacterium, *A. cellulolyticus* (AF155197); and plant (eukaryotic) cellulases from *Prunus persica* (X96853), *Populus alba* (D32166), *Citrus sinensis* (AF000135), *Persea americana* (M17634), *Pinus radiata* (X96853), *Arabidopsis thaliana* (X98543), *Phaseolus vulgaris* (M57400), *Capsicum annuum* (X97189), *Lycopersicon esculentum* (U20590).

Theme B enzymes: CelF Clotm, endoglucanase F from *Clostridium thermocellum* (X60545); CelZ Closr, exoglucanase Z from *Clostridium stercorarium* (X55299); CelA Calsa, cellulase A from Caldocellum saccharolyticum (L32742); CelG Cloce, endoglucanase G from *Clostridium cellulolyticum* (M87018); CelI Clotm, endoglucanase I from *Clostridium thermocellum* (L04735); CelB Celfi, endoglucanase B from *Cellulomonas fimi* (M64644); E4 Thefu, endo/exoglucanase E4 from *Thermomonospora fusca* (M73322).

Theme C enzymes: CelJ Clotm, cellulase J from *Clostridium thermocellum* (D83704); CelD Clotm, endoglucanase D from *Clostridium thermocellum* (X04584); CelC Butfi, endoglucanase C from *Butyrivibrio fibrisolvens* (X55732).

Theme D enzymes: CbhA Clotm, cellobiohydrolase A from *Clostridium thermocellum* (X80993); CelA Psefl, endoglucanase A from *Pseudomonas fluorescens* (X12570); CelC Celfi, endoglucanase C from *Cellulomonas fimi* (X57858); CelI Strre, endoglucanase I from *Streptomyces reticuli* (X65616); E1 Thefu, endoglucanase E1 from *Thermomonospora fusca* (L20094).

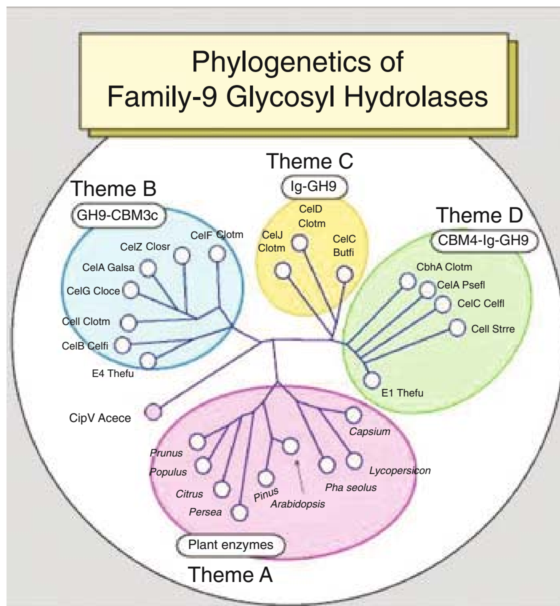

Fig. 8. Phylogenetic analysis of the *N*-terminal family-9 catalytic module of CipV and its relationship with other family-9 members. The various theme groupings roughly follow the groups shown in Fig. 7. Theme A (group A) enzymes lack associated helper modules. Theme B (group B) enzymes carry a fused family-3c cellulose-binding domain (CBD) downstream to the catalytic module. Theme C (group C) and theme D (group D) enzymes carry an immunoglobulin-like (Ig) domain upstream to the catalytic module, the theme D enzymes having an additional *N*-terminal family-4 CBM.

The analysis of the designated catalytic modules was performed using GenBee, based on the respective GenBank sequences (accession codes in parentheses).

Family-9 Crystal Structures. Two crystal structures of family-9 cellulases have been elucidated, representing two subtypes of this particular family of glycosyl hydrolase. These are cellulase E4 from Thermomonospora fusca (recently reclassified as *Thermobifida fusca*; Sakon et al., 1997) and CelD from *Clostridium thermocellum* (Juy et al., 1992). These two examples are architecturally distinct—the E4 cellulase being an example of a theme B family-9 enzyme (see Figs. 7B and 8) and the CelD cellulase being a theme C enzyme. Fortunately, in both cases, one of the neighboring modules co-crystallized with the catalytic module, thus providing primary insight into their combined structures. In the case of *T. fusca* E4, the catalytic domain and neighboring family-3c CBM were found to be interconnected by a long, rigid linker sequence, which envelops about half of the catalytic domain until it connects to the adjacent CBM (Fig. 9A). In contrast, in the *C. thermocellum* CelD, the catalytic domain is adjoined at its *N*-terminus by a 7-stranded immunoglobulin-like (Ig) domain of unknown function. The comparison between the E4 and CelD cellulases indicates that a given type of catalytic module can be structurally and functionally modulated by different types of accessory domains.

Helper Modules. The family-3c CBM is special. To date, this particular type of CBM has been found in nature associated exclusively with the family-9 catalytic domain. Structurally, the CBM is homologous to the other family-3 CBMs, but contains substitutions in many important surface residues. The three-dimensional crystal structure of the E4 cellulase revealed the close interrelationship between the family-9 catalytic domain and the family-3c CBM, thus suggesting a functional role as a helper module. This CBM seems not to bind directly to crystalline cellulose but appears to act in concert with the catalytic domain by binding transiently to the incoming cellulose chain, which is then fed into the active-site cleft pending hydrolysis (Gal et al., 1997; Irwin et al., 1998; Sakon et al., 1997; Fig. 9B).

The information derived from the family-9 enzymes suggests that the activity of catalytic domains can be modulated by accessory modules. The accessory modules can either supplement or otherwise alter the overall properties of an enzyme (Bayer et al., 1998). The recurrent appearance in nature of a given type of module adjacent to a specific type of neighboring catalytic domain may indicate a functionally significant theme. These observations raise the possibility of a more selective role for certain types of CBM and other modules, whereby their association with certain types of catalytic domains could signify a "helper" role. The helper module would provide hydrolytic efficiency and alter the catalytic character of the enzyme.

New Developments in Cellulase Analysis

The biochemical characterization of cellulases is in many cases a difficult task owing to the large variety of enzyme types and modes of action. At first glance, it is an intriguing phenomenon that for such a simple reaction (i.e., the hydrolysis of the β-1,4-glucose linkage in a linear glucose chain), Nature has evolved so many types of cellulases. The vast varieties of enzymes are found not only among the different species of cellulolytic bacteria but also within the same organism. The reason for this extensive diversity comes from the insoluble nature of cellulose and the fact that, although the chemical com-

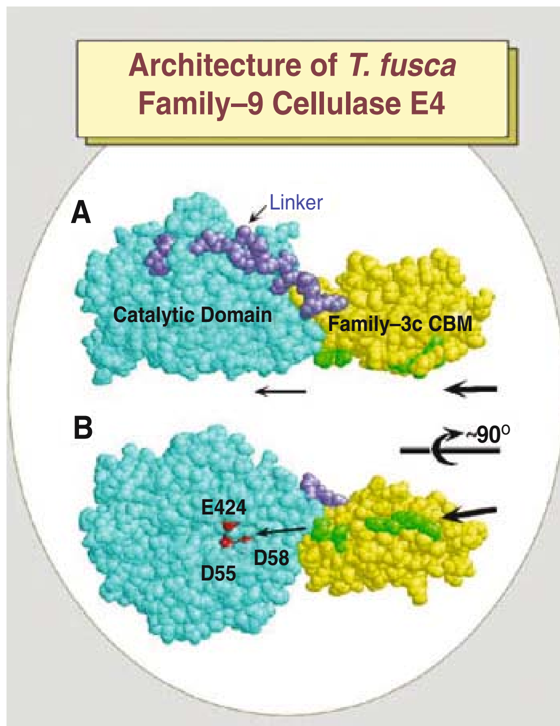

Fig. 9. Structural aspects of family-9 theme-B cellulase E4 from *Thermobifida fusca*. A. "Side view" of the E4 molecule, drawn using RasMol in spacefill mode. Shown are the family-9 catalytic module (turquoise, at left), the family-3c carbohydrate-binding module (CBM; in yellow, at right) and the intermodular linker (dark blue strip). The presumed path of a single cellulose chain, from the CBM to the catalytic domain, is shown at the bottom of the structure (arrows). The enzyme also possesses a fibronectin-like domain (FN3) and a cellulose-binding family-2 CBM (not shown). Note that the linker appears to serve a defined structural role by which the family-3c CBM is clamped tightly to the catalytic domain. Selected surface residues on the catalytic domain along the interface of both the linker and the CBM3c also serve to fasten both features tightly to the catalytic module. B. "Bottom view" of the E4 molecule (~90° rotation of "A"). From this perspective, the proposed catalytic residues (red), positioned in the active site cleft, are clearly visible. The path of the cellulose chain (arrows) passes through a succession of polar residues (green) on the bottom surface of the CBM, which would conceivably bind to the incoming cellulose chain and serve to direct it towards the active-site acidic residues of the catalytic domain.

position of the homopolymer is rather trivial, the physical and three-dimensional arrangement of the chains within the crystalline and amorphous regions of the microfibril can differ significantly.

Regarding the enzymes that degrade the substrate, the modular nature of the cellulases contributes additional degrees of complexity in our quest to characterize a given enzyme. Thus, the number, types, and arrangement of the accessory modules vis-à-vis the catalytic domain are important structural features that modulate the overall activity of the enzyme in question. This descriptive information should always be defined for a recombinant enzyme. Whenever possible, it is desirable to determine the relative contribution of the individual accessory modules to the activity of the enzyme. In this regard, the affiliation of a given module, e.g., CBM, into a defined family does not necessarily define its contribution to enzyme activity, as different specificities and functions have been attributed to different members of the same family of module. Moreover, sequences for almost 70 different "X" modules (i.e., modules for which the function remains undefined) are currently available (Coutinho and Henrissat, 1999b; Coutinho and Henrissat, 1999c), most of which probably play a binding or processing role in assisting the catalytic domain(s) in its capacity to hydrolyze the substrate.

A decade ago, the range of cellulases and hemicellulases within a given species was assessed mainly by biochemical techniques. In some cases, individual enzymes were isolated and their properties assessed using desired insoluble or soluble substrates. Another approach involved electrophoretic separation of cell-derived or cell-free extracts, and analysis of desired activities using zymograms. There are advantages and disadvantages with each of these strategies, and the employment of combined complementary approaches is always advisable.

More recently, molecular biology techniques have been used to reveal cellulase and hemicellulase genes, which can often be characterized on the basis of sequence homology with related, known genes (Béguin, 1990; Hazlewood and Gilbert, 1993). If further information is required on the structure or action of a given enzyme, the gene can then be expressed in an appropriate host organism, and the properties of the product can be characterized.

It is always instructive to compare the properties of an expressed gene product with those of the same protein isolated from the original bacterial culture. The results may be surprising; there are hazards inherent to both approaches. Expression of a gene may yield preparations with reduced or altered enzymatic properties. In this context, the expressed gene product may not have been folded properly. It is of course assumed that the investigator has taken the time and trouble to sequence the cloned gene to ensure no mutations have occurred. Unlike a gene expressed in a host cell environment, the native counterpart may have undergone post-translational modifications (e.g., glycosylation, proteolytic truncation, etc.) that improve its

physicochemical properties. Moreover, since the cellulase system in the native environment includes numerous enzyme types, often exhibiting similar molecular masses and other physical characteristics, the reputed purification of a given extracellular cellulase may still include contaminating enzymes that alter (usually increasing greatly due to synergistic action of two or more enzymes) the true enzymatic properties of the desired enzyme. The onus belongs to the conscience of the investigating scientist when publishing the properties of a given enzyme. Too often, erroneous data that enter the scientific literature are taken as fact. One should particularly be wary of comparing enzymatic activities of the same or similar types of enzymes (e.g., members of the same family) that have been published at different times and by different laboratories.

The assessment of cellulase activity is indeed a complicated undertaking, and there is no clear or standard methodology for doing so. This predicament apparently reflects a combination of factors, including the complex nature of the substrate, the multiplicity of enzymes and their synergistic action, and the variety of products formed. The fact that cellulose is an insoluble substrate converted to lower-order cello-oligosaccharide products is a further complication. It must be noted that as the cello-oligomers increase in length, they become less soluble, such that cello-octaose of 8 glucose units is no longer soluble in aqueous solutions. Moreover, the accumulation of one (particularly cellobiose) or more of the cellulose degradation products may be inhibitory towards enzymatic activity.

Today, the study of cellulase action usually includes, in addition to conventional biochemical assays, the analysis of the primary structure and the assignment of the various domains into known families. The catalytic domains can usually be assigned into one of the known glycoside hydrolase families (Henrissat and Bairoch, 1996; Henrissat and Davies, 1997). Whenever the sequence of a known polysaccharide-degrading enzyme failed to match a known family, a new family of glycosyl hydrolase was established. This approach was extensively developed in the last decade, owing to the increasing number of available DNA sequences and bioinformatics analysis tools. At the same time, an increasing number of crystal or solution structures of various catalytic and accessory domains were published that allow us to examine a new protein sequence in light of its structure. Sometimes, the publication of the structure of an accessory domain precedes determination of its function.

We can divide the analysis of a newly described prospective cellulase into several stages, such that a variety of complementary approaches are currently in use to classify the enzyme. Some of the questions one may ask are:

1) What is the primary structure (the amino acid sequence) of the enzyme? What are the binding residues and/or binding module(s) associated with the enzyme? What are its other accessory domains and their respective role(s) in catalysis or stability?
2) Is the enzyme a "true" cellulase, i.e., its preferred substrate is cellulose or cellulose degradation products, or whether the enzyme can act alone on insoluble cellulose.
3) What is the mode of action? Does the enzyme act as an endoglucanase, an exoglucanase or a processive enzyme?
4) What is the stereochemistry of the reaction? Does the enzyme exhibit an inverting or retaining mechanism?
5) What are the catalytic residues: the acid/base residue and the nucleophile that characterize a glycosyl hydrolase?

In the past ten years, several extensive reviews and book chapters dealing with different assays of cellulose degradation have been published (Ghose, 1987; Wood and Kellogg, 1988). In this treatise, we will briefly summarize the various approaches currently in use and direct the reader to the relevant literature.

While characterizing the activity of a new enzyme preparation, one has to bear in mind several secondary or indirect issues, such as the purity of the protein preparation, the sensitivity of the assay used, and the crossreactivity of the expected enzymatic activities. In some cases, only detailed kinetic analysis can provide appropriate characterization of the enzyme. As for many other types of glycosyl hydrolases, cellulases can exhibit crossreactivity with substrates of similar structure. This is particularly true when using, for example, *p*-nitrophenyl derivatized substrates that provide highly sensitive assays. However, in many cases such a soluble synthetic chromogenic substrate can fit the active-site pocket of a related but atypical enzyme, which catalyzes its hydrolysis. For example, family-10 glycosyl hydrolases are typically xylanases but can readily hydrolyze *p*-nitrophenyl cellobioside, which is a typical cellulase substrate. Without a detailed comparative kinetic analysis (kcat/Km) using different substrates, the true specificity of the enzyme might be overlooked. Given the amino acid sequence of the protein, its assignment to a given glycosyl hydrolase family will in many cases provide a reasonable general indication of its activity. The description of the modular structure provides additional knowledge that can imply how the catalytic function might be modulated, but this knowledge can also be

misleading. In the final analysis, there is no substitute for extensive biochemical and biophysical characterization of the given protein (recombinant or native) and its catalytic properties.

General procedures for assaying for cellulase and hemicellulase activities are very well documented in the *Methods in Enzymology* volume 160 (Wood and Kellogg, 1988). Conventional procedures for cellulase assay have been defined precisely by the International Union of Pure and Applied Chemists (IUPAC; Ghose, 1987). However, owing to the complexity of the substrate and enzyme systems, these procedures can only provide a starting point for understanding the true nature of the enzyme in question.

Since the publication of Part A of this treatise (Coughlan and Mayer, 1992), many of the previously reported assays of cellulase activity are still in common use. These include the use of soluble, derivatized forms of cellulose, e.g., carboxymethyl cellulose and hydroxymethyl cellulose as conventional substrates for determining endoglucanase activity. In addition, a derivatized, colored form of insoluble cellulose, i.e., azure cellulose, is frequently used as an indication of cellulase activity. Zymograms with such colored embedded substrates are useful in detecting endoglucanase or xylanase activities (Béguin, 1983). Individual soluble cello-oligomers (cellotetraose, cellopentaose, cellohexaose, etc.) are still used as substrates for analyzing enzyme action, but the reliance on these substrates as determinants for assessing cellulase activity is no longer a definitive approach. In the past decade or so, newly developed substrate analogues and reagents include thioglycoside substrates (Driguez, 1997), fluoride-derivatized sugars (Williams and Withers, 2000), chromophoric and fluorescent cello-oligosaccharides (Claeyssens and Henrissat, 1992; O'Neill et al., 1989; van Tilbeurgh et al., 1985). Recently, an ultraviolet-spectrophotometric method and an enzyme-based biosensor have been described (Bach and Schollmeyer, 1992; Hilden et al., 2001). In addition, a novel and intriguing bifunctionalized fluorogenic tetrasaccharide has been developed as an effective reagent for measuring the kinetic constants of cellulases by resonance energy transfer (Armand et al., 1997).

The thio-oligosaccharides serve as competitive inhibitors that mimic natural substrates but are enzyme resistant (Driguez, 1997). In this type of oligosaccharide, the oxygen of a bond to be cleaved is replaced by sulfur. The thio-oligodextrins are sometimes more soluble than the native cellodextrins and longer chains can be synthesized. The modified sugars can be used in biochemical studies or crystallographic studies to gain some information about the geometry of the active site or determine the mechanism of action of an enzyme.

Determination of "True" Cellulase Activity: Solubilization of Crystalline Cellulose Substrates True cellulase activity is usually defined as the ability to solubilize to an appreciable degree insoluble, "crystalline" forms of cellulose. The extent of hydrolysis can be evaluated by turbidity assays, weight loss of insoluble material, generation of reducing power, and accumulation of soluble sugars. It is important to realize that crystalline cellulose is not of uniform composition and therefore the rate of catalysis is in most cases not linear with time or enzyme concentration. Notably, the different preparations of crystalline cellulose contain varying levels of loosely associated loops and chains. The latter are readily accessible to hydrolysis by a given enzyme and lead to relatively high initial rates of activity, which do not reflect the actual degree of true cellulase activity. For example, such loose chains can be degraded by a relatively ineffectual enzyme, whereas the crystalline portions of the substrate will be immune to further hydrolysis by the same enzyme. To overcome these difficulties, IUPAC suggests determining the amount of enzyme required to achieve digestion of 5.2% of the insoluble substrate (e.g., filter paper) in 16 h (Ghose, 1987; Irwin et al., 1993).

Cellulose substrates commonly in use include Avicel, filter paper, cotton, Solka Floc, and more recently bacterial cellulose from *Acetobacter aceti* and algal cellulose prepared from *Valonia*. Consequently, these assays should be treated as a relative and not quantitative assessment. The nature of the original substrate selected—especially its extent of crystallinity—should always be taken into account. Proper controls and reference substrates should always be used. One should be wary about comparison among results reported by different laboratories and even by different researchers in the same laboratory. Nevertheless, such assays give an excellent indication of whether a given enzyme preparation exhibits substantial activity towards crystalline cellulose substrates.

Endoglucanase Versus Exoglucanase Activity As discussed earlier in this chapter, the cellulases have traditionally been divided into either endoglucanases or exoglucanases (Fig. 4). The biochemical or enzymatic assays that discriminate between these two modes of action usually involve soluble forms of cellulose, i.e., carboxymethyl or hydroxymethyl derivatives of cellulose. The action of a given enzyme on these substrates is followed by determining the

amount of reducing ends generated by the enzyme and the degree of polymerization (DP). The reducing power is usually determined either by using reagents such as 3,5-dinitrosalicylic acid (DNS; Miller et al., 1960), ferricyanide (Kidby and Davidson, 1973), or copper-arseno molybdate (Green et al., 1989; Marais et al., 1966).

Despite their traditional popularity, these two methods are intrinsically disadvantageous, owing to interference by metal ions and certain buffers. Moreover, such assays are sensitive to the chain length of the reducing end. A more recent approach involves the use of disodium 2,2′-bicinchoninate (BCA) for determination of reducing sugar. This procedure is more sensitive than the conventional methods and gives comparable values of reducing sugars for cellodextrins of different lengths (Doner and Irwin, 1992; Garcia et al., 1993; Vlasenko et al., 1998; Waffenschmidt and Jaenicke, 1987).

Viscosity-based measurements represent the most common approach for assessing the degree of polymerization. This approach is highly sensitive for internal bond cleavage, which leads to significant reduction of the average molecular weight of the substrate. The comparison between the amount of reducing sugars generated and the average molecular weight (i.e., viscosity or fluidity of the soluble cellulose substrate) gives a very good indication whether an enzyme is essentially exo- or endo-acting.

The average degree of polymerization also can be evaluated by size-exclusion chromatography either alone (Srisodsuk et al., 1998; Teeri, 1997) or combined with multiangle laser light scattering (Vlasenko et al., 1998). Mass spectometric procedures also can be applied to determine the identity and distribution of degradation products following hydrolysis of cellulosic substrates by an enzyme (Hurlbert and Preston, 2001; Rydlund and Dahlman, 1997). The mode of enzymatic action also can be appraised by determining the increase in reducing power associated with the insoluble versus the soluble fraction of the substrate. Increase in the proportion of reducing sugars associated with the soluble fraction indicates an exo-type of activity whereas a relatively large increase in the insoluble fraction would suggest an endo-type of activity (Barr et al., 1996).

Exocellulases can exhibit different specificities depending on their preference for the reducing or nonreducing end of the cellulose chain (Barr et al., 1996; Teeri, 1997). This feature of an exocellulase can be determined either by using oligosaccharide substrates labeled by tritium or 18O at the reducing end. Other procedures involve NMR, HPLC and/or mass spectrometric analysis of products released from native (unlabeled) cello-oligosaccharides. Within the past decade, the 3-D structures of enzyme-substrate complexes have been obtained, and the specificities of the enzyme can be interpreted directly from the data (Davies and Henrissat, 1995; Davies et al., 1998; Divne et al., 1998; Juy et al., 1992; Notenboom et al., 1998; Parsiegla et al., 1998; Rouvinen et al., 1990; Sakon et al., 1997; Zou et al., 1999).

Processivity One of the major recent conceptual advances in assessing the mode of enzymatic action of a cellulase is the concept of "processivity." Processive enzyme action can be defined as the sequential cleavage of a cellulose chain by an enzyme. In effect, exoglucanases are by nature and structure processive enzymes. Their tunnel-like active site thus allows processive action on the cellulose chain. Endoglucanases, however, were thought to be intrinsically nonprocessive. However, the traditional distinction between exo- and endocellulases was modified recently.

Experiments combining two or more purified cellulases have shown that synergism can even be detected upon mixing two different types of exo-acting enzymes. Such experiments led to the recognition that the exo enzymes can operate on both ends (i.e., the reducing and nonreducing ends) of the cellulose chain. Some enzymes, however, exhibit both endo and exo activities, although in such cases, the endocellulase activity is usually very low. In attempts to explain these phenomena, the concept of processivity was proposed, by which the activity of the enzyme is characterized by the sequential hydrolysis of the cellulose chain. Implicit in this concept is the notion that the catalytic site of the enzyme remains in continual and intimate contact with a given chain of the cellulose substrate.

A more complete mechanistic picture of the processive nature of such cellulases was revealed with the advent of high-resolution 3-D structures. It was thus demonstrated that the cellulose chain makes contact with the protein at multiple sites, either via a tunnel-shaped structural element (such as that observed in the family-48 enzymes) or by a special type of CBM (such as the family-9 theme B cellulases). These arrangements allow the threading of the cellulose chain into the active site, and, following initial cleavage at the end of the chain, the enzyme can move along the chain and position itself for the next cleavage. In addition to this processive nature of the active site, these enzymes also can make classic endo cleavages thus generating new ends.

Biochemically processive enzymes exhibit characteristics between endo- and exoenzymes. They have low but detectable endo activity towards soluble derivatives of cellulose (i.e.,

CMC), and may or may not possess exo activity on such substrates. With insoluble substrates, they will generate reducing power with a ratio between the soluble to the insoluble fractions of about 7. Endocellulases usually give a ratio of less than 2, whereas exocellulases produce a ratio of 12 to 23 (Irwin et al., 1998).

Once the processive nature of an enzyme has been indicated experimentally, molecular insight into the mechanisms responsible for this feature can be gained by determining the 3-D crystal structure of the active site together with model cellodextrins. In the case of the cellulases, the crystal structure of the catalytic domain together with the fused module, combined with accumulating enzymatic activity data, allowed further postulation as to the accessory role of the fused module. The fused CBM presumably interacts with a single cellulose chain and feeds it into the active site. Interestingly, this domain does not bind crystalline cellulose, but is inferred to act in dynamic binding of the single cellulose chain prior to its hydrolysis, thereby imparting the quality of processivity to the enzyme. Once such a property is associated with a given type of enzyme, the primary structure of the protein can now be used as an indication for all such enzymes. In the case of the family-9 theme B enzymes, it is now possible to identify the catalytic domain (e.g., glycosyl hydrolase family 9) and the additional accessory domains (in this case, family-3c CBM). Thus, the primary structure may by itself give a strong indication of the nature of the enzyme itself. Of course, the ultimate identification as to the mechanism of enzyme activity will come from the detailed 3-D structure of the enzyme-substrate complex.

An intriguing recent development in the analysis of the cellulolytic action of a given cellulase or a mixture of cellulase is the direct transmission electron microscopic (TEM) observation of the enzymatic action on bacterial cellulose ribbons. The approach provides information as to the endo or exo preference of the enzyme, the extent of processivity as well as the directionality of hydrolysis (i.e., from the reducing to the nonreducing ends or vice versa). This strategy has been used to study the hydrolysis of bacterial cellulose ribbons by individual purified enzymes, mixtures of purified enzymes, and intact cellulosomes.

Mechanism of Catalysis The mechanism of catalysis of cellulases address issues such as stereochemistry, binding and active-site residues and transition state intermediates. Excellent reviews have been published recently covering many of these (Ly and Withers, 1999; McCarter and Withers, 1994; Rye and Withers, 2000; Sinnott, 1990; White and Rose, 1997; Withers, 2001; Withers and Aebersold, 1995; Zechel and Withers, 2000). The fact that the stereochemistry and catalytic residues are conserved between members of the same family allows the putative identification of these elements if one member of the given (glycosyl hydrolase) has been characterized biochemically (Henrissat and Bairoch, 1996; Henrissat et al., 1995; Henrissat and Davies, 1997).

The sterochemistry of the reaction can in most cases be determined by proton NMR spectroscopy or by using chromatography systems that allow the resolution of anomeric species. In the case of NMR, the reaction between the test enzyme and its substrate is carried out in deuterated water (D_2O) and the appearance of the anomeric proton can be easily detected. Thus, for the degradation of cellulose, a retaining enzyme would produce a product in the β configuration whereas an inverting enzyme would yield the α-sugar.

The catalytic residues can be identified by performing site-directed mutagenesis on conserved acidic residues and studying the catalytic properties of the mutants with substrates bearing different leaving groups. Commonly used phenol substituents include the following, listed in order of leaving group ability (pKa values shown parenthetically): 2,4-dinitro (3.96) > 2,5-dinitro (5.15) > 3,4-dinitro (5.36) > 2-chloro-4-nitro (5.45) > 4-nitro (7.18) > 2-nitro (7.22) > 3,5-dichloro (8.19) > 3-nitro (8.39) > 4-cyano (8.49) > 4-bromo (9.34; Tull and Withers, 1994). In retaining enzymes, the nucleophilic residue can be identified directly by trapping the intermediate with an appropriate inhibitor. Such inhibitors include model saccharides containing a fluorine substituent in the 2- or 5-position and a good leaving group, such as fluoride or dinitrophenolate (Williams and Withers, 2000). The substituted substrate forms a relatively stable covalent substrate-enzyme complex, involving the nucleophile residues. The complex is then subjected to proteolytic cleavage and sequencing of the glycosylated peptide. Recently, the use of protocols involving combined liquid chromatography and mass spectrometry has facilitated the identification of the modified residues.

The acid-base residue in a retaining enzyme can be identified by a combination of kinetics-based methodologies. Mutation of this residue (usually to alanine) should affect the rate of both chemical steps, i.e., glycosylation and deglycosylation, though the effect on each step should be different. The effect on the glycosylation step will depend strongly on the leaving group ability of the aglycon. Thus, rates of hydrolysis for substrates with a poor leaving group should be affected much more strongly than those with a

good leaving group. The deglycosylation step, however, will be affected equally for all substrates carrying different leaving groups, because the same glycosyl enzyme intermediate is hydrolyzed during this step. Thus, detailed kinetic analysis (i.e., determination of k_{cat} and K_m) with substrates bearing different leaving groups can reveal whether the corresponding mutation is the acid-base residue. It should be noted that this approach requires synthetic substrates that are not necessarily recognized by all families of enzymes and are not necessarily commercially available. For example, the family-11 xylanases fail to hydrolyze *p*-nitrophenyl xylobioside, which is an excellent substrate for the family-10 xylanases. The assignment of the acid-base catalyst can also be examined by use of external nucleophilic anions, such as azide. In this approach, termed "azide rescue," the small azide anion enters the vacant space created by alanine replacement of the acidic amino acid residue. The azide reacts with the anomeric carbon instead of a water molecule to form the corresponding β-glycosyl azide product. In the absence of an acid-base catalyst, which normally provides general base catalysis during the second step, the deglycosylation step is severely affected. Thus, the acceleration of the reaction by the mutant enzymes in the presence of these external anions (provided that the second step is rate limiting) is a good indication that a mutant residue is the acid-base catalyst. Finally, the assignment of the acid-base catalyst can be tested by comparing the pH-dependence profiles for the wild-type and mutant enzymes. The profile for the native enzyme would approximate a perfect bell shape curve, reflecting the ionization of the two active site carboxylic acids, whereas the no reduction of activity at high pH values would be observed for the mutant. This pH dependency approach is also applicable for identifying the nucleophile residues and the catalytic residues in inverting enzymes.

Prokaryotic Cellulase Systems

The cellulolytic bacteria produce a variety of different cellulases and related enzymes, which together convert the plant cell wall polysaccharides to simple soluble sugars that can subsequently be assimilated. The complement of cellulases and hemicellulases that are synthesized by a given bacterium for this purpose is referred to as its "cellulase system." Different bacteria exploit different strategies for the ultimate degradation of their substrates. The given strategy is reflected by the complement and type(s) of enzymes produced by a given bacterium. The bacterial cellulase system may be characterized by free enzymes, cell-bound enzymes, multifunctional enzymes, cellulosomes, or any combination of the latter.

Cellulase enzyme systems are comprised of several different types of components, each type may exist in a multiplicity of forms. To add to the complexity, the same component may exist as free individual entities in the culture fluid, as individual entities bound to cellulose, or associated with the cell surface. Alternatively, an individual component may be organized as part of a multicomponent cellulosome complex attached to the cell surface, to the cellulose, to both, or as free complexes in the culture fluid. Furthermore, the situation existing during growth under one set of conditions (e.g., pH, temperature, distribution of carbon source, etc.) may not exist under another, or may change considerably during the course of cultivation. The bacterium reacts to these changes and its production of cellulases and/or cellulosomes may reflect the dynamics of the growth conditions.

Free Enzymes

As mentioned earlier in this chapter, the free enzymes in their simplest form comprise a catalytic module alone with no accessory domains or modules. Such enzymes often specialize in degrading soluble oligosaccharide breakdown products. Alternatively, such single-modular enzymes may rely on an intrinsic association with insoluble polysaccharide substrate such as cellulose, perhaps related to the active site of the enzyme.

A higher order level of organization and activity are free enzymes composed of a polypeptide chain that includes both a catalytic domain together with a CBM. This basic bi-modular arrangement can be further extended by the inclusion of additional types of modules or repeating units of the same module, all of which serve to modulate the activity of the catalytic domain on the substrate. The intact free enzyme, however, remains unattached to other enzymes and can work in an independent manner on a given substrate.

Cell-Bound Enzymes

Some enzymes are connected directly to the cell wall. In Gram-positive bacteria, this is frequently accomplished via a specialized type of module, the SLH (S-layer homology) module, previously shown to be associated with the cell surface of Gram-positive bacteria (Lupas et al., 1994). This arrangement may have evolved to provide a more economic degradation of insoluble substrates and to reduce competition with other bac-

teria for the soluble products, subject to diffusion in the media. As opposed to free enzymes, diffusion of an attached enzyme would itself be prevented.

Examples of enzymes, which are bound to the cell surface via an SLH module include, a family-5 cellulase and family-13 amylase-pullulanase from *Bacillus*, a family-10 xylanase from *Caldicellulosiruptor* (Saul et al., 1990), a family-5 endoglucanase from *Clostridium josui*, a family-16 lichenase and family-10 xylanase from *Clostridium thermocellum* (Jung et al., 1998), and a variety of enzymes (family-10 xylanases, a family-5 mannanase and a family-13 amylase-pullulanase) from different species of *Thermoanaerobacter* (Matuschek et al., 1996). The modular architecture of these enzymes may be particularly complicated, containing several different modules in a single polypeptide chain, thus forming extremely large enzymes sometimes comprising over 2,000 amino acids (Fig. 10).

Multifunctional Enzymes

Some cellulases exhibit a more complex architecture in that more than one catalytic domain and/or CBD may be included in the same protein. Examples of such enzymes are the very similar cellulases from *Anaerocellum thermophilum* (Zverlov et al., 1998) and *Caldocellum saccharolyticum* (Te'o et al., 1995), both of which contain a family-9 and a family-48 catalytic domain. Other paired catalytic domains include those from family 44 and either family 5 or 9. Such an arrangement might indicate a close cooperation between two particular catalytic domains, which may lead to synergistic action on the cellulosic substrate, thus portending on a smaller scale the advent of cellulosomes.

Like the cellulases, xylanases also tend to exhibit a modular structure, being composed of multiple domains joined by linker sequences. Family-10 and -11 xylanases may be linked in the same polypeptide chain either to each other, to catalytic domains from families 5, 16 and 43 or to carbohydrate esterases (Flint et al., 1993; Laurie et al., 1997). One particularly interesting combination of multifunctional catalytic modules that appear in the same polypeptide chain is a typical xylanase together with a feruloyl esterase. Such a combination would allow the rapid cleavage of hemicellulose from the lignin in natural systems, i.e., the plant cell wall (see Fig. 3). In this manner, the xylan chain would be severed by the xylanase component (Xyn in Fig. 3) and the lignin-xylan association would be disconnected simultaneously by the feruloyl acid esterase (Fae in Fig. 3).

Indeed, some xylanases are extremely complex in their modular architecture (Fig. 11). In addition to multiple catalytic modules, these enzymes often contain several different types of CBMs. Why would such a xylanase contain several types of CBM? And why would a xylanase contain a cellulose-specific CBD? Unlike the case of various cellulases, for which the CBD is usually essential for degrading insoluble crystalline cellulose, the CBMs of a hemicellulase do not necessarily bind the hemicellulose component (xylan). In some cases, its CBM is in fact an authentic CBD that situates the hemicellulase on the insoluble plant cell wall material by utilizing the most abundant and most stable cell-wall component—cellulose. Indeed, the three family-3 CBDs (CBM3) shown in Fig. 11 apparently bind to crystalline cellulose. Why would this xylanase require three tandem copies of the same type of CBD is yet another mystery that should eventually be addressed experimentally. At any rate, once bound via the

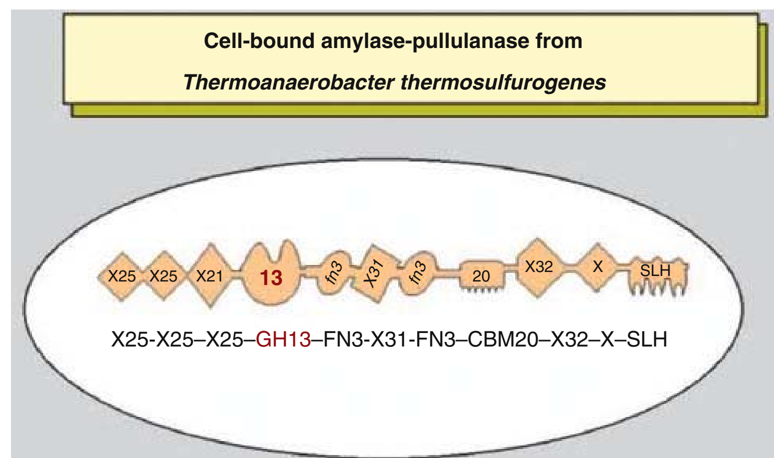

Fig. 10. A very large, cell-surface enzyme from *Thermoanaerobacter thermosulfurogenes*. The 1861-residue enzyme contains an SLH module, which is believed to mediate the attachment of the enzyme to the cell surface in Gram-positive bacteria. The enzyme contains a multiplicity of modules, which apparently serve to regulate the hydrolytic action of its single family-13 catalytic module with the complex substrate. Several X domains of unknown function may either represent as yet undescribed catalytic functions, carbohydrate-binding activities or structural entities.

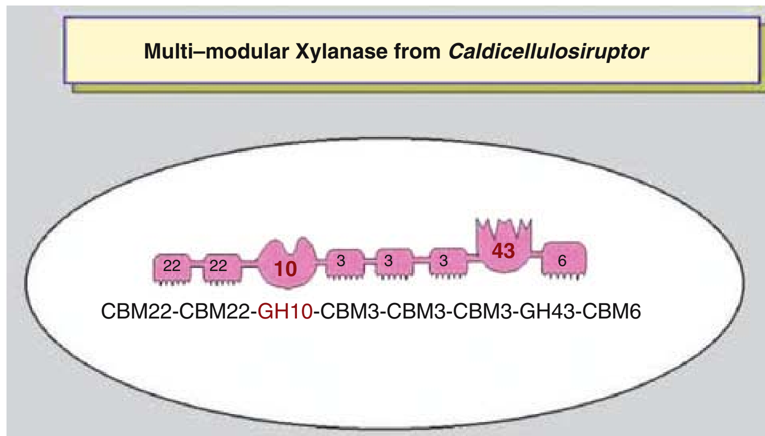

Fig. 11. A very large, multimodular xylanase from *Caldicellulosiruptor*. The 1,795-residue enzyme contains 8 separate modules, including 2 catalytic modules from families 10 (invariably a xylanase) and 43 (frequently an arabinofuranosidase). These are modulated by numerous carbohydrate-binding modules, which include 3 from family 3 (likely for binding to crystalline cellulose), 2 from family 22 (newly classified and shown to function in xylan binding and one from family 6.

cellulose component of the plant cell wall composite substrate, the immobilized enzyme then acts on the accessible and appropriate hemicellulose components. Once thus situated on the plant cell wall, another type of CBM on the same molecule would then assist in the binding to the xylan (or mannan, etc.) component to direct the appropriate catalytic module to its true substrate. Hence, the modular proximity of the xylanase shown in Fig. 11 would presumably indicate that the two CBM22s would modulate the action of the family-10 catalytic module, and the C-terminal CBM6 would facilitate the catalysis by the family-43 module. Together, the two catalytic modules would act synergistically to degrade susceptible plant cell wall components. In this context, the complex architecture of a xylanase would reflect the complex chemistry of its substrate and the neighboring polymers of its immediate environment in the plant cell wall.

Cellulosomes

Cellulosomes are multienzyme complexes, which bind to and catalyze the efficient degradation of cellulosic substrates. The first cellulosome was discovered while studying the anaerobic thermophilic bacterium, *Clostridium thermocellum* (Bayer et al., 1983; Lamed et al., 1983). Since its initial description in the literature, the cellulosome concept has been subject to numerous reviews (Bayer et al., 1996; Béguin and Lemaire, 1996; Belaich et al., 1997; Doi et al., 1994; Doi and Tamura 2001; Felix and Ljungdahl, 1993; Karita et al., 1997; Lamed and Bayer, 1988; Lamed and Bayer, 1991; Lamed and Bayer, 1993; Lamed et al., 1983; Shoham et al., 1999).

Cellulosomes in *C. thermocellum* exist in both cell-associated and extracellular forms, the cell-associated form being associated with polycellulosomal protuberance-like organelles on the cell surface. Later, cellulosomes were detected in other cellulolytic organisms (Lamed et al., 1987; Mayer et al., 1987), including *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Clostridium cellulovorans* and *Ruminococcus albus*, all of which contained protuberance-like organelles on their surfaces (Bayer et al., 1994; Lamed and Bayer, 1988; Fig. 12).

The cellulosomes contain numerous components, many of which were shown to display enzymatic activity. They also contain a characteristic nonenzymatic high-molecular-weight component. This component proved to be highly antigenic and glycosylated (Bayer et al., 1985). The cellulosomal enzymatic subunits from this organism showed a broad range of different cellulolytic and xylanolytic activities (Morag et al., 1990). Ultrastructural evidence indicated the multisubunit nature of the cellulosome (Fig. 13).

Eventually, genetic engineering techniques led to the sequencing of cellulosomal genes in *C. thermocellum* and several other bacteria, thus confirming the existence of cellulosomes as a major paradigm of prokaryotic degradation of cellulose and related plant cell wall polysaccharides.

Clostridium Thermocellum Cellulosomal Subunits and Their Modules

A simplified schematic view of the cellulosome from *C. thermocellum* and its interaction with its substrate is shown in Fig. 14. The cellulosomal enzyme subunits were found to be united into a complex by means of a unique class of nonenzymatic, multimodular polypeptide subunit, termed “scaffoldin” (Bayer et al., 1994). The

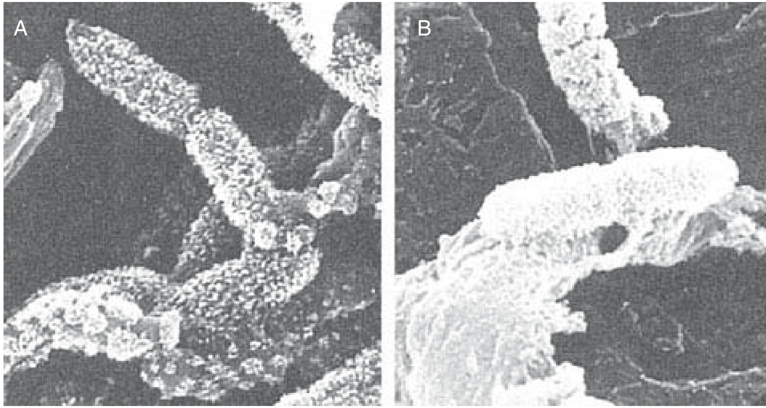

Fig. 12. Scanning electron microscopy (SEM) of *Acetivibrio cellulolyticus* showing the presence of large characteristic protuberance-like structures on the cell surface. Cells are shown in the free state (A) or bound to cellulose (B). Cell preparations were treated with cationized ferritin before processing. Cationized ferritin has been shown to stabilize such surface structures, thus allowing their ultrastructural visualization (Lamed et al., 1987a; Lamed et al., 1987b). Without pretreatment with cationized ferritin, these structures are invisible. In (B), the cellulose-bound cells appear to be connected to the substrate via structural extensions of the cell-surface protuberances. Such a mechanism was originally observed for other cellulolytic prokaryotes, e.g., *C. thermocellum* (Bayer and Lamed, 1986).

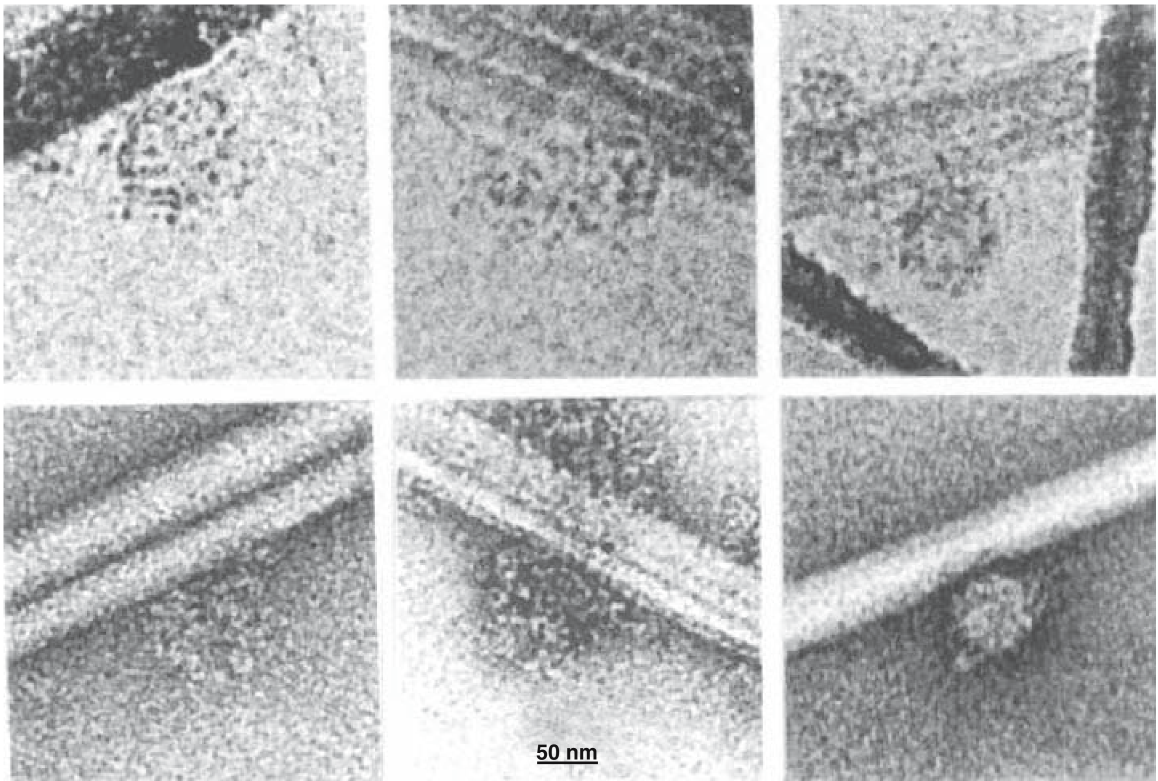

Fig. 13. Comparison between negative staining (bottom) and cryo images (top) of the purified cellulosome from *C. thermocellum*, adsorbed on cellulose microcrystals from the algae, *Valonia ventricosa*. The images illustrate the diversity of shapes of the cellulosomes, which adopt either compact or loosely organized ultrastructure. In the cryo images, the subunits of the cellulosomes (i.e., the individual enzymatic components) are clearly visible. Micrographs courtesy of Claire Boisset and Henri Chanzy (CNRS—CERMAV, Grenoble, France).

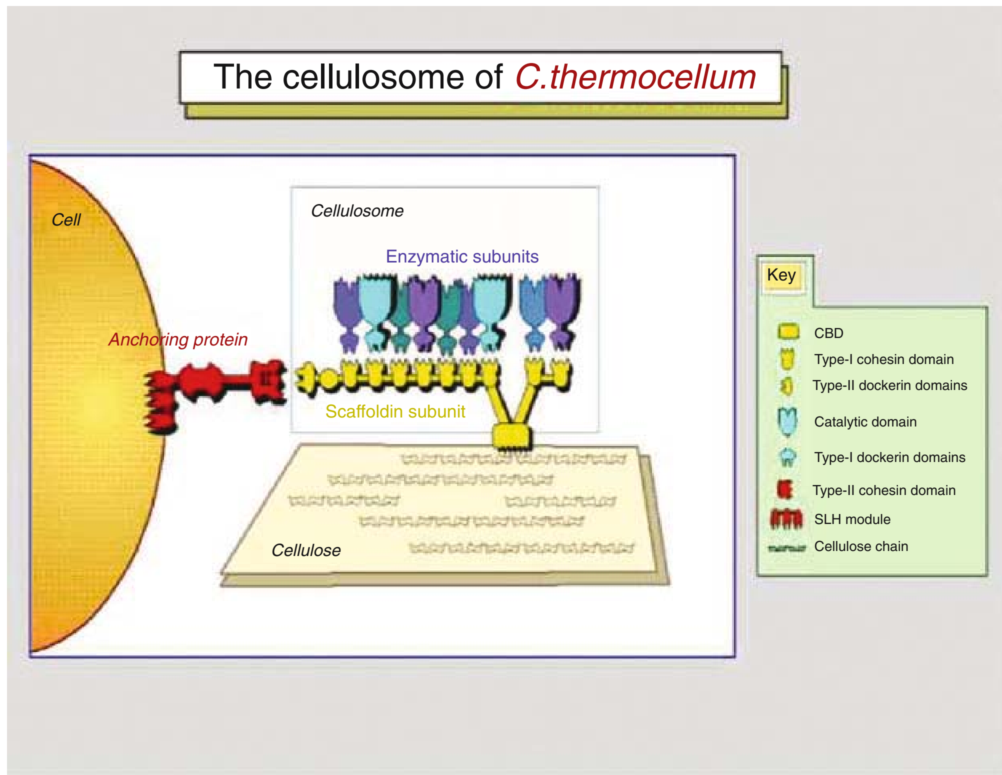

Fig. 14. Simplified schematic view of the molecular disposition of the cellulosome and one of the associated anchoring proteins on the cell surface of *C. thermocellum*. The key defines the symbols used for the modules, from which the different cellulosomal proteins are fabricated. The progression of cell to anchoring protein to cellulosome to cellulose substrate is illustrated. The SLH module links the parent anchoring protein to the cell. The cellulosomal scaffoldin subunit performs three separate functions, each mediated by its resident functional domains: 1) its multiple type-I cohesins integrate the cellulosomal enzymes into the complex via their resident type-I dockerins, 2) its family-IIIa CBD binds to the cellulose surface, and 3) its type-II dockerin interacts with the type-II cohesin of the exocellular anchoring protein.

scaffoldins usually contain a family-3 CBD that provides the cellulose-binding function. The scaffoldins also contain multiple copies of a definitive type of module, called "the cohesin domain." The cellulosomal enzyme subunits, on the other hand, contain a complementary type of module, called "the dockerin domain." The interaction between the cohesin and dockerin domains provides the definitive molecular mechanism that integrates the enzyme subunits into the cellulosome complex (Salamitou et al., 1994; Tokatlidis et al., 1991; Tokatlidis et al., 1993). Cohesin and dockerins are considered to be cellulosome "signature sequences"—i.e., their presence is a good indication of a cellulosome in a given bacterium (Bayer et al., 1998).

The major difference between free enzymes and cellulosomal enzymes is that the free enzymes usually contain a CBD for guiding the catalytic domain to the substrate, whereas the cellulosomal enzymes carry a dockerin domain that incorporates the enzyme into the cellulosome complex. Otherwise, both the free and cellulosomal enzymes contain very similar types of catalytic domains. The cellulosomal enzymes rely on the Family-3a CBD of the scaffoldin subunit for collective binding to crystalline cellulose.

The incorporation of the multiplicity of enzyme subunits into the cellulosome complex is a function of the repeated copies of the cohesin module borne by the scaffoldin subunit. For most species of scaffoldin, the cohesins have been classified as type-I on the basis of sequence homology. The cohesin module is composed of about 150 amino acid residues. The basic structure of the cohesin is known and comprises a nine-stranded β sandwich with a jelly-roll topology (Shimon et al., 1997; Spinelli et al., 2000; Tavares et al., 1997).

The dockerin domain contains about 70 amino acids and is distinguished by a 22-residue duplicated sequence (Chauvaux et al., 1990), which bears similarity to the well-characterized EF-hand motif of various calcium-binding proteins (e.g., calmodulin and troponin C). Within this repeated sequence is a 12-residue calcium-binding loop, indicating that calcium-binding is an important characteristic of the dockerin

domain. This assumption was eventually confirmed experimentally (Yaron et al., 1995). The specificity characteristics of the cohesin-dockerin interaction also have been investigated. The results showed that four suspected residues may serve as recognition codes for interaction with the cohesin domain (Mechaly et al., 2000; Mechaly et al., 2001; Pagès et al., 1997). The three-dimensional solution structure of the 69-residue dockerin domain of a *Clostridium thermocellum* cellulosomal cellulase subunit was recently determined (Lytle et al., 2001). As predicted earlier (Bayer et al., 1998; Lytle et al., 2000; Pagès et al., 1997), the structure consists of two Ca^{2+}-binding loop-helix motifs connected by a linker; the E helices entering each loop of the classical EF-hand motif are absent from the dockerin domain.

The scaffoldin of *C. thermocellum* also contains a special type of dockerin domain. This dockerin failed to bind to the cohesins from the same scaffoldin subunit, but instead interacted with a different type of cohesin—termed "type-II cohesins"—identified on the basis of sequence homology (Salamitou et al., 1994). These cohesins are somewhat different than those of type I, having an additional segment and diversity in the latter half of the sequence. The type-II cohesins were discovered as component parts of a group of noncatalytic cell-surface "anchoring" proteins on *C. thermocellum* (Leibovitz and Béguin, 1996; Leibovitz et al., 1997; Lemaire et al., 1995; Salamitou et al., 1994). The three known anchoring proteins in *C. thermocellum* contain different copy-numbers of the type-II cohesins as illustrated in Fig. 15. Each of these anchoring proteins also contains an S-layer homology (SLH) module, analogous to those of the cell-bound enzymes mentioned above. The intervening sequences, however, between the cohesins and SLH domains are different. In any case, the type-II cohesins selectively bind the type-II dockerins, and the cellulosome (i.e., the scaffoldin subunit together with all of its enzyme subunits) is thereby incorporated into the cell surface of *C. thermocellum*.

Similarity and Diversity of Scaffoldins from Different Species

The modular architecture of the known scaffoldins and their comparison to that of *Clostridium thermocellum* is presented in Fig. 16. Two new scaffoldins have recently been described for *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens* that, like *C. thermocellum*, carry dockerin domains at their C terminus (Ding et al., 1999; Ding et al., 2000). The *A. cellulolyticus* genome also includes a gene (immediately downstream of the scaffoldin gene) that contains type-II

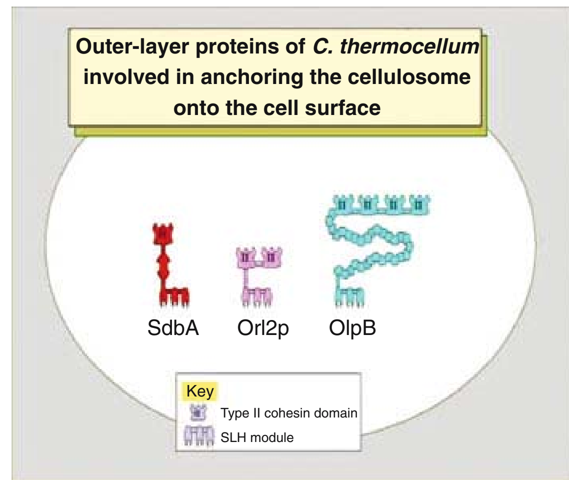

Fig. 15. Schematic representation of the known anchoring proteins of the *C. thermocellum* cell surface. Each protein bears an SLH domain that connects the protein to the cell surface via yet undefined surface components. The different proteins carry different numbers of type-II cohesins. SdbA has one cohesin, Orf2p has 2 and OlpB has 4, presumably allowing the corresponding number of scaffoldins (i.e., cellulosomes) to be attached to the given protein.

cohesins that may represent an anchoring protein. It thus seems that the arrangement of the cellulosome on the cell surface of these latter strains may be analogous to that of *C. thermocellum*. It is interesting to note that the cohesins of the *Bacteroides cellulosolvens* scaffoldin are clearly type-II cohesins and not of type I. This infers that there is not a clear linkage between the type-II cohesins and anchoring proteins.

The scaffoldins from the other clostridial species thus far described all lack "type-II dockerin" domains, the inference being that cells of *C. cellulovorans*, for example, would apparently not bear anchoring proteins that contain type-II cohesins. It thus follows that either their cellulosomes are not surface bound or, if indeed they are surface components, then their anchoring thereto is accomplished via an alternative molecular mechanism. Recently (Doi and Tamura, 2001; Tamaru and Doi, 1999a; Tamaru et al., 1999b), a cell-surface binding function has been proposed for a domain of unknown function, designated "X2" (Coutinho and Henrissat, 1999b; Coutinho and Henrissat, 1999c) of the scaffoldin from *C. cellulovorans*. On the basis of sequence alignment of a few conserved identical amino acids with S-layer proteins from Mycoplasma hyorhinis and Plasmodium reichenowi, the authors consider that this domain may be recognized as an SLH domain. The four X2 domains of the *C. cellulovorans* scaffoldin are very similar in sequence to the X-domains from the scaffoldins of *C. cellulolyticum* and *C. josui*,

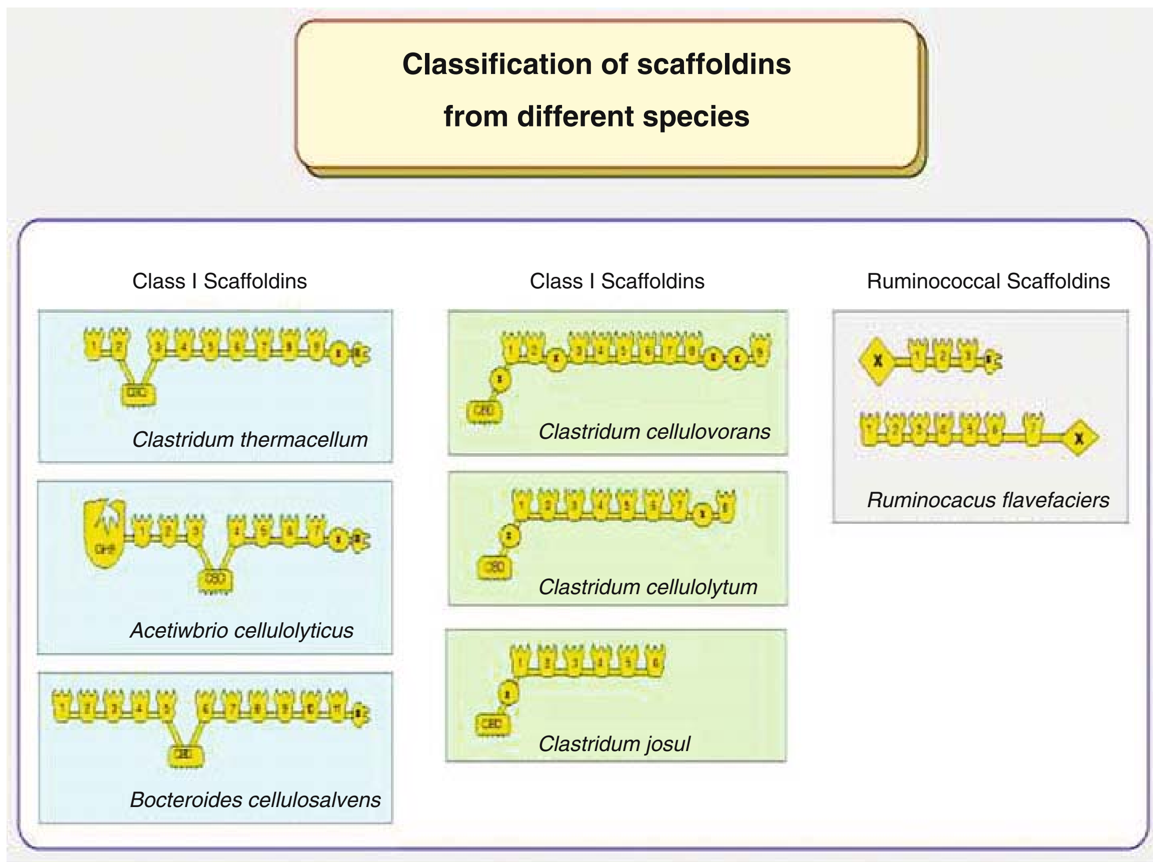

Fig. 16. Schematic view of the modular similarity and diversity of scaffoldins from different cellulosome species. Class-I scaffoldins feature an internal CBD and a *C*-terminal type-II dockerin domain. Class-II scaffoldins exhibit an *N*-terminal CBD and lack a dockerin domain. The newly described scaffoldins from *Ruminococcus flavefaciens* lack a defined CBD. The functional role of the two different X domains in the two *R. flavefaciens* scaffoldins is currently unknown. All of these scaffoldins contain multiple copies of cohesin domains.

which contain only two and one copies of this domain, respectively. If this domain functions in attaching the scaffoldin with its complement of enzymes to the cell surface, it is unclear why there would be different copy numbers of the domain in the different scaffoldins. Likewise, one of the *C. cellulovorans* cellulosomal enzyme components (EngE) also contains a triplicated segment of unknown function, designated "X48" (Coutinho and Henrissat, 1999b; Coutinho and Henrissat, 1999c) that the authors consider to be involved in cell-surface attachment (Tamaru and Doi, 1999a). In any case, final proof of the function of the X2 and X48 domains awaits biochemical examination, as has been clearly achieved for the SLH domain of the *C. thermocellum* anchoring proteins (Chauvaux et al., 1999; Lemaire et al., 1998).

Finally, two new scaffoldins have recently been sequenced from the rumen bacterium, *Ruminococcus flavefaciens* (Ding et al., 2001). Although each of the two proteins contains multiple cohesins, their sequences indicate that they are neither of type-I or type-II, but occupy their own phylogenetic branch. Interestingly, the ruminococcal scaffoldins lack a known type of CBD. Both have dissimilar X domains of unknown function, the sequences of which bear no resemblance to any other known module. Both X domains were expressed, but the resultant proteins failed to bind to cellulose. The lack of a scaffoldin CBD raises the question as to how the ruminococcal cellulosome(s) and/or the bacterium bind to the substrate. Perhaps it does so like another closely related species, *R. albus*, which binds cellulose via a noncellulosomal cell-surface protein (Pegden et al., 1998).

Schematic Comparison of Prokaryotic Cellulase Systems

In this section, we will describe schematically the similarity and diversity of representative enzyme systems, demonstrating different strategies, from

different plant cell wall degrading bacteria. It is emphasized that the accumulating information is based on what is known currently from biochemical data combined with gene sequencing and bioinformatics. The information is still rather sketchy but quite revealing when compared among different bacteria. As time progresses and the entire genomes of cellulolytic microorganisms become known, the data concerning the complement of enzymes produced by a given bacterium will be complete, and we will be able to speculate with heightened certainty how the various cellulase systems might have evolved. A survey of genes, however, does not inform us how a given bacterial system is regulated and what role(s) the bacterium and its enzyme system may play in nature. The explosive development of molecular biology techniques, however revealing, cannot supplant the fundamental contribution of biochemical and ecological approaches to the study of microbial degradation of cellulose and other plant cell wall polysaccharides.

Free Enzyme Systems

Many cellulolytic microorganisms show a very similar pattern in the types of enzymes that comprise the complement of their cellulase system. For the purposes of this discussion, the concept of "cellulase system" will include the complement of all plant cell wall hydolyzing enzymes and other glycosyl hydrolases, including the different cellulases per se, the hemicellulases (e.g., xylanases and mannanases), etc.

The cellulase system of the mesophilic cellulolytic aerobe, *Cellulomonas fimi*, is one of the first studied, and has since been one of the most studied bacterial cellulase systems (O'Neill et al., 1986; Shen et al., 1995; Whittle et al., 1982). The enzymes of this bacterium are essentially free enzymes, which allowed their early isolation and characterization. Moreover, the genes of the cellulases from this bacterium were of the earliest to have been sequenced. To date, about 10 glycosyl hydrolases have been sequenced from *Cellulomonas fimi*. Their modular composition and family associations are shown symbolically in Fig. 17. As an example of a free enzyme system, most of the enzymes bear a substrate-targeting CBM—in this bacterium, most of the CBMs are from family 2. Several of the enzymes have multiple copies of the fibronectin 3 (FN3) domain, the function of which is still unknown.

The *Cellulomonas* system includes two family-6 enzymes—an endoglucanase and an exoglucanase (cellobiohydrolase) of the types described in Fig. 4. The modularity of the endoglucanase is very simple, having the family-6 catalytic module together with a family-2 CBM. The cellobiohydrolase is a bit more complex with three additional FN3 domains that separate the same two types of modules. Another cellobiohydrolase (that exhibits processive cleavage of the substrate) is from family 48. Its general modular architecture is similar to that of the family-6 cellobiohydrolase with the substitution of the catalytic module from a different family. The cellulase system from this organism also includes two family-9 cellulases with modular themes B and D, familiar to us from the earlier description (Fig. 7). In addition, a simple family-5 cellulase and an interesting cell-borne family-26 mannanase are components of the system. The fact that an enzyme bears an SLH domain and is presumably cell-associated would underscore its importance to the cell. Finally, three xylanases are currently known for Cellulomonas fimi. One of these xylanases is a simple enzyme consisting of a family-10 catalytic domain connected to a family-2 CBM. The other two are more complicated, each containing two catalytic domains—either a family-10 or -11 domain and a carbohydrate esterase (in both cases, probably an acetyl xylan esterase; Fig. 3)—plus several CBMs. This rather complex system is probably not nearly complete, and more enzymes will inevitably be described in the future.

A second example of a free enzyme system, from the aerobic thermophilic bacterium *Thermobifida fusca* (formerly classified as *Thermomonospora fusca*), has also been studied extensively (Wilson, 1992; Wilson and Irwin, 1999). A brief comparison of its known enzyme components (Fig. 18) shows a striking resemblance to those of *Cellulomonas* (compare Figs. 17 and 18). According to known data, both species produce similar types of cellulases from families 5, 6, 9 and 48 plus xylanases from families 10 and 11. Nevertheless, the modular repertoire of the corresponding enzyme in *T. fusca* is generally somewhat simpler. For example, two of the *T. fusca* cellulases include single FN3 domains, whereas several *Cellulomonas* cellulases harbor multiple copies of the same domain. Some *T. fusca* enzymes lack accessory modules other than a cellulose-binding CBM, whereas the corresponding *Cellulomonas* enzyme is elaborated by multiple copies of accessory modules. In some cases though, the respective CBMs appear on opposite termini of the polypeptide chain (i.e., the family-48 and family-5 cellulases).

The complement of enzymes and their modular content of the free enzyme systems from *Cellulomonas* and *T. fusca* are not necessarily similar in other free enzyme systems. Many free enzyme systems, such as those of *Butyrivibrio fibrisolvens*, *Pseudomonas fluorescens*, *Fibro-*

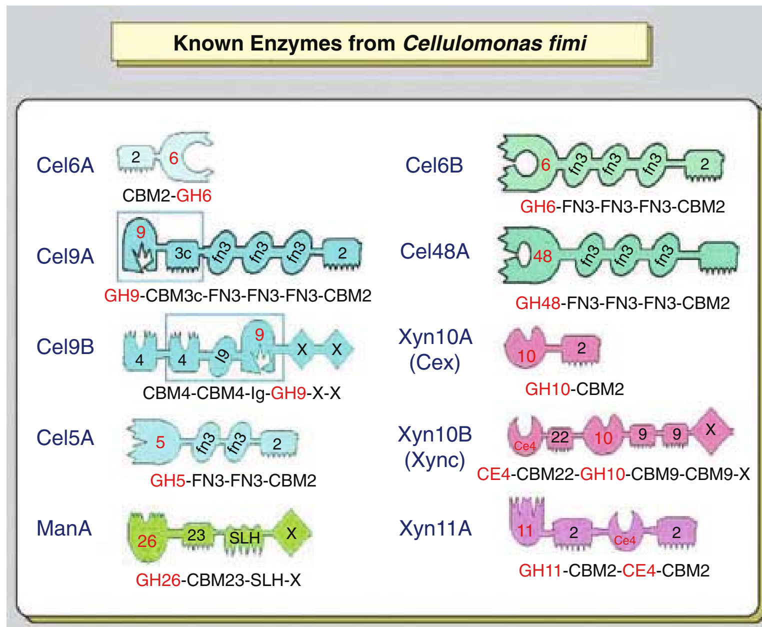

Fig. 17. *Cellulomonas fimi* cellulase system: Symbolic view of the enzyme components and their modular architecture. An example of a cell-free enzyme system. The modular content of the enzymes in this and subsequent figures is shown from (left to right) the *N*-terminus to the *C*-terminus of the polypeptide chain. The family numbers of the given domains are enumerated, the catalytic modules given in red. Key to symbols: GH, glycosyl hydrolase (e.g., cellulase, xylanase and mannanase); CE, carbohydrate esterase (e.g., acetyl xylan esterase and ferulic acid esterase); CBM, carbohydrate-binding module (e.g., CBD, cellulose-binding domain); SLH, S-layer homology (domain); FN3, fibronectin-3 (domain); Ig, immunoglobulin-like domain; and X, domain of unknown function.

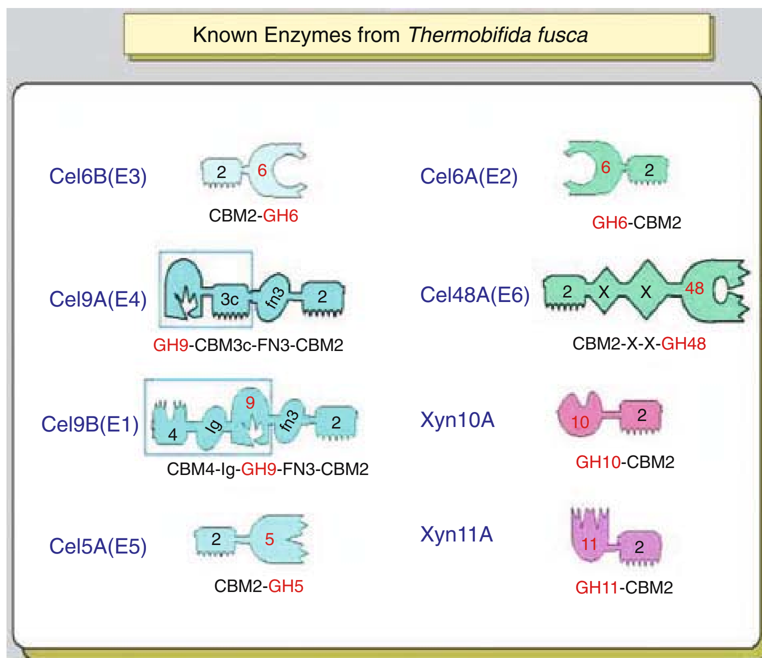

Fig. 18. *Thermobifida fusca* cellulase system. A cell-free enzyme system. The modular content of the enzymes is shown from (left to right) the *N*-terminus to the *C*-terminus of the polypeptide chain. Compare with the *Cellulomonas* system (Fig. 17). Key to symbols: GH, glycosyl hydrolase (e.g., cellulase, xylanase and mannanase); CBM, carbohydrate-binding module (e.g., CBD, cellulose-binding domain); FN3, fibronectin-3 (domain); Ig, immunoglobulin-like domain; and X, domain of unknown function.

bacter succinogenes, various species of *Streptomyces*, *Erwinia* and *Thermatoga*, appear to have several cellulases, xylanases and mannanases from the common families, together with other glycosyl hydrolases, e.g., arabinosidases, lichenases, amylases, pullulanases, galactanases, polygalacturonase, glucuronidases and pectate lyases. In many of these bacterial enzymes, the family-2 CBM appears to predominate as a common cellulose-binding domain, but in others (e.g., *Erwinia*) relevant enzymes usually bear a cellulose-binding CBM from family-3. Nevertheless, in many of the free systems, many enzymes are characterized by CBMs from other families as well as other noncatalytic domains of unknown function (X domains). Once again, until the genome sequences of cellulolytic prokaryotes are widely available, we are still lim-

ited in our capacity to compare among the enzyme systems because our knowledge of their enzyme sequences is incomplete.

Multifunctional Enzyme Systems

In an extremely thermophilic bacteria, classified as *Caldicellulosiruptor*, the enzymes currently characterized in this system also appear to be free enzymes, but their modular organization is of a higher order (Daniel et al., 1996; Gibbs et al., 2000; Reeves et al., 2000). Many of the enzymes of this system are bifunctional in that they contain two separate catalytic modules in the same polypeptide chain (Fig. 19). As mentioned earlier, the appearance of two catalytic modules in the same enzyme would infer a distinctive synergistic action between the two. Thus, in CelA, the family-9 and -48 catalytic modules would be expected to work in concerted fashion on crystalline cellulose. In another type of enzyme, the family-10 xylanase and family-5 cellulase would likely be most effective on regions of the plant cell wall that are characterized by cellulose-xylan junctions. The diversity in the modular architecture of the family-10 xylanases is particularly striking, and the various combinations of this type of catalytic module are apparently important to the sustenance of the bacterium in its environment. One of these xylanases appears to be attached to the cell surface via SLH domains. In contrast to the *Cellulomonas* and *T. fusca* enzymes that often harbor a family-2 CBM, the module responsible for binding to cellulosic substrates in *Caldicellulosiruptor* enzymes is usually one or more copies of a family-3 CBM.

Other bacterial strains that include at least one free bifunctional enzyme in their enzyme systems are *Anaerocellum thermophilum*, *Bacillus stearothermophilus*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Streptomyces chattanoogensis* and the thermophilic anaerobe NA10. Unlike the *Caldicellulosiruptor* system, most of the free bifunctional enzymes in the latter strains appear to be isolated cases in the given system, rather than being a common character of their enzymes.

Cellulosomal Systems

The inclusion of enzymes into a cellulosome via the noncatalytic scaffoldin subunit represents a higher level of organization. The association of complementary enzymes into a complex is considered to contribute sterically to their synergistic action on cellulose and other plant cell wall polysaccharides. As mentioned earlier, in the case of *Clostridium thermocellum*, *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens*, the cellulosomes appear to be attached to the cell surface. The cellulosomes of *C. cellulolyticum*, *C. cellulovorans* and *C. josui* may also be cell-associated, but if so, the lack of a scaffoldin-borne dockerin and reciprocal anchoring protein would suggest an alternative mechanism.

The cellulosomes of *C. cellulolyticum*, *C. cellulovorans* and *C. josui* are very similar. The genes encoding for many or most of the enzymes in all

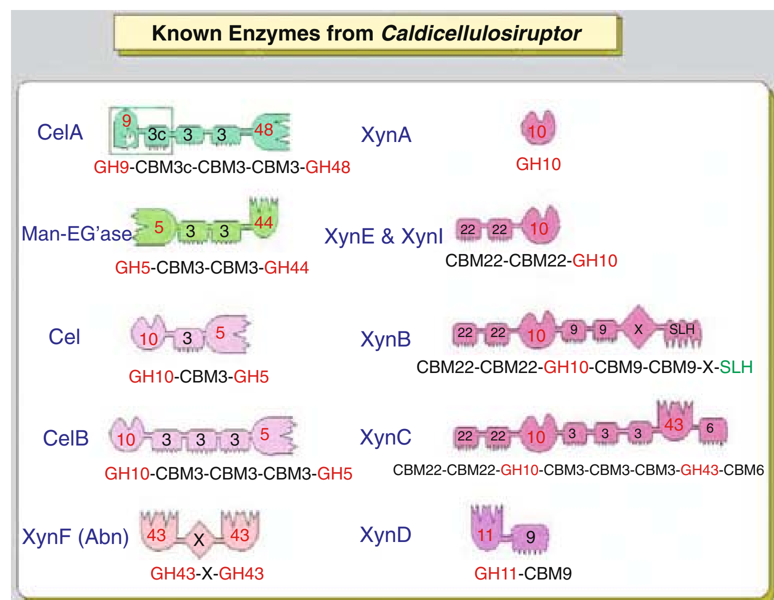

Fig. 19. Caldicellulosiruptor enzyme system. An example of a cell-free enzyme system that includes several multifunctional enzymes. The modular content of the enzymes is shown from (left to right) the *N*-terminus to the *C*-terminus of the polypeptide chain. Key to symbols: GH, glycosyl hydrolase (e.g., cellulase, xylanase and mannanase); CBM, carbohydrate-binding module (e.g., CBD, cellulose-binding domain); and SLH, S-layer homology (domain).

three cellulosomal systems are arranged in a large cluster on the chromosome. Some of the cellulosomal genes, however, are located outside of the cluster in other regions of the chromosome. The majority of the cellulosome gene clusters from *C. cellulolyticum* and *C. cellulovorans* have been sequenced (Bagnara-Tardif et al., 1992; Belaich et al., 1999; Tamaru et al., 2000b). In contrast, the cellulosomal genes from *C. thermocellum* are generally scattered over a large portion of the chromosome (Guglielmi and Béguin, 1998). A few small clusters of cellulosomal genes are apparent in the genome, including a scaffoldin-containing cluster that also contains several cell-surface anchoring proteins (Fujino et al., 1993). The following descriptive analysis serves to compare the cellulosomal system of these three microorganisms.

Cellulosomal components from *Clostridium cellulolyticum*. All of the sequenced enzymes from this organism are relatively common cellulases (Belaich et al., 1999). None of the known cellulosomal enzymes yet described for this species contains more than one catalytic module (Fig. 20). The largest one, CelE (estimated at 94 kDa), is a theme-D family-9 cellulase (Gaudin et al., 2000). The critical family-48 cellulase (CelF) is also a major cellulosome component (Reverbel-Leroy et al., 1997). Interestingly, the gene cluster of *C. cellulolyticum* contains three copies of other family-9 cellulases (CelG, CelH and CelJ), all of which contain the theme-B fused family-3c CBM (Belaich et al., 1998; Fig. 8). The currently known cellulosome system in this bacterium also contains two family-5 cellulases (CelA and CelD), a family-5 mannanase (ManK, which bears an *N*-terminal rather than *C*-terminal dockerin) and a family-8 cellulase (CelC).

Biochemical characterization of the *C. cellulolyticum* cellulosome demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) a 160-kDa scaffoldin band and up to 16 smaller bands, representing putative enzyme subunits (Gal et al., 1997). Many of these were clearly identified as known gene products. Only two cellulosomal cellulase genes are currently known to be located outside of the gene cluster. Further work on the enzyme system of this species may yet provide more complicated multimodular enzymes and/or other types of enzymes, such as hemicellulases. In this context, recent biochemical evidence has suggested that xylanases from *C. cellulolyticum* are also organized in a cellulosome-like complex, but defined xylanase sequences are still lacking from this organism (Mohand-Oussaid et al., 1999). The known activity of this organism on other plant cell wall polysaccharides would indicate that numerous other enzymes, either cellulosomal or not, remain as yet undiscovered.

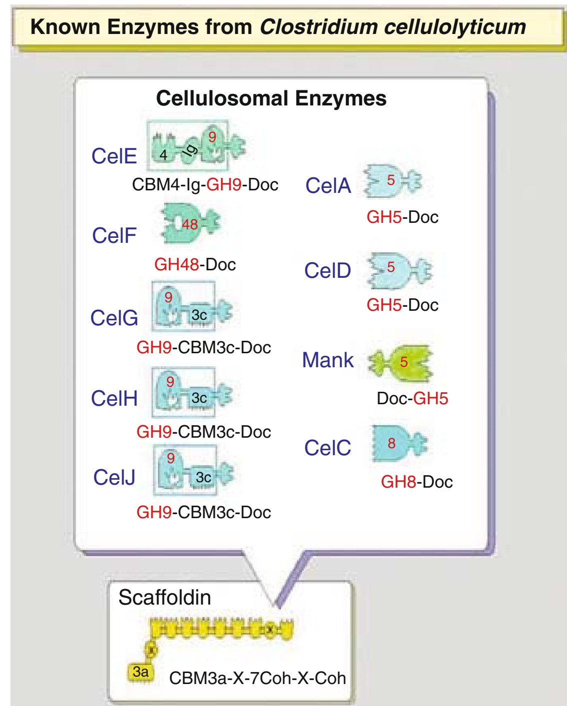

Fig. 20. *Clostridium cellulolyticum* enzyme system. An example of a cellulosomal system. The modular content of the enzymes is shown from (left to right) the *N*-terminus to the *C*-terminus of the polypeptide chain. Key to symbols: GH, glycosyl hydrolase (e.g., cellulase, xylanase and mannanase); CBM, carbohydrate-binding module (e.g., CBD, cellulose-binding domain); and Doc, dockerin domain.

Cellulosomal components from *Clostridium cellulovorans*. Like *C. cellulolyticum*, the cellulases from this organism are relatively simple (Fig. 21). In addition to the cellulosomal enzymes thus described, at least three non-cellulosomal endoglucanases have also been partially or totally sequenced (Doi et al., 1998; Tamaru et al., 1999b).

Several of the cellulosomal enzymes are architecturally synonymous to those of the *C. cellulolyticum* system (compare Figs. 20 and 21). This includes the critical family-48 cellulase (ExgS; Liu and Doi, 1998), two copies of the theme-B family-9 cellulase (EngH and EngY), a family-5 endoglucanase and a family-5 mannanase that bears an N-terminal dockerin (Tamaru and Doi, 2000a). Rather than a single theme-D family-9 cellulase as in *C. cellulolyticum*, the *C. cellulovorans* system contains two such enzymes (EngK and EngM). The *C. cellulovorans* cellulosome also appears to contain an unusual theme-A

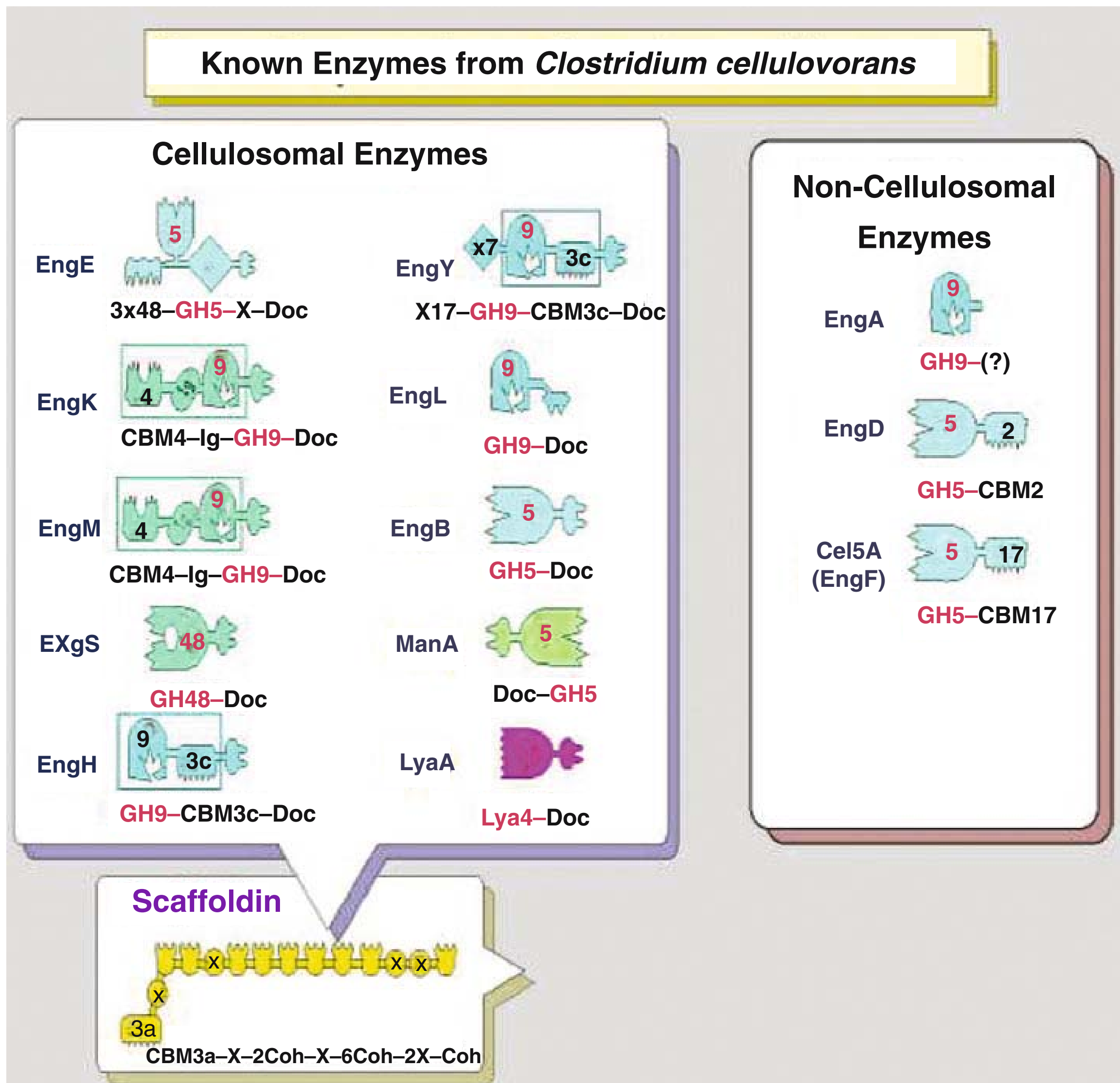

Fig. 21. *Clostridium cellulovorans*: A second cellulosomal system. The modular content of the enzymes is shown from (left to right) the *N*-terminus to the *C*-terminus of the polypeptide chain. Key to symbols: GH, glycosyl hydrolase (e.g., cellulase, xylanase and mannanase); CBM, carbohydrate-binding module (e.g., CBD, cellulose-binding domain); Doc, dockerin domain; SLH, S-layer homology (domain); Ig, immunoglobulin-like domain; and X, domain of unknown function.

family-9 cellulase (EngL) that lacks helper domains. The remaining two known cellulosomal enzymes are thus far unique to *C. cellulovorans*. A dockerin-bearing pectate lyase (LyaA) infers that the bacterium would degrade pectin (Tamaru and Doi, 2001). Indeed, early evidence (Sleat et al., 1984) indicated that, in addition to cellulose, *C. cellulovorans* is capable of assimilating a wide variety of other plant cell wall polysaccharides, including, xylans, pectins and mannans. As in the case of *C. celluolyticum*, it seems that future work will yield new sequences of many other types of cellulosomal and noncellulosomal enzymes.

More significant to the cellulosomal system of *C. cellulovorans*, perhaps, is the large family-5 enzyme that purportedly comprises both an *N*-terminal SLH domain and a *C*-terminal dockerin (Tamaru and Doi, 1999a). This arrangement may imply that the entire cellulosome is bound to the cell surface via this enzyme. If this proves to be the case, it is interesting to speculate whether the *C. cellulolyticum* and *C. josui* cellulosomes are also connected to the cell surface by a similar, but as yet undiscovered enzyme that bears both SLH and dockerin domains.

Cellulosomal components from Clostridium thermocellum. Compared to the cellulosomal

systems of *C. cellulovorans* and *C. cellulolyticum*, the enzymes from *C. thermocellum* are relatively large proteins, ranging in molecular size from about 40–180 kDa (Bayer et al., 1998; Bayer et al., 2000; Béguin and Lemaire, 1996; Felix and Ljungdahl, 1993; Lamed and Bayer, 1988; Shoham et al., 1999). Examination of Fig. 22 reveals why these enzymes are so big—many of the larger ones contain multiple types of catalytic domains as well as other functional modules as an integral part of a single polypeptide chain (see Table I in Bayer et al., 1998, for a list of relevant references). In addition to the cellulosomal enzymes, several noncellulosomal enzymes have also been described from this organism (Morag et al., 1990). These include two free enzymes (one of which lacks a CBM) and two cell-associated (SLH-containing) enzymes. Consequently, the potent cellulose- and plant cell wall-degrading activities of *C. thermocellum* are clearly reflected in its cellulase system, which displays an exceptional wealth, diversity and intricacy of enzymatic components, thus representing the premier cellulose-degrading organism currently known.

Many of the *C. thermocellum* cellulosomal enzymes are cellulases, which include both endo- and exo-acting β-glucanases. Some of the important exoglucanases and processive cellulases include CelS, CbhA, CelK and CelF. The CelS subunit is a member of the family-48 glycosyl hydrolases, and this particular family is now recognized as a critical component of bacterial cellulosomes (Morag et al., 1991; Morag et al., 1993; Wang et al., 1993; Wang et al., 1994; Wu et al., 1988). Several other processive cellulases are members of the family-9 glycosyl hydrolases. CelF and CelN are theme-B family-9 enzymes (Navarro et al., 1991; Fig. 7). The other two are remarkably similar theme-D enzymes, which exhibit nearly 95% similarity along their common regions (Kataeva et al., 1999a; Kataeva et al., 1999b; Zverlov et al., 1998; Zverlov et al., 1999). The main difference between CbhA and CelK is the presence in the former of three extra modules (a family-3 CBD and two modules of unknown function). The functional significance of these supplementary modules to the activity of CbhA has not been elucidated.

The fact that the cellulosome from this organism contains many different types of cellulases is, of course, to be expected if we consider that growth of *C. thermocellum* is restricted to cellulose and its breakdown products, particularly cellobiose. Consequently, it is surprising to discover, in addition to the cellulases, at least five classic xylanases, i.e., those belonging to glycosyl hydrolase families 10 and 11. In addition, two of the larger enzymes, CelH and CelJ, contain hemicellulase components, i.e., family-26 and -44 catalytic modules (a mannanase and a xylanase, respectively), together with a standard cellulase module in the same polypeptide chain (Ahsan et al., 1996; Yagüe et al., 1990). It is also interesting to note the presence of carbohydrate esterases together with xylanase or cellulase modules in some of the enzyme subunits (i.e., XynU/A, XynY, XynZ and CelE), thus conferring the capacity to hydrolyze acetyl or feruloyl groups from hemicellulose substrates (Blum et al., 2000; Fernandes et al., 1999). Finally, the *C. thermocellum* cellulosome includes a typical family-16 lichenase, a family-26 mannanase and a family-18 chitinase.

The non-cellulosomal enzymes include another theme-B family-9 cellulase (CelI), and cell-bound forms of a xylanase (XynX) and a lichenase (LicA), both of which contain multiple CBMs adjacent to the catalytic module. In the midst of all this complexity, the *C. thermocellum* non-cellulosomal cellulase system includes a simple family-5 cellulase, CelC, which is completely devoid of additional accessory modules.

Why does this bacterium—which subsists exclusively on cellulosic substrates—need all these hemicellulases? The inclusion of such an impressive array of non-cellulolytic enzymes in a strict cellulose-utilizing species would suggest that their major purpose would be to collectively purge the unwanted polysaccharides from the milieu and to expose the preferred substrate—cellulose. The ferulic acid esterases, in concert with the xylanase components of the parent enzymes, could grant the bacterium a relatively simple mechanism by which it could detach the lignin component from the cellulose-hemicellulose composite. The lichenase (LicB) and chitinase (ChiA) are also intriguing components of the cellulosome. The former would provide the bacterium with added action on cell-wall β-glucan components from certain types of plant matter. It is not clear whether the presence of the latter cellulosomal enzyme would reflect chitin-derived substrates from the exoskeletons of insects and/or from fungal cell walls. Whatever the source, the chitin breakdown products, like those of the hemicelluloses, would presumably not be utilized by the bacterium itself, but would be passed on to appropriate satellite bacteria for subsequent assimilation.

Phylogenetics of Cellulase and Cellulosomal Systems

Early in the history of the development and establishment of the cellulosome concept, it was noted that the apparent occurrence of cellulo-

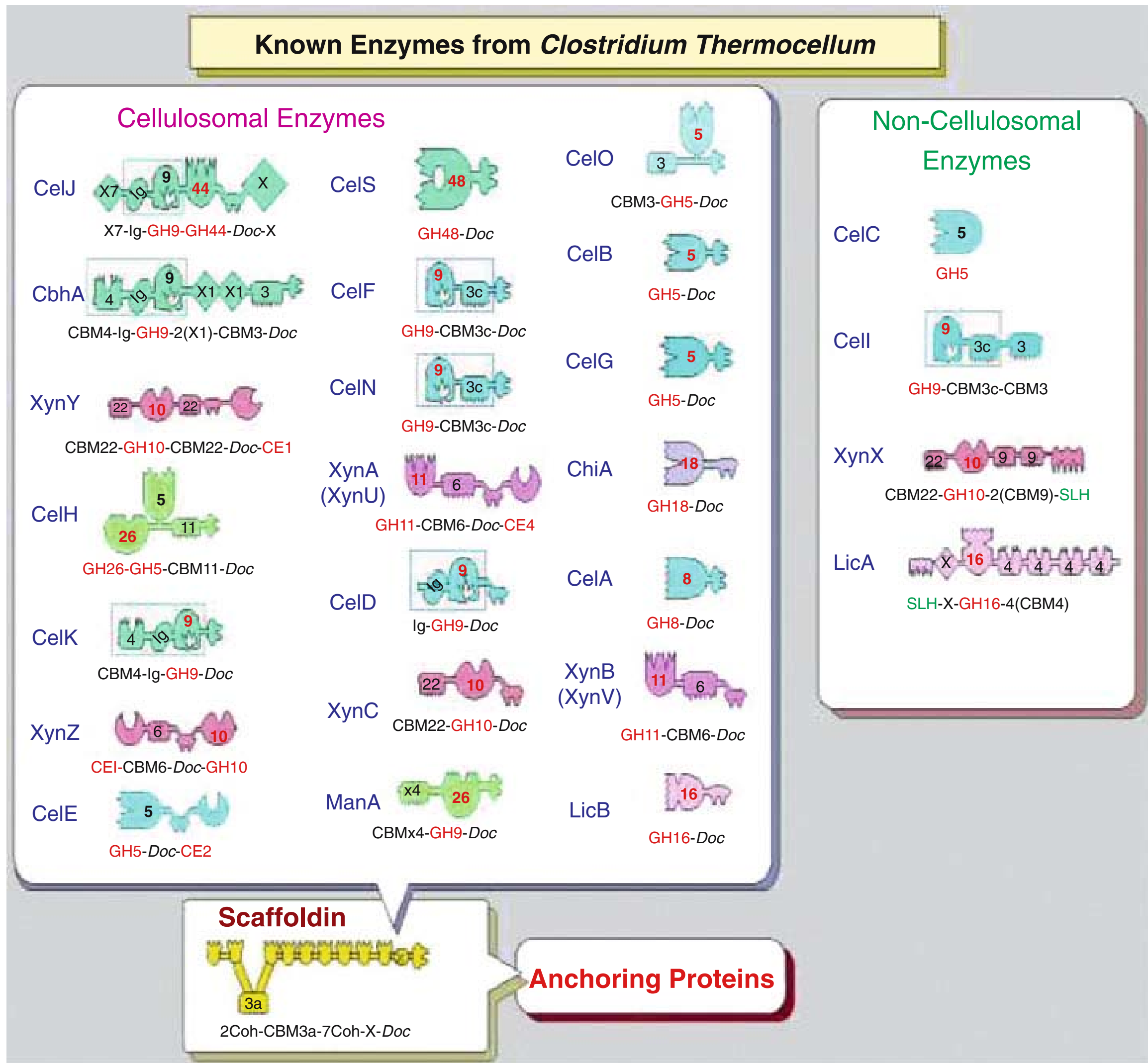

Fig. 22. *Clostridium thermocellum*: A very complex cellulosomal system. The modular content of the enzymes is shown from (left to right) the *N*-terminus to the *C*-terminus of the polypeptide chain. Key to symbols: GH, glycosyl hydrolase (e.g., cellulase, xylanase and mannanase); CE, carbohydrate esterase (e.g., acetyl xylan esterase and ferulic acid esterase); CBM, carbohydrate-binding module (e.g., CBD, cellulose-binding domain); Doc, dockerin domain; SLH, S-layer homology (domain); Ig, immunoglobulin-like domain; and X, domain of unknown function.

somes in different microorganisms tended to cross ecological, physiological and evolutionary boundaries (Lamed et al., 1987). Initial biochemical and immunochemical evidence to this effect has been supported by the accumulated molecular biological studies.

Various lines of evidence indicate that the modular enzymes that degrade plant cell wall polysaccharides have evolved from a restricted number of common ancestral sequences. Much of the information in this direction remains as a legacy, inherently encoded in the sequences of the functional domains that comprise the different enzymes. By comparing sequences of the various cellulosomal and noncellulosomal enzymes within and among the different strains, we can gain insight into the evolutionary rationale of the multigene families that comprise the glycosyl hydrolases.

Horizontal Gene Transfer

It is clear that very similar enzymes which comprise a given glycosyl hydrolase family are prevalent among a variety of different bacteria and fungi, thus indicating that they were not inherited through conventional evolutionary processes. The widespread occurrence of such conserved enzymes among phylogenetically different species argues that horizontal transfer of

genes has been a major process by which a given microorganism can acquire a desirable enzyme. Once such a transfer event has taken place, the newly acquired gene would then be subjected to environmental pressures of its new surroundings, i.e., the genetic and physiological constitution of the cell itself. Following such selective pressure, the sequence of the gene would be adjusted to fit the host cell.

Gene Duplication

Sequence comparisons have also revealed the presence of very similar genes within a genome that may have very similar or even identical functions. One striking example is the tandem appearance of *cbhA* and *celK* genes in the chromosome of *Clostridium thermocellum*. Other examples are *xynA* and *xynB* also of *C. thermocellum* and *xynA* of the anaerobic fungus *Neocallimastix patriciarum*, which includes two very similar copies of family-11 catalytic modules within the same polypeptide chain. These examples imply a mechanism of gene duplication (Chen et al., 1998; Gilbert et al., 1992), whereby the duplicated gene can serve as a template for secondary modifications that could result in two very similar enzymes with different properties, such as substrate and product specificities. A similar process could also account for the multiplicity of other types of modules (i.e., CBDs, cohesins or helper modules) within a polypeptide chain. Comparison of the modular architectures of similar genes from different species would suggest that individual modules can undergo a duplication process. This is exemplified by the multiple copies of FN3 in CelB from *Cellulomonas fimi* versus the single copy of the same domain in cellulase E4 from *Thermobifida fusca*. But innumerable other examples are evident from the databases, whenever multiple copies of the same modular type exist in the same protein.

Domain Shuffling

Another observation from the genetic composition of the glycosyl hydrolases argues for an alternative type of process, which would propagate new or modified types of enzymes. It is clear that many microbial enzyme systems contain individual hydrolases that carry very similar catalytic domains but include different types of accessory modules (Gilkes et al., 1991). An example that demonstrates this phenomenon is the observed species preference of otherwise very similar glycosyl hydrolases for a given family of crystalline cellulose-binding CBD, which is entirely independent of the type of catalytic module borne by the complete enzyme. In this context, as we have seen above, the free enzymes of some bacteria, such as *Cellulomonas fimi*, *Pseudomonas fluorescens* and *Thermomonospora fusca*, invariably include a family-2 CBD, irrespective of the type of catalytic domain. In contrast, those of other bacteria, e.g., *Bacillus subtilis*, *Caldocellum saccharolyticum*, *Erwinia carotovora* and various clostridia, appear to prefer family-3 CBDs. Moreover, the position of the CBD in the gene may be different for different genes. For example, the CBD may occur upstream or downstream from the catalytic domain; it may be positioned either internally (sandwiched between two other modules) or at one of the termini of the polypeptide chain. The same pattern is characteristic of several other kinds of modules associated with the plant cell wall hydrolases. This is particularly evident in family-9 cellulases and family-10 xylanases, where the number and types of accessory modules may vary greatly within a given species. It seems that individual domains can be transferred en bloc and incorporated independently into appropriate enzymes. Once again, the modular architectures and sequence similarities between *Clostridium thermocellum* cellulosomal enzyme pairs (CbhA and CelK; XynA and XynB) are particularly revealing: in both cases, following an apparent gene duplication event, one or more additional modules appear to have been incorporated into the duplicated enzyme. Taken together, the information suggests that domain shuffling is an important process by which the properties of such enzymes can be modified and extended.

Proposed Mechanisms for Acquiring Cellulase and Cellulosomal Genes

Like the free enzyme systems, the phylogeny of cellulosomal components seems to have been driven by processes that include horizontal gene transfer, gene duplication and domain shuffling. In cellulolytic/hemicellulolytic ecosystems, the resident microorganisms are usually in close contact, often under difficult conditions and in competition or cooperation with one another toward a common goal: the rapid degradation of recalcitrant polysaccharides and assimilation of their breakdown products.

A possible scenario for the molecular evolution of a cellulase/hemicellulase system in a prospective bacterium could involve the initial transfer of genetic material from one microbe to another in the same ecosystem. The size and type of transferred material could vary, such as a gene or part of gene (e.g., selected functional modules) or even all or part of a gene cluster. The process could then be sustained by gene duplica-

tion, which would propagate the insertion of repeated modules, e.g., the multiple cohesin domains in the scaffoldins, or even smaller units, such as the linker sequences or the duplicated calcium-binding loop of the dockerin domain. Domain shuffling can account for the observed permutations in the arrangement of domains in scaffoldin subunits from different species (Fig. 16). Finally, conventional mutagenesis would then render such products more suitable for the cellular environment or for interaction with other components of the cellulase system.

The available data suggest that there are no set of rules, which would, at this stage, enable us to anticipate the nature of a given cellulase system from a given microorganism. It seems that phylogenetically dissimilar organisms can possess similar types of cellulosomal or non-cellulosomal enzyme systems, whereas phylogenetically related organisms that inhabit similar niches may be characterized by different types of enzyme systems. It is clear that to shed further light on this apparent enigma, we require more information about more types of enzyme systems. In addition to more sequences and structures, we will need more information—biochemical, physiological and ecological—to sharpen existing notions regarding the enzymatic degradation of plant cell wall polysaccharides or to formulate new ones.

Acknowledgments. Grants from the Israel Science Foundation (administered by the Israel Academy of Sciences and Humanities, Jerusalem) and the Minerva Foundation (Germany) are sincerely appreciated.

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Prokaryotes (2006) 2:618–634
DOI: 10.1007/0-387-30742-7_20

CHAPTER 1.20

Aerobic Methylotrophic Prokaryotes

MARY E. LIDSTROM

Introduction

Methylotrophic bacteria are those organisms with the ability to utilize (as their sole source of carbon and energy) reduced carbon substrates with no carbon-carbon bonds. By this definition the group includes bacteria that can grow on substrates such as methane, methanol, methylated amines, halogenated methanes and methylated sulfur species. Methylotrophic bacteria are quite widespread in nature, being found in a variety of aquatic and terrestrial habitats (King, 1992). They appear to play an important role in the cycling of carbon in specific habitats (King, 1992), and they comprise the principal biological sink for methane and other methylated greenhouse gases, highlighting an important role in global warming (King, 1992; Oremland and Culbertson, 1992). Although many anaerobic methylotrophic bacteria are known, especially among the methanogens, this chapter will cover only the aerobic and facultatively anaerobic methylotrophs (for convenience, termed "aerobic methylotrophs"). Table 1 lists the major groups of aerobic methylotrophs with examples of the genera that have been described to contain methylotrophs.

Aerobic methylotrophic bacteria are phylogenetically diverse, with representatives found among the Proteobacteria as well as the high and low G+C Gram-positive bacteria (Firmicutes; Table 1). Many of the known strains of methylotrophic bacteria are obligately methylotrophic species, that is, they are incapable of growing on any compounds containing carbon-carbon bonds. However, especially among the group of bacteria that grow on methanol, a variety of facultative organisms are known that can grow either on multi-carbon compounds or on one-carbon (C_1) compounds (Table 1). Two functional groups of methylotrophs may be distinguished: those capable of growth on methane, called "methanotrophs," and those capable of growth on methanol and/or other methylated compounds but not on methane. The methanotrophs are characterized by the presence of internal membrane systems (Hanson and Hanson, 1996). Many but not all of the methylotrophs also can use N_2 as a nitrogen source and therefore are considered to be diazotrophs (Table 1). In addition, several of the methylotrophs also affect the nitrogen cycle by carrying out transformations of ammonia and nitrate (Anthony, 1982). Some methylotrophs are known that can use methylated sulfur species, and these appear to play an important role in sulfur cycling (DeBont et al., 1981; Kelly and Murrell, 1999). A number of methylotrophs can grow on halogenated methanes (Leisinger and Braus-Stromeyer, 1995) and have the potential to play an important role in the detoxification of these pollutants.

The ability to grow on reduced C_1 compounds requires the presence of unique biochemical pathways for both energy and carbon metabolism. So far, a limited number of variations of these metabolic pathways are known. Figure 1 gives an outline of methylotrophic metabolism, showing how different methylotrophic substrates are fed into central metabolic pathways. A key feature of aerobic methylotrophy is the role of formaldehyde as a central intermediate. In most methylotrophs, the pool of formaldehyde generated from methylotrophic substrates is split, with part being oxidized to CO_2 for energy and part being assimilated into cell carbon via one of two unique pathways, the serine cycle or the ribulose monophosphate cycle. Other methylotrophs, sometimes called "pseudomethylotrophs" or "autotrophic methylotrophs" (Anthony, 1982), are capable of growth on reduced C_1 compounds by oxidizing these compounds to CO_2 and then assimilating the CO_2 via the classical Calvin-Benson-Bassham cycle. The diagram shown in Fig. 1 is an amalgam of the known diversity of methylotrophic metabolism, and no single methylotroph can carry out all of these types of metabolism. In fact, the major phylogenetic divisions mirror distinct physiological classes. For instance, all of the known methylotrophs containing the serine cycle for formaldehyde assimilation are clustered in the α-Proteobacteria, all of the restricted obligate methylotrophs that do not use methane are clustered in the β-

Table 1. Characteristics of aerobic methylotrophic bacteria.

Group	Major assimilation pathway	N_2 fixing	Phylogenetic position[a]	References
Obligate methylotrophs				
Type I methanotrophs				
Methylomonas	RuMP	Yes	γ-Proteobacteria	Anthony, 1982
Methylobacter	RuMP	Yes	γ-Proteobacteria	Anthony, 1982
Methylococcus	RuMP	Yes	γ-Proteobacteria	Anthony, 1982
Methylomicrobium	RuMP	No	γ-Proteobacteria	Bowman et al., 1995
Methylosphaera	RuMP	No	γ-Proteobacteria	Bowman et al., 1997
Methylocaldum	RuMP	No	γ-Proteobacteria	Bodrossy et al., 1997
Type II methanotrophs				
Methylosinus	Serine	Yes	α-Proteobacteria	Anthony, 1982
Methylocystis	Serine	Yes	α-Proteobacteria	Anthony, 1982
Methylocella	Serine	Yes	α-Proteobacteria	Dedysh et al., 2000
Restricted facultative methylotrophs				
Methanol utilizers				
Hyphomicrobium	Serine	No	α-Proteobacteria	Harder and Attwood, 1978; Stackebrandt et al., 1988
Methylophilus	RuMP	No	β-Proteobacteria	Jenkins and Jones, 1987
Methylobacillus	RuMP	No	β-Proteobacteria	Bratina et al., 1992
Methylophaga	RuMP	No	γ-Proteobacteria	Janvier and Grimont, 1995
Facultative methylotrophs				
Methylobacterium (*Pseudomonas*)	Serine	No	α-Proteobacteria	Green and Bousfield, 1983
Aminobacter	Serine	No	α-Proteobacteria	Urakami et al., 1992
Methylorhabdus	Serine	No	α-Proteobacteria	Doronina et al., 1995
Methylopila	Serine	No	α-Proteobacteria	Doronina et al., 1998
Methylosulfonomonas	Serine	No	α-Proteobacteria	Holmes et al., 1997
Marinosulfonomonas	Serine	No	α-Proteobacteria	Holmes et al., 1997
Paracoccus	CBB	No	α-Proteobacteria	Anthony, 1982
Xanthobacter	CBB	Yes	α-Proteobacteria	Jenni et al., 1987
Ancylobacter (*Microcyclus*)	CBB	Yes	α-Proteobacteria	Raj, 1989
Thiobacillus	CBB	No	α-Proteobacteria	Chandra and Shethna, 1977
Rhodopseudomonas	CBB	No	α-Proteobacteria	Anthony, 1982
Rhodobacter	CBB	No	α-Proteobacteria	Anthony, 1982
Acetobacter	RuMP	ND	γ-Proteobacteria	Yamada et al., 1997
Bacillus	RuMP	ND	Gram-positive (low G+C)	Dijkhuizen et al., 1988
Mycobacterium	RuMP	ND	Gram-positive (high G+C)	Reed and Dugan, 1987
Arthrobacter	RuMP	ND	Gram-positive (high G+C)	Levering et al., 1981
Amycolatopsis (*Nocardia*)	RuMP	ND	Gram-positive (high G+C)	De Boer et al., 1990

Abbreviations: RuMP, ribulose monophosphate; CBB, Calvin-Benson-Bassham; and ND, no data.
[a]Many phylogenetic affiliations can be found at the Ribosomal Database Project (http://www.cme.msu.edu/RDP/html/index.html) or National Center for Biotechnology Information websites and in Bratina et al. (1992).

Proteobacteria (with one exception, *Methylophaga*), all of the methanotrophs that use the ribulose monophosphate cycle for formaldehyde assimilation are clustered in the γ-Proteobacteria, and all of the known Gram-positive methylotrophs contain the ribulose monophosphate cycle (Table 1). For the methanotrophs, the α-Proteobacteria containing the serine cycle are referred to as type I strains, whereas the γ-Proteobacteria containing the ribulose monophosphate cycle are referred to as type II strains (Hanson and Hanson, 1996). Because the natural diversity of methylotrophs is still under investigation, the current clustering of phylogenetic

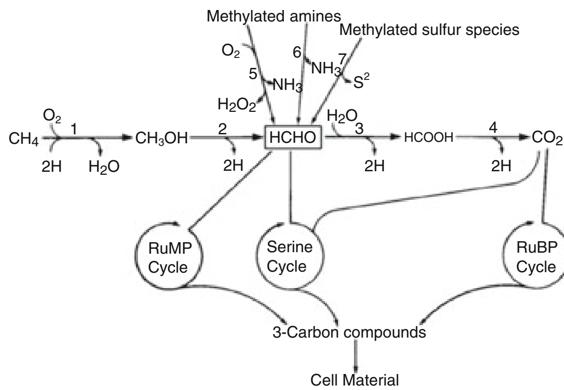

Fig. 1. Metabolism of one-carbon compounds in aerobic methylotrophic bacteria. 1, methane monooxygenase; 2, methanol dehydrogenase; 3, formaldehyde oxidation system; 4, formate dehydrogenase; 5, halomethane oxidation system; 6, methylated amine oxidases; 7, methylated amine dehydrogenase; and 8, methylated sulfur dehydrogenase or oxidase. RuMP is ribulose monophosphate, and CBB is Calvin-Benson-Bassham. Adapted from Anthony (Anthony, 1982; Anthony, 1996) and DeBont et al. (1981).

and physiological groups may not hold up, as new strains are identified and characterized.

Dissimilatory Metabolism

Aerobic methylotrophs contain specialized pathways for dissimilatory metabolism during methylotrophic growth. In general, the methyl groups of methylotrophic substrates are oxidized to the level of formaldehyde by oxidases and/or dehydrogenases. The dehydrogenases are generally coupled to energy metabolism at the level of cytochromes and the oxidases are usually non-energy conserving. Formaldehyde is then further oxidized to the formyl level by one of a number of formaldehyde oxidation systems, which usually generate a reduced pyridine nucleotide. Carbon at the level of formate is then oxidized to CO_2 via another pyridine nucleotide-linked step.

Methane Oxidation

The enzyme that oxidizes methane to methanol in the methanotrophic bacteria is a mixed-function oxidase called "methane monooxygenase" (MMO; Fig. 1). Two different enzymes are known, a membrane-bound form, known as "the particulate MMO" (pMMO), and a soluble form, called "the soluble MMO" (sMMO; Hanson and Hanson, 1996). The soluble MMO has so far been documented in only a few strains and it is not yet known how widely distributed it is in methanotrophs. It has been found in all tested strains of *Methylosinus* and *Methylococcus*, and in a few strains of *Methylomonas Methylomicrobium* and *Methylocystis* (Hanson and Hanson, 1996; Fuse et al., 1998; Shigematsu et al., 1999; Grosse, 1999). However, pMMO appears to be present in all known strains of methanotrophs.

The sMMO has been purified from both type I and type II methanotrophs (Lipscomb, 1994), and it is similar in all cases. It consists of three components: a hydroxylase (consisting of three polypeptides and a non-heme iron center), component B (with no cofactors), and a reductase that contains FAD and and an Fe_2S_2 cluster (Lipscomb, 1994). The sMMO uses NADH as a source of reducing power, and contains an hydroxo-bridged di-iron center in its active site (Lipscomb, 1994). It is characterized by an extremely broad substrate specificity, being able to oxidize or hydroxylate a wide variety of aliphatic straight chain, branched, aromatic, and halogenated hydrocarbons (Lipscomb, 1994; Hanson and Hanson, 1996). The broad substrate range of this enzyme has attracted a great deal of attention as a result of the use of methanotrophs for bioremediation of a variety of toxic hydrocarbons (Hanson and Hanson, 1996). Crystal structures are available for the hydroxylase and component B from two different methanotrophs (Walters et al., 1999; Elango et al., 1997; Chang et al., 1999; Rosenzweig et al., 1997). Genes for the subunits of the sMMO (*mmo* genes) have been cloned and sequenced from a number of methanotrophs, and they have a similar organization with high similarity at the amino acid level (Murrell, 1994; Shigematsu et al., 1999; McDonald et al., 1997).

The pMMO is highly unstable and has proven more difficult to analyze. However, recently, pMMO was purified in an active state from *Methylococcus capsulatus* Bath by two groups (Zahn and DiSpirito, 1996; Nguyen et al., 1998). In both cases, the pMMO had 3 subunits, PmoABC, of approximately 27, 45 and 22 kDa, respectively, and was a copper-containing enzyme. In one case no other metals were present (Nguyen et al., 1998), whereas in the other iron was also present (Zahn and DiSpirito, 1996). The pMMO has a narrower substrate range than the sMMO (Hanson and Hanson, 1996). The genes encoding the pMMO are present in multiple copies in most methanotrophs (Semrau et al., 1995). In both a type I and type II methanotroph, evidence exists that the copies are nearly identical in sequence (Stolyar et al., 1999; Gilbert et al., 2000), and mutant evidence has shown that in *Methylococcus capsulatus* Bath the copies are functionally redundant (Stolyar et al., 1999).

In methanotrophs containing both pMMO and sMMO, the expression of each enzyme is regulated by copper. In copper sufficiency, pMMO is expressed, and in copper limitation,

sMMO is expressed. In *Methylococcus capsulatus* Bath and *Methylosinus trichosporium*, it has been shown that this regulation occurs at the transcriptional level (Nielsen et al., 1997).

Methanol Oxidation

Methanol is widespread, produced in nature as a result of demethylation reactions (Anthony, 1982), especially from plants (Holland and Polacco, 1994). Methanol is oxidized to formaldehyde by three classes of enzymes, a quinoprotein methanol dehydrogenase (MDH) found in the Gram-negative methylotrophs (Goodwin and Anthony, 1998), an NAD-linked enzyme found in the *Bacillus* strains (Arfman et al., 1997), and a methanol:*N*,*N'*-dimethyl-4-nitrosoaniline oxidoreductase (MNO) found in other Gram-positive strains (Bystrykh et al., 1993; Bystrykh et al., 1997). In general, methanol oxidation is an energy-conserving step, either generating reduced cytochromes or reduced pyridine nucleotides.

Quinoprotein Methanol Dehydrogenase

All of the known Gram-negative methanol- and methane-utilizing bacteria contain a periplasmic enzyme for oxidizing methanol called "methanol dehydrogenase." This enzyme, which oxidizes primary alcohols to their corresponding aldehydes, has an $\alpha_2\beta_2$ structure and contains the cofactor pyrroloquinoline quinone (PQQ; Goodwin and Anthony, 1998). Electrons from the oxidation of PQQ are transferred from PQQ to a specific cytochrome *c*, and from there through other carriers to the terminal oxidase (Goodwin and Anthony, 1998). The primary sequences and structures for methanol dehydrogenase from diverse methylotrophs are highly conserved (Goodwin and Anthony, 1998). These enzymes contain a Ca^{2+} near the active site and also have an unusual disulfide bridge in the same region (Goodwin and Anthony, 1998; Anthony and Ghosh, 1998).

NAD-Linked Methanol Dehydrogenases

An NAD-linked methanol dehydrogenase has been purified and characterized from methylotrophic *Bacillus* strains (Arfman et al., 1997). This enzyme oxidizes C_1–C_4 primary alcohols, and is composed of ten identical 43,000-Mr subunits. Each MDH subunit contains a tightly, but noncovalently bound NAD(H) molecule, in addition to 1 Zn^{2+} and 1 or 2 Mg^{2+} ions. This MDH also interacts with a 50,000-Mr activator protein, which appears to facilitate the oxidation of the reduced NADH cofactor of MDH (Arfman et al., 1997). The structural gene for this MDH shows identity with type II alcohol dehydrogenases (de Vries et al., 1992).

Methanol:N,N′-dimethyl-4-nitrosoaniline Oxidoreductase (MNO)

Other Gram-positive methylotrophs (*Amycolatopsis* and *Mycobacterium*) oxidize methanol via a methanol:*N*,*N'*-dimethyl-4-nitrosoaniline oxidoreductase (MNO), which is a decameric protein with 50-kDa subunits, each carrying a tightly bound NADPH (Bystrykh et al., 1997). This protein also has been isolated as a complex containing two other components that impart a tetrazolium-dye-linked methanol dehydrogenase activity (Bystrykh et al., 1997).

Oxidation of Methylated Amines

Methylated amines are also widespread in the environment, being produced as degradation products of some pesticides, of carnitine and lecithin derivatives, and of trimethylamine oxide. The latter is especially prevalent in fish and in marine environments (Anthony, 1982). A variety of bacteria are known that are capable of growing on methylated amines. In general, the methyl groups of methylated amines are oxidized to formaldehyde, either by an oxidase or a dehydrogenase, with energy conservation occurring in the latter case. Growth on formaldehyde occurs via normal methylotrophic assimilatory and dissimilatory pathways (Fig. 1).

Trimethylamine and Dimethylamine

Trimethylamine is oxidized to dimethylamine and formaldehyde by trimethylamine dehydrogenase (Fig. 2). This enzyme is a flavoprotein, that also contains two Fe_2S_2 clusters and two molecules of ADP (McIntire, 1990). A second pathway for utilization of trimethylamine occurs in which a trimethylamine monooxygenase oxidizes trimethylamine to trimethylamine *N*-oxide. The *N*-oxide is subsequently demethylated by trimethylamine demethylase to dimethylamine and formaldehyde (Anthony, 1982). Dimethylamine is oxidized to methylamine and formaldehyde by dimethylamine monooxygenase (Fig. 2). Gene sequences suggest that trimethylamine and dimethylamine dehydrogenases are evolutionarily related (Yang et al., 1995).

Methylamine

Four possible routes are known in bacteria for utilizing methylamine (Fig. 2). The first of these involves the periplasmic enzyme, methylamine dehydrogenase (MADH), which is another quinoprotein shown to contain the cofactor,

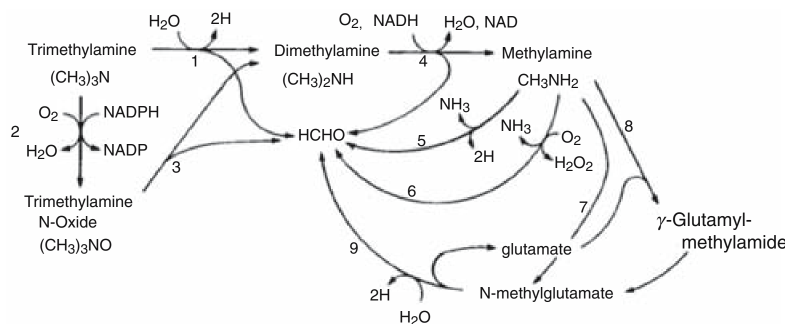

Fig. 2. Pathways for converting methylated amines to formaldehyde in methylotrophic bacteria. 1, trimethylamine dehydrogenase; 2, trimethylamine monooxygenase; 3, trimethylamine *N*-oxide demethylase; 4, dimethylamine monooxygenase; 5, methylamine dehydrogenase; 6, methylamine oxidase; 7, *N*-methylglutamate synthase; 8, γ-glutamylmethylamide synthetase; and 9, *N*-methyl glutatmate dehydrogenase. From Anthony (1982).

tryptophan tryptophylquinone (TTQ), instead of the PQQ found in MDH. This TTQ is formed by covalent crosslinking of two tryptophan residues in the small subunit of MADH, and incorporation of two oxygen atoms into one of the indole rings to form a quinone (Davidson, 1999). The MADH converts primary amines to their corresponding aldehydes plus ammonia, and electrons are transferred to a small copper protein, amicyanin. These electrons are transferred to the respiratory chain via a *c*-type cytochrome (Davidson, 1999). Structural, kinetic and site-directed mutagenesis studies have characterized protein-protein interactions, and mechanisms of catalysis and electron transfer by TTQ. In addition, the genes encoding the functions required for active MADH (*mau* genes) have been studied from several bacteria, and they are similar in both amino acid sequence and genetic organization (van der Palen et al., 1995; Chistoserdov, 1994a; Chistoserdov, 1994b; Gak et al., 1997; Graichen et al., 1999).

In *Arthrobacter* P1, methylamine is utilized by another quinoprotein, methylamine oxidase. This enzyme is a blue copper amine oxidase similar to mammalian copper amine oxidases, which generate hydrogen peroxide (Levering et al., 1981; McIntire and Hartman, 1993; Fig. 2). This enzyme contains the cofactor 6-hydroxydopa quinone, which is formed posttranslationally from a tyrosine residue in the amino acyl chain (McIntire and Hartman, 1993). A few methylotrophs contain indirect pathways, which involve the conversion of methylamine to *N*-methylglutamate, and finally to formaldehyde; *N*-methylglutamate can be synthesized directly, via *N*-methylglutamate synthase, or indirectly via a γ-glutamylmethylamide intermediate, as shown in Fig. 2, although the latter pathway is still uncertain (Anthony, 1982).

Utilization of Methylated Sulfur Species

A few organisms have been isolated that are capable of utilizing methylated sulfur compounds such as dimethylsulfoxide (DMSO), dimethylsulfide (DMS), and dimethyldisulfide (DMDS). Most of these strains have been *Hyphomicrobium* species (DeBont et al., 1981; Suylen and Kuenen, 1986), but a few *Thiobacillus* strains and a *Methylophaga* strain have been reported (Kanagawa and Kelly, 1986; De Zwart et al., 1996). The *Hyphomicrobium* strains apparently reduce DMSO to DMS and then convert the DMS to methanethiol and formaldehyde (Fig. 3). The methanethiol is then oxidized by an oxidase to H_2S and formaldehyde with the production of hydrogen peroxide (DeBont et al., 1981). Formaldehyde is utilized by standard methylotrophic pathways (Fig. 1).

DMS arises in marine environments through the cleavage of dimethyl-β-propiothetin, one of the products of sulfur metabolism of marine algae (Andreae, 1980). Also, DMS has been thought to play an important role in the transport of reduced sulfur compounds between aquatic and terrestrial environments and from terrestrial environments into the atmosphere (Andreae and Raemdonck, 1983; Banwart and Bremner, 1976). The distribution of bacteria capable of utilizing methylated sulfur compounds has not been well studied. However, it seems likely that these organisms are widespread and are present in many environments in which DMS is produced.

Some specialized methylotrophs (including *Methylosulfonomonas*, *Marinosulfonomonas*, and strains of *Hyphomicrobium* and *Methylobacterium*) can use methanesulfonate as a carbon and energy substrate to support growth (Kelly and Murrell, 1999; Pol et al., 1994). Methane-

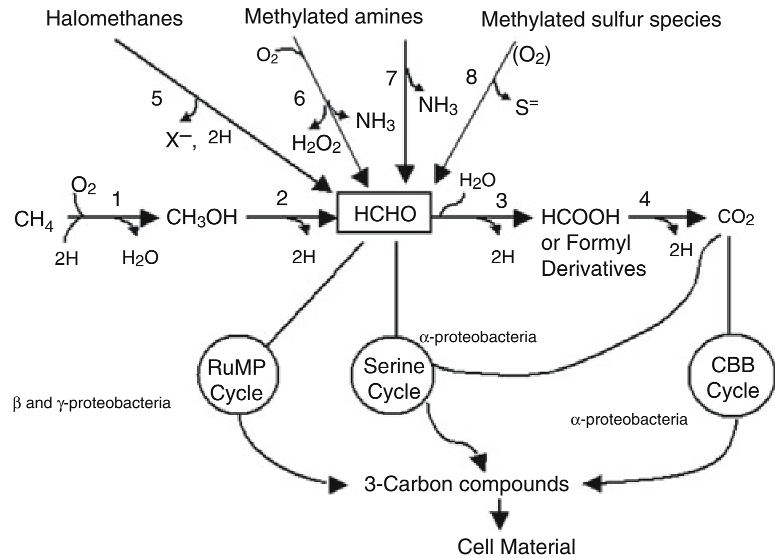

Fig. 3. Proposed pathway for converting methylated sulfur species to formaldehyde in *Hyphomicrobium* species Adapted from DeBont et al. (1981).

sulfonate is oxidized to sulfite and formaldehyde by NADH-dependent methanesulfonate monooxygenase, and utilization of formaldehyde proceeds by normal serine-cycle-dependent methylotrophic metabolism (Kelly and Murrell, 1999). The methanesulfonate monooxygenase has been shown to consist of three components: 1) a 200-kDa hydroxylase complex containing two major polypeptides of around 50 and 20 kDa with a Rieske [2Fe-2S] center; 2) a 16-kDa ferredoxin component; and 3) the putative reductase component, a 36-38 kDa-monomeric protein catalyzing the NADH-dependent reduction of several electron acceptors, including cytochrome *c* (Kelly and Murrell, 1999).

Halomethanes

A number of methylotrophic bacteria are known that are capable of aerobic growth on halomethanes such as chloromethane, bromomethane and dimethylchloride (Leisinger, 1994; Leisinger and Baus-Stromeyer, 1995; Hancock et al., 1998). These bacteria are generally found in the genera *Methylobacterium*, *Hyphomicrobium* or *Methylophilus*, although two strains using monohalomethanes also have been identified that class together in a new subgroup of α-proteobacterial methylotrophs within a clade of rhizobia (Schaefer, 1999; Coulter, 1999). Dichloromethane degradation involves a glutathione-linked dehalogenase that produces formaldehyde (Leisinger, 1994), and the rest of metabolism proceeds by general methylotrophic pathways. Chloromethane degradation has been shown to involve a corrinoid-dependent methyltransferase with sequence identity to methanogen methyltransferases (Studer et al., 1999; Coulter, 1999). In *Methylobacterium* strain CM4, the methyltransferase reaction is coupled to tetrahydrofolate derivatives to produce formate, followed by formate oxidation (Vannelli, 1999; Fig. 4). Assimilation occurs via methylene tetrahydrofolate and the serine cycle. In strain CC495, which is one of the chloromethane utilizers that classes near rhizobia, evidence is presented for a bisulfide-coupled reaction in which methanethiol is the product (Coulter, 1999). In that case, it has been proposed that methanethiol is oxidized to formaldehyde, and metabolism proceeds by general methylotrophic pathways.

Formaldehyde Oxidation

Although it is theoretically possible for methylotrophs to grow on formaldehyde, this substrate is usually too toxic to sustain growth in batch cultures. A few cultures of both methane and methanol utilizers have been reported to grow on formaldehyde (Whittenbury et al., 1981; Hirt et al., 1978), but the growth is poor and usually requires that the substrate be provided in the gas phase. *Arthrobacter* P1 has been grown in a formaldehyde-limited chemostat by first establishing cultures on choline, then adding low levels of formaldehyde, and finally eliminating the choline gradually (Levering et al., 1986). It seems likely that other methylotrophs could be grown on formaldehyde using a similar technique.

A number of formaldehyde oxidation systems are known in methylotrophs (Figs. 4 and 5). The simplest of these is formaldehyde dehydrogenase, which converts formaldehyde to formate. A number of NAD-linked and dye-linked (presumably PQQ-containing and cytochrome-linked) formaldehyde dehydrogenases have been identified from methylotrophs, but the low activity and general lack of inducibility of these

NAD NADH$_2$
A. Formaldehyde → Formate
NAD-dependent FaDH

GSH NAD NADH$_2$ GSH
B. Formaldehyde → S-hydroxy methyl GSH → S-formyl GSH → Fomate
(spontaneous) NAD-GSH dependent FaDH S-formyl GSH hydrolase

MySH NAD NADH$_2$ MySH
C. Formaldehyde → S-hydroxy methyl MySH → S-formyl MySH → Formate
(spontaneous) NAD-MySH dependent FaDH

D. Formaldehyde
H_4MPT → Methylene H_4MPT — NAD(P) NAD(P)H$_2$ — Methylene H_4MPT dehydrogenase → Methenyl H_4MPT — Methenyl H_4MPT cyclohydrolase → N^5-Formyl H_4MPT — MFR? H_4MPT — Formyl MF R:H_4MPT formyltransferase → Formyl MFR ? — 2e-, MFR? — (Formyl MFR dehydrogenase) → CO_2
H_4F → Methylene H_4F — NADP NADPH — Methylene H_4F dehydrogenase → Methenyl H_4F — Methenyl H_4F Cyclohydrolase → N^{10}-Formyl H_4F — ATP, H_4F — Formyl H_4F synthetase → Formate — NAD NADH — Formate dehydrogenase → CO_2

Fig. 4. Linear pathways for formaldehyde oxidation in aerobic methylotrophic bacteria. A, NAD-linked formaldehyde dehydrogenase (FaDH); B, glutathione (GSH)-linked FaDH; C, Mycothiol (MySH)-linked FaDH; D, the two formate-linked pathways, one (upper) involving tetrahydromethanopterin (H_4MPT) and the other (lower) involving tetrahydrofolate (H_4F). Adapted from Anthony (1982); Misset-Smiths et al. (1997); Harms (1996); Chistoserdova et al. (1998).

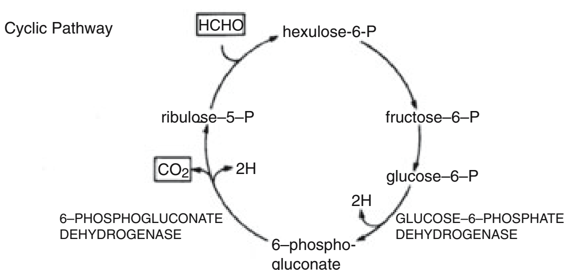

Fig. 5. Cyclic pathway of formaldehyde oxidation, involving enzymes of the RuMP pathway. Adapted from Anthony (1982).

enzymes has called their physiological role into question (Hirt et al., 1978; Stirling, 1978; Anthony, 1982; Marison and Attwood, 1982; Weaver and Lidstrom, 1985; Van Ophem and Duine, 1990; Chistoserdova et al., 1991; Attwood et al., 1992; Speer et al., 1994). It is likely that these enzymes are involved in formaldehyde detoxification rather than playing a major dissimilatory role (Chistoserdova et al., 1991; Vorholt et al., 1999).

NAD- and Mycothiol-Linked Formaldehyde Dehydrogenase

Recent studies have suggested that alternate routes are involved in formaldehyde dissimilation. In Gram-positive methylotrophs, the major formaldehyde dehydrogenase appears to be an enzyme that had previously been described as NAD-linked factor-dependent formaldehyde dehydrogenase (Van Ophem et al., 1992; Van Ophem and Duine, 1994; Duine,

1999; Fig. 4). It is now known that this factor is mycothiol (1-*O*-(2′-[*N*-acetyl-L-cysteinyl]amido-2′-deoxy-α-D-glucopyranosyl)-D-myoinositol), a compound also found in *Mycobacterium* strains (Misset-Smiths et al., 1997; Duine, 1999). This trimeric enzyme consists of a single type of subunit containing Zn (Van Ophem et al., 1992).

NAD- and GSH-Linked Formaldehyde Dehydrogenase

An analogous enzyme coupled to glutathione (GSH) is involved in formaldehyde dissimilation in a variety of Gram-negative methylotrophs, including *Paracoccus* and *Rhodobacter* (Ras et al., 1995; Harms et al., 1996; Barber and Donohue, 1998; Fig. 4). In this case, two enzymes act in concert, an NAD- and GSH-linked dehydrogenase that generates the formyl-GSH derivative, and a hydrolase that releases GSH and formate. Analysis of the genes encoding these enzymes (*flh* genes) suggests they are similar to genes involved in formaldehyde detoxification in a variety of organisms (Harms et al., 1996; Barber and Donohue, 1998).

Folate-Linked Formaldehyde Oxidation Pathways

Two additional linear formaldehyde oxidation pathways are known in methylotrophs, both linked to folates. The first of these involves a standard tetrahydrofolate (H_4F) oxidation pathway, similar to the C_1 interconversion pathways found in most organisms, which oxidizes methylene tetrahydrofolate to formate and tetrahydrofolate (Fig. 4). This pathway was suggested to be the major dissimilatory route for formaldehyde oxidation in serine cycle methylotrophs (Marison and Attwood, 1982). However, in *Methylobacterium extorquens* AM1, this pathway appears to be a minor one (Chistoserdova et al., 1998). In addition, the methylene H_4F dehydrogenase is unusual for bacteria, in that it only carries out this first step instead of both steps as does the normal, coupled enzyme (encoded by *folD*). The gene sequence is highly divergent from other methylene H_4F dehydrogenases (Chistoserdova and Lidstrom, 1994b), and *Methylobacterium extorquens* also contains an unusual methenyl H_4F cyclohydrolase (Pomper et al., 1999).

The major dissimilatory pathway has been suggested to be an analogous tetrahydromethanopterin-linked (H_4MPT-linked) pathway (Fig. 4), similar to that found in the archeaon *Archaeoglobus fulgidis*, and to the reversal of the first few steps of the CO_2 reduction pathway found in archaeal methanogens. This pathway involves a cofactor that had been thought to be specific to archaea, tetrahydromethanopterin (H_4MPT), and the genes encoding the archaeal-like enzymes show significant identity to the corresponding archaeal genes (Chistoserdova et al., 1998). Therefore, it has been suggested that this pathway was acquired by an early methylotroph by horizontal gene transfer from an archaea (Chistoserdova et al., 1998). The archaeal H_2- or F_{420}- enzymes that interconvert methylene and methenyl H_4MPT are not found in the aerobic methylotrophs. Instead, two enzymes have been identified that oxidize methylene H_4MPT to methenyl H_4MPT, one linked to NAD and the other to NADP (Vorholt et al., 1998; Hagemeier et al., 2000). The NAD-linked enzyme is specific to H_4MPT and shows similarity to the NADP-linked enzyme (Hagemeier et al., 2000). The NADP-linked enzyme has activity with both H_4MPT and H_4F, although the activity with the latter is 10% that of the former, and it appears to be the only methylene H_4F-dehydrogenase during methylotrophic growth (Vorholt et al., 1998). Activity and genes are present for methanofuran-utilizing enzymes, but no evidence exists for the presence of methanofuran in methylotrophs. Therefore, the details of the final oxidation step in this pathway are still not known.

Cyclic Formaldehyde Oxidation Pathway

Another formaldehyde pathway is cyclic and involves the condensation of the C_1 compound with a five-carbon acceptor molecule, followed by oxidation of the resulting six-carbon compound (Fig. 5). The enzymes carrying out these reactions are those of the ribulose monophosphate cycle for formaldehyde assimilation, with the exception of one novel enzyme, the 6-phosphogluconate dehydrogenase. A second enzyme is also needed, glucose-6-phosphate dehydrogenase, but this may or may not be a part of the RuMP cycle depending upon the variant utilized. These genes have been cloned and sequenced from *Methylobacillus flagellatum* (Chistoserdova et al., 2000). In those organisms that carry out the cyclic pathway of formaldehyde oxidation, glucose-6-phosphate dehydrogenase activity utilizes both NADP and NAD. However, in *Methylophilus methylotrophus* and *Methylobacillus flagellatum*, two different 6-phosphogluconate dehydrogenases have been found, one of which is active with both NADP and NAD, and the other specific for NAD only (Beardsmore et al., 1982; Kiriuchin et al., 1988). It has been speculated that the flow of carbon at the branch point between oxidation and assimilation in the cyclic pathway is regulated allosterically by these two isoenzymes (Beardsmore et al., 1982).

Distribution of Formaldehyde Oxidation Pathways in Methylotrophs

Although some methylotrophs appear to have only one dissimilatory formaldehyde oxidation pathway, others have multiple routes. Many methylotrophs contain low activities of one or more formaldehyde dehydrogenases, and these may play a largely protective role in formaldehyde detoxification. Of the main dissimilatory pathways, the mycothiol-linked formaldehyde dehydrogenase has so far been found only in Gram-positive methylotrophs (Duine, 1999). The GSH-linked formaldehyde oxidation system has been found mainly in the Gram-negative autotrophic methylotrophs (Harms, 1996; Barber and Donohue, 1998), whereas the H_4F-linked pathway is found in the serine cycle methylotrophs (Vorholt et al., 1999). The cyclic formaldehyde oxidation pathway occurs mainly in the obligate methylotrophs containing the RuMP cycle (Anthony, 1982; Grundig and Babel, 1987). Although it also is found in *Arthrobacter* P1 (Levering et al., 1981), it has been found not to be effective in formaldehyde resistance and may not be a major dissimilatory pathway (Grundig and Babel, 1989). Likewise, although enzyme activities of both pathways exist in the obligate methanotrophs containing the RuMP cycle, the low activities of the glucose-6-phosphate and 6-phosphogluconate dehydrogenases suggest that an alternate pathway must dominate in vivo (Zatman, 1981). This latter conclusion is supported by the fact that the H_4MPT-linked pathway is found in both serine-cycle and RuMP-cycle methanotrophs, at high activity (Vorholt et al., 1999). This pathway has a broad distribution, being found in all tested Gram-negative methylotrophs with either the serine cycle or the RuMP cycle (Vorholt et al., 1999). It was not found in the Gram-positive methylotrophs tested, nor in most of the autotrophic methylotrophs. However, it was present in the autotrophic *Xanthobacter* strains (Vorholt et al., 1999). These results suggest that methylotrophs either have one of the thiol-linked formaldehyde oxidation systems (mainly Gram-positive and autotrophic methylotrophs), or they have the H_4MPT-linked formaldehyde oxidation system (all other methylotrophs). In addition, they may have the H_4F-linked pathway or the cyclic oxidation pathway. In the β-proteobacterial obligate-RuMP-cycle methylotroph *Methylobacillus flagellatum* KT, mutational analysis suggested that the cyclic oxidation pathway was the major dissimilatory pathway, whereas the H_4MPT-linked pathway played a detoxification role (Chistoserdova et al., 2000). An analysis of partial sequences of genes encoding one of the diagnostic enzymes for the H_4MPT-linked pathway, methenyl H_4MPT cyclohydrolase, showed that the bacterial genes grouped in a cluster separate from the cluster of archaeal genes, and the branching pattern for the bacterial genes roughly mirrored the branching pattern of the 16S rRNA genes (Vorholt et al., 1999).

Formate Oxidation

In methylotrophs that have one of the linear oxidation pathways, formate is generally thought to be oxidized to CO_2 by an NAD-linked formate dehydrogenase (Anthony, 1982). Two classes of soluble formate dehydrogenase have been identified in methylotrophs, a dimeric enzyme described in a *Mycobacterium* strain (Galkin, 1995) and in an unidentified Gram-negative methylotroph (Lamzin et al., 1992); these show similar properties and gene sequences (Galkin et al., 1995). However, most methylotrophs appear to have an enzyme (composed of 4 subunits, containing iron, molybdenum and flavin; Jollie and Lipscomb, 1991) similar in properties and at the gene sequence level to a formate dehydrogenase found in *Ralstonia eutropha* (Friedebold and Bowien, 1993). A membrane-bound formate dehydrogenase has been reported in *Amycolatopsis methanolica*, but it has not been characterized (Khmelenina et al., 1997).

No mutants have been reported in formate dehydrogenase in a methylotroph, and so the physiological role of this enzyme has not yet been confirmed.

Assimilatory Metabolism

Three main pathways of assimilatory metabolism are known in aerobic methylotrophs: two that assimilate carbon at the level of formaldehyde (the serine cycle and the ribulose monophosphate cycle), and one that assimilates carbon at the level of CO_2 (the Calvin-Benson-Bassham or CBB cycle). Both pathways for the assimilation of formaldehyde involve cycles in which a condensation reaction between a C_1 compound and a multicarbon compound occurs, followed by regeneration of the acceptor molecule and production of a C_3 compound (Figs. 1, 6 and 7).

Serine Cycle

The serine cycle for formaldehyde assimilation is shown in Fig. 6. This pathway initiates with the condensation of methylene tetrahydrofolate and glycine to form serine. This 3-carbon compound then undergoes a series of transformations to phosphoenolpyruvate, which is carboxylated to form malate. The malate is cleaved into two 2-carbon compounds, which are then converted

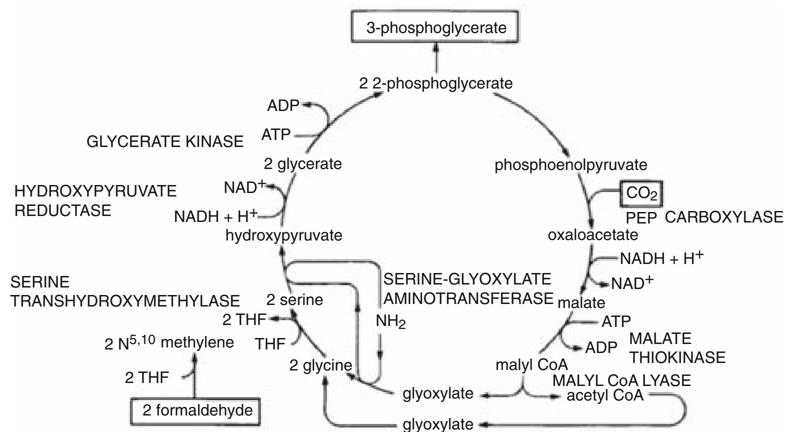

Fig. 6. The serine cycle for formaldehyde assimilation. Adapted from Anthony (1982).

back into glycine, thus completing the cycle. In most organisms that have the serine cycle, it is not clear how acetyl CoA is converted to glyoxylate. The usual route for this conversion involves isocitrate lyase, but this enzyme is only present in a few strains (Anthony, 1982; Chistoserdova and Lidstrom, 1996).

The enzymes specific to the serine cycle are noted in the figure and most of the genes encoding these enzymes have been cloned and sequenced from *Methylobacterium extorquens* AM1 (Chistoserdova and Lidstrom, 1994a; Chistoserdova and Lidstrom, 1994b; Chistoserdova and Lidstrom, 1996; Chistoserdova and Lidstrom, 1997) or *Hyphomicrobium methylovorum* (Yoshida et al., 1994; Hagishita et al., 1996; Tanaka et al., 1997). Two isoenzymes are known to exist for phosphenol pyruvate (PEP) carboxylase (Newaz and Hersh, 1975; McNerney and O'Connor, 1980). The C_1-specific PEP carboxylase is acetyl-CoA independent, unlike the classical acetyl-CoA-dependent anapleurotic enzyme (Newaz and Hersh, 1975), and the gene sequence for this enzyme is on the order of 30% identical to genes encoding the anapleurotic enzyme (Chistoserdova and Lidstrom, 1997). For each C_3 compound that is generated by the serine cycle two carbons are derived from formaldehyde and one from CO_2.

Ribulose Monophosphate Cycle

The ribulose monophosphate cycle (RuMP cycle) is shown in Fig. 7. Formaldehyde is condensed with the acceptor molecule (ribulose monophosphate) by the enzyme hexulose phosphate synthase to produce hexulose phosphate. The six-carbon molecule is then isomerized to fructose 6-phosphate by phosphohexulose isomerase, and a series of interconversions occur that regenerate the five-carbon acceptor molecule. The condensation of three formaldehyde molecules results in the net production of one C_3 compound (Anthony, 1982). As shown in Fig. 7, four different variants of the ribulose monophosphate pathway are possible. However, only three of the possible combinations have been shown to exist. In those obligate methylotrophs that use the RuMP pathway, the combination that appears to occur is that involving 6-P-gluconate (right) and not dihydroxyacetone phosphate (left). The two key enzymes for this variant are 2-keto, 3-deoxy, 6-phosphogluconate aldolase and transaldolase. In the facultative methylotrophs the existing evidence suggests that some strains such as *Bacillus* PM6 (Colby and Zatman, 1975) contain the combination involving fructose bisphosphate (center) and sedoheptulose bisphosphate (lower), and the two key enzymes in this case are fructose bisphosphatase and sedoheptulose bisphosphatase. Other facultative strains, such as *Arthrobacter* P1, contain the combination involving the enzymes fructose bisphosphatase (center) and transaldolase (left; Levering et al., 1982).

Genes encoding hexulose phosphate synthase and phosphohexuloisomerase (*rmpA* and *rmpB*) have been cloned and sequenced from both Gram-positive and Gram-negative methylotrophs (Yanase et al., 1996; Sakai et al., 1999; Mitsui et al., 2000). Surprisingly, genes with identity to hexulose phosphate synthase are common in non-methylotrophic bacteria and in archaea, and for the most part their role is not known (Reizer et al., 1997). However, *Bacillus subtilis* contains orthologs to both *rmpA* and *rmpB*, and evidence has been presented that these genes encode functional enzymes involved in protection of the cell from formaldehyde (Yasueda et al., 1999).

Those organisms that utilize the Calvin-Benson-Bassham pathway for CO_2 fixation appear to utilize the standard pathway without alternations (Anthony, 1982).

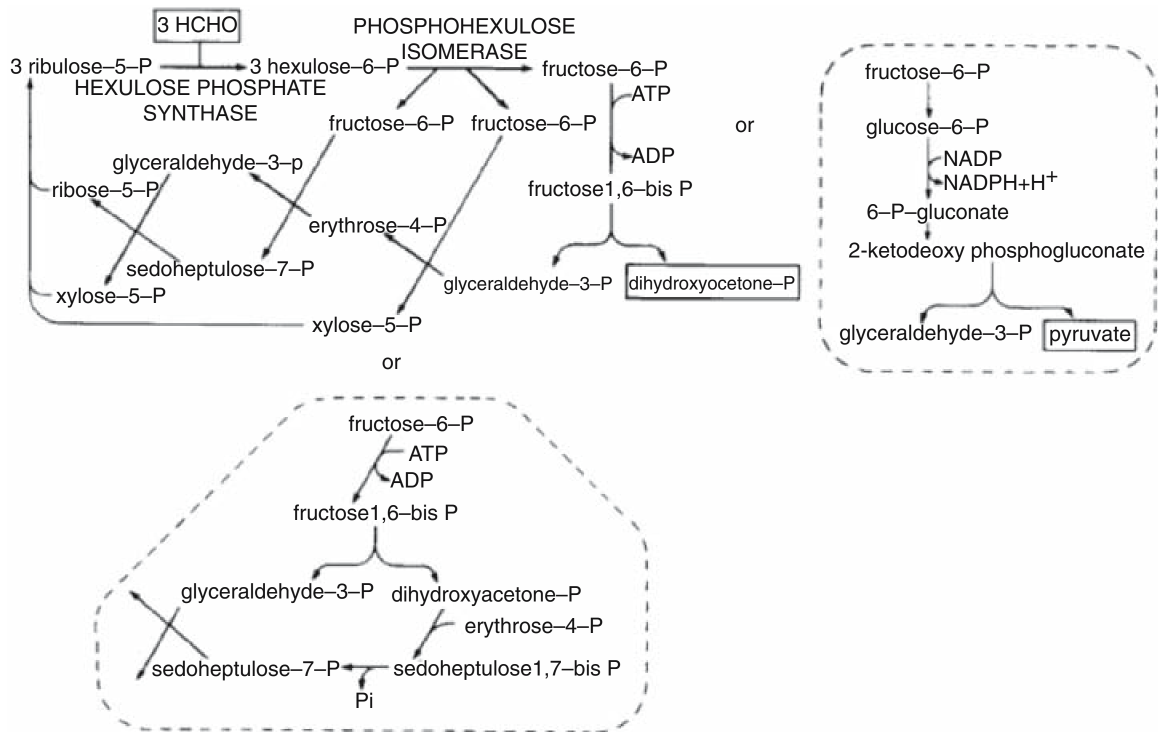

Fig. 7. The ribulose monophosphate (RuMP) cycle for formaldehyde assimilation, showing the two variants for cleavage and the two variants for acceptor regeneration. Adapted from Anthony (1982).

Methylotrophic Bacteria

Methanotrophs

Methanotrophs are a subgroup of the methylotrophic bacteria, which have the ability to grow on methane as sole carbon and energy source. They are found in most environments in which methane and O_2 meet, and have been isolated from a variety of environments including those with extremes of pH and temperature (Hanson and Hanson, 1996; Bodrossy et al., 1997; Bowman et al., 1997; Dedysh et al., 2000). They contain characteristic intracytoplasmic membrane systems (Hanson and Hanson, 1996), either stacks of membrane disks in the type I strains (γ-Proteobacteria), rings of membranes at the periphery of the cell in the type II strains (*Methylosinus* and *Methylocystis*), or vesicular membranes in *Methylocella* (Dedysh et al., 2000). So far, all well-studied methanotrophs have been obligate methylotrophs, unable to grow on compounds with C–C bonds, but reports have been made of a facultative *Mycobacterium* strain capable of growth on methane (Reed and Dugan, 1987) and a mutant of a type I methanotroph has been described that is capable of growth on glucose (Zhao and Hanson, 1984). Some methanotrophs are capable of growth on methanol (Anthony, 1982; Hanson and Hanson, 1996). Some methanotrophs contain nitrogenase and are capable of growth with N_2 as a nitrogen source, mainly *Methylococcus* and *Methylosinus* strains (Hanson and Hanson, 1996). Methanotrophs contain either the serine cycle or RuMP cycle, and so far no autotrophic methanotrophs have been identified (Table 1). Methanotrophs exist as symbionts in mussels, clams and *Pogonophora*, and although the 16S rRNA sequences class with type I methanotrophs, they have not yet been isolated in pure culture (Distel and Cavanaugh, 1994).

Non-Methane Utilizing Methylotrophs

The bacteria capable of growth on methanol and other methylated compounds but not on methane are more diverse than those capable of growing on methane. So far, all of the Gram-positive and α-proteobacterial strains are facultative methylotrophs, whereas the β-proteobacterial and γ-proteobacterial strains are either obligate methylotrophs or restricted facultative methylotrophs (capable of poor growth on a restricted range of multicarbon compounds; Anthony, 1982; Table 1). Most of these bacteria can grow on methanol and may grow on other methylated compounds, but a few strains are known that grow on methylated amines and in some cases also grow on other methylated compounds, but

do not grow on methanol (Anthony, 1982). These include the *Aminobacter* (Urakami et al., 1992) and some of the strains that grow on halogenated methanes (Hancock et al., 1998). The non-methane-utilizing methylotrophic bacteria do not generate intracytoplasmic membrane systems characteristic of the methanotrophs, with the exception of the photosynthetic membranes in the phototrophs. The phototrophs that grow on methanol use it as an electron donor for photosynthesis and in some cases as a carbon source (Quayle and Pfennig, 1975). A number of the Gram-negative methylotrophs contain nitrogenase and are capable of growth with N_2 as a nitrogen source (Table 1).

These bacteria are widely distributed in terrestrial, freshwater and marine habitats (Anthony, 1982). Bacteria capable of utilizing methylated amines are particularly prevalent in the marine environment where it is postulated that they may play a role in carbon cycling in the photic zone (Strand and Lidstrom, 1984). The pink-pigmented *Methylobacterium* strains are common epiphytes on plant leaves, and some evidence exists to suggest a mutualistic symbiosis (Holland and Polacco, 1994).

Genetics in Aerobic Methylotrophs

Genetic Capabilities

A variety of genetic capabilities are available in Gram-negative methylotrophic bacteria, mostly based on broad-host range vectors of the incompatibility (Inc)PI or IncQ groups, including both general cloning vectors and promoter probe vectors based on either *lacZ* or *xylE* as reporters (Holloway et al., 1987; Lidstrom and Sterling, 1990; Harms and van Spanning, 1991; De Vries et al., 1990; Barta and Hanson, 1993; Murrell et al., 2000). However, IncQ vectors are unstable in the *Methylobacterium* strains, and serve as suicide vectors (Biville et al., 1989). Targeted mutants can be generated from cloned genes by recombinational insertion in these strains using suicide vectors, most of them based on ColE1 replicons (Harms, 1996; Barta and Hanson, 1993; Chistoserdov and Lidstrom, 1994a; Murrell et al., 2000). The most common mode of transfer of these vectors into these methylotrophs is by conjugation using a helper plasmid, but electroporation protocols also have been reported for some of the non-methanotrophs (Ueda et al., 1991; Kim and Wood, 1997; Gliesche et al., 1997; Toyama et al., 1998). So far, electroporation has not been successful for methanotrophs (Murrell et al., 2000). Random transposon mutagenesis has been reported for a number of Gram-negative methylotrophs (Whitta et al., 1985; Gliesche and Hirsch, 1992; Studer, 1999; Kang et al., 1999), but not for methanotrophs (Murrell et al., 2000).

In the Gram-positive methylotrophs, cloning vectors have been developed for individual strains. The IncQ vectors and electroporation have been used for *Brevibacterium methylicum* (Nesvera et al., 1994), whereas in *Bacillus methanolicus* and *Amycolatopsis methanolica*, shuttle plasmids were developed using replicons from endogenous plasmids (Vrijbloed et al., 1995; Cue et al., 1997). In the latter two cases, genetic transformation systems were developed for vector transfer.

Genomics

A number of genome sequencing projects are underway for methylotrophic bacteria, including *Methylobacterium extorquens* AM1, *Methylococcus capsulatus* Bath, *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*.

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Prokaryotes (2006) 2:635–658
DOI: 10.1007/0-387-30742-7_21

CHAPTER 1.21

Dissimilatory Fe(III)- and Mn(IV)-Reducing Prokaryotes

DEREK LOVLEY

Introduction

Dissimilatory Fe(III) reduction is the process in which microorganisms transfer electrons to external ferric iron [Fe(III)], reducing it to ferrous iron [Fe(II)] without assimilating the iron. A wide phylogenetic diversity of microorganisms, including archaea as well as bacteria, are capable of dissimilatory Fe(III) reduction. Most microorganisms that reduce Fe(III) also can transfer electrons to Mn(IV), reducing it to Mn(II).

As detailed in the next section, dissimilatory Fe(III) and Mn(IV) reduction is one of the most geochemically significant events that naturally takes place in soils, aquatic sediments, and subsurface environments. Dissimilatory Fe(III) and Mn(IV) reduction has a major influence not only on the distribution of iron and manganese, but also on the fate of a variety of other trace metals and nutrients, and it plays an important role in degradation of organic matter. Furthermore, dissimilatory Fe(III)-reducing microorganisms show promise as useful agents for the bioremediation of sedimentary environments contaminated with organic and/or metal pollutants. Despite their obvious environmental significance, Fe(III) and Mn(IV)-reducing microorganisms are among the least studied of any of the microorganisms that carry out important redox reactions in the environment.

The Fe(III)- and Mn(IV)-reducing microorganisms are also of intrinsically interesting because they have unique metabolic characteristics. Foremost is the ability of these microorganisms to transfer electrons to external, highly insoluble electron acceptors such as Fe(III) and Mn(IV) oxides, as well as extracellular organic compounds such as humic substances. Furthermore, microbiological and geological evidence suggests that dissimilatory Fe(III) reduction was one of the earliest forms of microbial respiration. Thus, insights into Fe(III) reduction mechanisms may aid in understanding the evolution of respiration in microorganisms.

Significance of Fe(III)- and Mn(IV)-Reducing Microorganisms

Some claims for the significance of Fe(III)-reducing microorganisms may be exaggerated, such as the assertion that “if it were not for the bacterium GS-15 [a Fe(III)-reducing microorganism] we would not have radio and television today” (Verschuur, 1993). However, it is also clear that Fe(III)-reducing microorganisms are of vitally important to the proper functioning of a variety of natural ecosystems and have practical applications. Detailed reviews of the literature covering many of these aspects of Fe(III) and Mn(IV) reduction are available (Lovley, 1987a; Lovley, 1991a; Lovley, 1993a; Nealson and Saffarini, 1994; Lovley, 1995a; Lovley et al. 1997c). Therefore only highlights of the significance of Fe(III)-reducing microorganisms, abstracted from these reviews, will be briefly summarized here.

Oxidation of Organic Matter in Anaerobic Environments

Microbial oxidation of organic matter coupled to the reduction of Fe(III) and Mn(IV) is an important mechanism for organic matter oxidation in a variety of aquatic sediments, submerged soils, and in aquifers. Depending on the aquatic sediments or submerged soils considered, Fe(III) and/or Mn(IV) reduction have been estimated to oxidize anywhere from 10% to essentially all of the organic matter oxidation in the sediments (Lovley, 1991a; Canfield et al., 1993; Lovley, 1995b; Lovley et al., 1997c). An important factor that enhances the significance of Fe(III) and Mn(IV) reduction in aquatic sediments is bioturbation which leads to the reoxidation of Fe(II) and Mn(II) so that each molecule of iron and manganese can be used as an electron acceptor multiple times prior to permanent burial. In deep pristine aquifers, there are often extensive zones exist in which Fe(III) reduction is the predominant mechanism for organic matter oxidation

(Chapelle and Lovley, 1992; Lovley and Chapelle, 1995c). The ability of Fe(III)-reducing microorganisms to outcompete sulfate-reducing and methanogenic microorganisms for electron donors during organic matter degradation is an important factor limiting the production of sulfides and methane in some submerged soils, aquatic sediments, and the subsurface (Lovley, 1991a; Lovley, 1995b).

A model for the oxidation of organic matter in sedimentary environments in which Fe(III) reduction is the predominant terminal electron-accepting process has been suggested (Lovley et al., 1997c). This model is based upon the known physiological characteristics of Fe(III)- and Mn(IV)-reducing microorganisms available in pure culture as well as on studies on the metabolism of organic matter metabolism by natural communities of microorganisms living in various sedimentary environments in which Fe(III) reduction is the terminal electron-accepting process (TEAP). In this model (Fig. 1), complex organic matter is hydrolyzed to simpler components by the action of hydrolytic enzymes from a variety of microorganisms. Fermentative microorganisms are the principal consumers of fermentable compounds such as sugars and amino acids and these compounds are converted primarily to fermentation acids and, possibly to hydrogen. Acetate is by far the most important fermentation acid produced (Lovley and Phillips, 1989a). Acetate also may be produced as the result of incomplete oxidation of some sugars by some Fe(III)-reducing microorganisms (Coates et al., 1999a). Other Fe(III)-reducing microorganisms oxidize the acetate and other intermediary products. Some Fe(III)-reducing microorganisms also can oxidize aromatic compounds and long-chain fatty acids. Thus, through the activity of diverse microorganisms, complex organic matter can be oxidized to carbon dioxide with Fe(III) serving as the sole electron acceptor. A similar model probably is probably appropriate for organic matter oxidation in sediments in which Mn(IV) reduction is the TEAP. This model emphasizes that acetate is likely to be the major electron donor for Fe(III) or Mn(IV) reduction in environments in which naturally occurring, complex organic matter is the major substrate for microbial metabolism. However, when otherwise organic-poor environments, such as sandy aquifers, are contaminated with a specific class of organic compounds, such as aromatics, then these contaminants may be the most important direct electron donors for Fe(III) or Mn(IV) reduction.

Influence on Metal and Nutrient Geochemistry and Water Quality The reduction of Fe(III) to Fe(II) is one of the most important geochemical changes as anaerobic conditions develop in submerged soils and aquatic sediments (Ponnamperuma, 1972). The Fe(II) produced as the result of Fe(III) reduction is the primary reduced species responsible for the negative redox potential in many anaerobic freshwater environments. The reduction of Fe(III) oxides and of the structural Fe(III) in clays typically results in a change in soil color from the red-yellow of Fe(III) forms to the green-gray of Fe(II) minerals (Lovley, 1995c). The oxides of Fe(III) and Mn(IV) oxides bind trace metals, phosphate, and sulfate, and Fe(III) and Mn(IV) reduction is associated with the release of these compounds into solution (Lovley, 1995a). Also, typically the pH, ionic strength of the pore water, and the concentration of a variety of cations are increased (Ponnamperuma, 1972; 1984). All of these changes influence water quality in aquifers and can affect the growth of plants in soils.

The solubility of Fe(II) and Mn(II) is greater than that of Fe(III) and Mn(IV) and thus Fe(III) and Mn(IV) reduction result in an increase in dissolved iron and manganese in pore waters. Undesirably high concentrations of iron and manganese may be toxic to plants (Lovley, 1995b) and are particularly significant in groundwaters sources of drinking water, being one of the most prevalent groundwater quality problems (Anderson and Lovley, 1997).

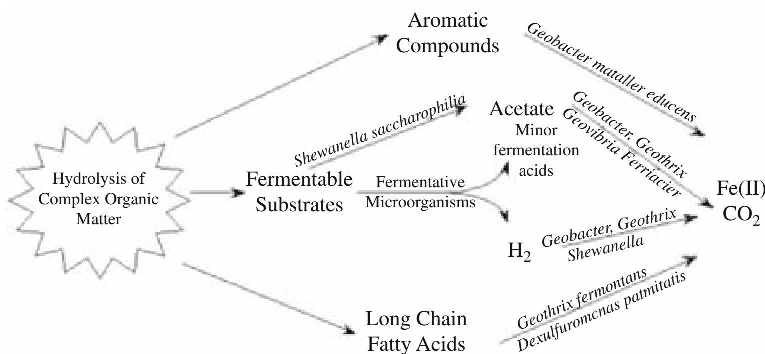

Fig. 1. Proposed pathways for organic matter degradation in mesophilic environments in which Fe(III) reduction is the predominant terminal electron-accepting process.

Most of the Fe(II) and Mn(II) produced from microbial Fe(III) and Mn(IV) reduction is found in solid phases, often in the form of Fe(II) and Mn(II) minerals of geochemical significance (Lovley, 1995c). The most intensively studied mineral that is formed during microbial Fe(III) reduction is the magnetic mineral magnetite (Fe_3O_4) (Lovley et al., 1987c; Lovley, 1990a; Lovley, 1991a). The magnetite produced during microbial Fe(III) reduction can be an important geological signature of this activity. For example, large quantities of magnetite at depths up to 6.7 km below the Earth's surface provided some of the first evidence for a deep, hot biosphere (Gold, 1992). The massive magnetite accumulations that comprise the Precambrian Banded Iron Formations provide evidence for the possible activity of Fe(III)-reducing microorganisms on early Earth. Formation of magnetite as the result of microbial Fe(III) reduction may contribute to the magnetic remanence of soils and sediments. The magnetic anomalies that aid in the localization of subsurface hydrocarbon deposits may result from the activity of hydrocarbon-degrading Fe(III) reducers. Formation of other Fe(II) and Mn(II) minerals such as siderite ($FeCO_3$) and rhodochrosite ($MnCO_3$) also may provide geological signatures of microbial Fe(III) and Mn(IV) reduction.

As detailed below, many Fe(III)- and Mn(IV)-reducing microorganisms can use other metals and metalloids as electron acceptors. Microbial reduction of the soluble oxidized form of uranium, U(VI), to insoluble U(IV) may be an important mechanism for the formation of uranium deposits and the reductive sequestration of uranium in marine sediments, the process which prevents dissolved uranium from building up in marine waters (Lovley et al., 1991a; Lovley and Philips, 1992). Reduction of other metals such as vanadium, molybdenum, copper, gold, and silver, as well as metalloids such as selenium and arsenic, can affect the solubility and fate of these compounds in a variety of sedimentary environments and may contribute to ore formations (Lovley, 1993a; Oremland, 1994a; Newman et al., 1998; Kashefi and Lovley, 1999).

Bioremediation of Organic and Metal Contaminants Iron [Fe(III)]-reducing microorganisms have been shown to play a major role in removing organic contaminants from polluted aquifers. For example, Fe(III)-reducing microorganisms naturally remove aromatic hydrocarbons from petroleum-contaminated aquifers (Lovley et al., 1989b; Lovley, 1995c; Lovley, 1997a; Anderson et al., 1998) and this process can be artificially enhanced with compounds that make Fe(III) more available for microbial reduction (Lovley et al., 1994a; Lovley, 1997a). The Fe(II)-minerals formed as the result of microbial Fe(III) reduction can be important reductants for the reduction of nitroaromatic contaminants (Heijman et al., 1993; Hofstetter et al., 1999). Minerals containing Fe(II) also may serve to reductively dechlorinate some chlorinated contaminants (Fredrickson and Gorby, 1996).

The ability of Fe(III)-reducing microorganisms to substitute other metals and metalloids in their respiration may be exploited for remediation of metal contamination (Lovley, 1995a; Lovley, 1995b; Fredrickson and Gorby, 1996; Lovley and Coates, 1997b). Reduction of soluble U(VI) to insoluble U(IV) can effectively precipitate uranium from contaminated groundwaters and surface waters. Microbial uranium reduction can be coupled with a simple soil-washing procedure to concentrate uranium from contaminated soils. Iron [Fe(III)]-reducing microorganisms can precipitate technetium from contaminated waters by reducing soluble Tc(VII) to insoluble Tc(IV). Soluble radioactive Co(III) complexed to EDTA can be reduced to Co(II) which is less likely to be associated with the EDTA found in contaminated groundwaters and more likely to adsorb to aquifer solids. Some Fe(III) reducers convert soluble, toxic Cr(VI) to less soluble less toxic Cr(III). Reduction of soluble selenate to elemental selenium can effectively precipitate selenium in sediments or remove selenate from contaminated waters in bioreactors.

A Possible Early Form of Microbial Respiration Iron [Fe(III)] reduction may have been one of the earliest forms of microbial respiration (Vargas et al., 1998). Biological evidence for this hypothesis is the finding from 16S rRNA phylogenies that all of microorganisms that are the most closely related to the last common ancestor of extant microorganisms are Fe(III)-reducing microorganisms. All of the deeply branching bacteria and archaea that have been examined can oxidize hydrogen with the reduction of Fe(III). Several that have been examined in more detail can conserve energy to support growth from this metabolism. Of most interest in this regard is *Thermotoga maritima*, which was previously considered to be a fermentative organism because it could not conserve energy to support growth from the reduction of other commonly considered electron acceptors. However, *T. maritima* it does grow via Fe(III) respiration. This result and the apparent conservation of the ability to reduce Fe(III) in all these deeply branching organisms suggests that the last common ancestor was a hydrogen-oxidizing, Fe(III)-reducing microorganism.

The concept that Fe(III) reduction is an early form of respiration agrees with geological scenarios that suggest the presence of large quanti-

ties of Fe(III) on prebiotic Earth (Cairns-Smith et al., 1992; de Duve, 1995) and elevated hydrogen levels (Walker, 1980)—conditions that would be conducive to the evolution of a hydrogen-oxidizing, Fe(III)-reducing microorganism. The large accumulations of magnetite in the Precambrian iron formations (discussed above) indicate that the accumulation of Fe(III) on prebiotic Earth was biologically reduced early in the evolution of life on Earth. This and other geochemical considerations suggest that Fe(III) reduction was the first globally significant mechanism for organic matter oxidation (Walker, 1987; Lovley, 1991a).

Fe(III)- and Mn(IV)-Reducing Microorganisms Available in Pure Culture

Dissimilatory Fe(III)- and Mn(IV)-reducing microorganisms can be separated into two major groups, those that support growth by conserving energy from electron transfer to Fe(III) and Mn(IV) and those that do not. Early investigations on Fe(III) and Mn(IV) reduction in pure culture were conducted exclusively with organisms that are not considered to be conservers of energy from Fe(III) or Mn(IV) reduction (Lovley, 1987a). However, within the last decade, a diversity of microorganisms has been described in which Fe(III) and Mn(IV) reduction are linked to respiratory systems capable of ATP generation. It is these Fe(III)- and Mn(IV)-respiring microorganisms (abbreviated here as FMR) that are likely to be responsible for most of the Fe(III) and Mn(IV) reduction in many sedimentary environments (Lovley, 1991a). A brief description of the known metabolic and phylogenetic diversity of dissimilatory Fe(III)- and Mn(IV)-reducing microorganisms follows.

Fermentative Fe(III)- and Mn(IV)-Reducing Microorganisms Many microorganisms which grow via fermentative metabolism can use Fe(III) or Mn(IV) as a minor electron acceptor during fermentation (Table 1). Growth is possible in the absence of Fe(III) or Mn(IV). In this form of Fe(III) and Mn(IV) reduction, most of the electron equivalents in the fermentable substrates are recovered in organic fermentation products and hydrogen. Typically, less than 5% of the reducing equivalents are transferred to Fe(III) or Mn(IV) (Lovley, 1987a; Lovley and Phillips, 1988b). However, significant amounts of Fe(II) and Mn(II) can accumulate in cultures of these fermentative organisms when Fe(III) or Mn(IV) is provided as a potential electron sink. Although thermodynamic calculations have demonstrated that fermentation with Fe(III) reduction [electron transfer to Fe(III)] is more energetically favorable than fermentation without Fe(III) reduction (Lovley and Phillips, 1989a), it has not been demonstrated that the minor transfer of electron equivalents to Fe(III) or Mn(IV) during fermentation causes any increase in cell yield. In contrast to these fermentative microorganisms, several microorganisms can partially or completely oxidize fermentable sugars and amino acids with the reduction of Fe(III) and conserve energy from this metabolism, as discussed below.

Sulfate-Reducing Microorganisms Many respiratory microorganisms that grow anaerobically with sulfate serving as the electron acceptor also have the ability to enzymatically reduce iron [Fe(III); Table 1]. Electron donors that support Fe(III) reduction are the same ones that support sulfate reduction by sulfate-reducing microorganisms. However, none of these sulfate reducers have been shown to grow with Fe(III) serving as the sole electron acceptor (Lovley et al., 1993b). This is true despite the fact that sulfate reducers have a higher affinity for hydrogen, and possibly for other electron donors, than for sulfate when Fe(III) serves as the electron acceptor (Coleman et al., 1993; Lovley et al., 1993c).

The advantage to sulfate reducers in reducing Fe(III), if there is one, has not been thoroughly investigated. Because it has been found that the intermediate electron carrier, cytochrome c_3, can function as an Fe(III) reductase (Lovley et al., 1993), intermediate electron carriers involved in sulfate reduction may inadvertently reduce Fe(III) because it has been found that the intermediate electron carrier, cytochrome c_3 can function as an Fe(III) reductase (Lovley et al., 1993b). Alternatively, Fe(III) reduction by sulfate reducers may be a strategy to hasten Fe(III) depletion and enhance conditions for sulfate reduction. Furthermore, the possibility that sulfate-reducing microorganisms may be able to generate ATP as the result of Fe(III) reduction, even if they can not grow with Fe(III) as the sole electron acceptor, has not been ruled out (Lovley et al., 1993c).

In contrast to the sulfate-reducing microorganisms discussed above, which could not be grown with Fe(III) as the sole electron acceptor, it has been suggested (Tebo and Obraztsova, 1998) that the sulfate-reducing microorganism "Desulfotomaculum reducens" could also conserve energy to support growth by reducing Fe(III), Mn(IV), U(VI), and Cr(VI) (Tebo and Obraztsova, 1998). However, the data supporting the claim that energy is gained from electron transport to metals is curious. For example, when the culture was grown on 400 μM U(VI), the cell yield was greater than when the culture reduced 8 mmol Fe(III). This occurs despite the fact that the number of electrons transferred to Fe(III)

Table 1. Organisms known to reduce Fe(III) but not known to conserve energy from Fe (III) reduction.

Organism	Electron donor	Form of Fe(III) reduced[a]	Reference
Fermentative bacteria			
Actinomucor repens	Glucose	Hematite	Ottow and von Klopotek, 1969
Aerobacter aerogenes	Glucose-asparagine	Hematite	Ottow, 1970
Aerobacter sp.	Glucose	PCIO	Bromfield, 1954
Alternaria tenuis	Glucose	Hematite	Ottow and von Klopotek, 1969
Bacillus cereus	Glucose-asparagine	Hematite	Ottow, 1970
Bacillus circulans	Sucrose	PCIO	Bromfield, 1954
	Glucose-asparagine	Hematite	Ottow, 1970
	Sucrose	Ferro-manganese ore	Troshanov, 1968
Bacillus mesentericus	Sucrose	Ferro-manganese ore	Troshanov, 1968
Bacillus polymyxa	Glucose	PCIO	Roberts, 1947
	Glucose	Hematite	Hammann and Ottow, 1974
	Sucrose	PCIO	Bromfield, 1954
Bacillus pumilus	Glucose-asparagine	Hematite	Ottow, 1970
Bacillus sp.	Glucose	Limonite, goethite, hematite	De Castro and Ehrlich, 1970
Bacillus subtilis	Glucose	Hematite	Ottow and Glathe, 1971
Bacteroides hypermegas	Glucose-tryptone	Fe(III)-Cl_3	Jones et al., 1984a
Clostridium butyricum	Glucose	Hematite	Hammann and Ottow, 1974
Clostridium polymyxa	Sucrose	Ferro-manganese ore	Troshanov, 1968
Clostridium saccarobutyricum	Glucose	Hematite	Hammann and Ottow, 1974
Clostridium sporogenes	Glucose, peptone	PCIO	Starkey and Halvorson, 1927
Escherichia coli	Glucose, peptone	PCIO	Starkey and Halvorson, 1927
Escherichia coli	Glucose-asparagine	Hematite	Ottow, 1970
Fusarium oxysporum	Glucose	Ferric ammonium citrate	Gunner and Alexander, 1964
Fusarium oxysporum	Glucose	Hematite	Ottow and von Klopotek, 1969
Fusarium solani	Glucose	Hematite	Ottow and von Klopotek, 1969
Paracolobactrum sp.	Glucose	PCIO	Bromfield, 1954
Pseudomonas aeruginosa	Glucose-asparagine	Hematite	Ottow, 1970
Pseudomonas denitrificans	Glucose	Fe(III)-Cl_3	Jones et al., 1984a
Pseudomonas liquefaciens	Sucrose	Ferro-manganese ore	Troshanov, 1968
Pseudomonas (several species)	Glucose-asparagine	Hematite	Ottow and Glathe, 1971
Rhodobacter capsulatus	Malate	Fe(III)-NTA	Dobbin et al., 1996
Serratia marcescans	Glucose-asparagine	Hematite	Ottow, 1970
Sulfolobus acidocaldarius	Elemental sulfur	Fe(III)-Cl_3	Brock and Gustafson, 1976
Thiobacillus thiooxidans	Elemental sulfur	Fe(III)-Cl_3	Brock and Gustafson, 1976 Kino and Usami, 1982
Vibrio sp.	Glucose	Fe(III)-Cl_3	Jones et al., 1983
Vibrio sp.	Malate, pyruvate	Fe(III)-Cl_3	Jones et al., 1984b
Wolinella succinogenes	Formate	Fe(III)-Cit	Lovley et al., 1998
Sulfate-reducing bacteria			
Desulfobacter postgatei	Acetate	Fe(III)-NTA	Lovley et al., 1993
Desulfobacterium autotrophicum	H_2	Fe(III)-NTA	Lovley et al., 1993
Desulfobulbus propionicus	Propionate	Fe(III)-NTA	Lovley et al., 1993
Desulfovibrio baarsii	Butyrate, caproate, octanoate	Fe(III)-NTA	Lovley et al., 1993
Desulfovibrio desulfuricans	Lactate	Fe(III)-Cl_3	Jones et al., 1984a Coleman et al., 1993
Desulfovibrio baculatus	Lactate	Fe(III)-NTA	Lovley et al., 1993
Desulfovibrio sulfodismutans	Lactate	Fe(III)-NTA	Lovley et al., 1993
Desulfovibrio vulgaris	Lactate	Fe(III)-NTA	Lovley et al., 1993
Desulfotomaculum nigrificans	Lactate	Fe(III)-Cl_3	Jones et al., 1984a
Archaea			
Archaeoglobus fulgidus	H_2	Fe(III)-Cit	Vargas et al., 1998
Methanococcus thermolithotrophicus	H_2	Fe(III)-NTA	Vargas et al., 1998
Methanopyrus kandleri	H_2	Fe(III)-Cit	Vargas et al., 1998
Pyrococcus furiosus	H_2	Fe(III)-Cit	Vargas et al., 1998
Pyrodictium abyssi	H_2	Fe(III)-Cit	Vargas et al., 1998

[a]Fe(III) forms: Poorly crystalline Iron Oxide (PCIO), Ferric citrate [Fe(III)-Cit], Ferric nitriloacetic acid [Fe(III)-NTA], Ferric pyrophosphate [Fe(III)-P], Fe(III) chloride [Fe(III)-Cl_3].

was ten-fold higher than the electron transfer to U(VI) and that Fe(III) reduction is energetically more favorable than U(VI) reduction. Cell yields with metals as the electron acceptor were comparable to those during sulfate reduction even though electron transfer to sulfate was at least 250-fold, and in some instances 2500-fold, greater than electron transfer to the metals. These results suggest that the presence of the metals had some additional influence on growth other than just serving as an electron acceptor.

Several sulfate-reducing microorganisms can oxidize S° to sulfate, with Mn(IV) serving as the electron acceptor, but were not found to conserve energy to support growth from this reaction (Lovley and Phillips, 1994a). Enrichment cultures that are established at circumneutral pH with S° as the electron donor and Mn(IV) or Fe(III) as the electron acceptor typically yield microorganisms which that disproportionate S° to sulfate and sulfide (Thamdrup et al., 1993). The Fe(III) or Mn(IV) serve to abiotically reoxidize the sulfide produced.

Microorganisms that Conserve Energy to Support Growth from Fe(III) and Mn(IV) Reduction

The Fe(III)- and Mn(IV)-respiring microorganisms (FMR) which are known to conserve energy to support growth from Fe(III) and Mn(IV) reduction (Table 2) are phylogenetically (Fig. 2) and morphologically (Fig. 3) diverse. Most of the FMR grow by oxidizing organic compounds or hydrogen with the reduction of Fe(III) or Mn(IV), but S° oxidation coupled to Fe(III) reduction also can provide energy to support growth of microorganisms growing at low pH. The various types of FMR are briefly described below.

Geobacteraceae Most of the known FMR, available in pure culture, that can oxidize organic compounds completely to carbon dioxide with Fe(III) or Mn(IV) serving as the sole electron acceptor are in the family *Geobacteraceae* in the delta *δ-Proteobacteria* (Fig. 2; Table 2). The family *Geobacteraceae* is comprised of the genera *Geobacter*, *Desulfuromonas*, *Desulfuromusa* and *Pelobacter.* With the exception of the *Pelobacter* species, all of the *Geobacteraceae* genera contain microorganisms that oxidize acetate to carbon dioxide. This metabolism is significant because, as discussed above, acetate is probably the primary electron donor for Fe(III) reduction in most sedimentary environments. Many of these *Geobacteraceae* also can use hydrogen as an electron donor for Fe(III) reduction. Various species in the *Geobacteraceae* oxidize a variety of other organic acids, including in some instances long-chain fatty acids (Table 2). Several species of *Geobacter* have the ability to anaerobically oxidize aromatic compounds, including the hydrocarbon toluene.

0.1 substitutions/site
Desulfuromonas chloroethenica
Desulfuromonas aetexigens
Pelobacter renetianus
Pelobacter carbinolicus
Desulfuromonas polmitatis
Desulfuromonas succinoxidans
Desulfuromonas bakii
Desulfuromonas kyisingii
Desulfuromonas acetoxidans
Pelobacter proplonicus
Geobacter chapellel
Geobacter sulfurreducens
Geobacter hydrogenopiholus
Geobacter metallireducens
Geobacter grbicium
Sulfurospirillum barnesii
Thiobacillus ferrooxidans
Ferribacterium limneticum
Ferrimanas balearica
Aeromonas hydrophila
Shewanella saccharophilia
Shewanella putrefaciens
Shewanella alga
Geobacter fementans
Deferribacter themophilus
Geovibria femreucens
Bacillus infenus
Themnoterrahacterium ferrireducens
Themus sp.SA-01
Thenmotoga marthma
Pyrobaculum aerophilum
Pyrobaculum islandicum

Fig. 2. Phylogenetic tree, based on 16S rDNA sequences, of microorganisms known to conserve energy to support growth from Fe(III) reduction. The tree was inferred using the Kimura two-parameter model in TREECON for Windows (Van der Peer and De Wachter, 1994). Bootstrap values at nodes were calculated from one hundred replicates.

Table 2. Organisms known to conserve energy to support growth from Fe(III) reduction.

Organism	Source	Electron donors oxidized with Fe(III)[a]	Oxidation with Fe(III)[b]	Fe forms reduced[c]	Other electron acceptors[d]	Growth Temp (°C)	Morphology	Reference[e]
Aeromonas hydrophila	Freshwater and sewage	Glyc, Lac, Succ	Complete	PCIO, Fe(III)-Cit	U(VI), Co(III), selenate, nitrate, Fum, O_2	37	Rod	Knight and Blakemore, 1998
Bacillus infernus	Deep subsurface	For, Lac	Incomplete	Fe(III)-Cl_3	Mn(IV), nitrate, TMAO	60	Rod	Boone et al., 1995
Deferribacter thermophilus	North Sea oil field	Ac, CAA, H_2, Lac, Mal, Pept, Pyr, Succ, Try, Valr, YE	ND[f]	PCIO, Fe(III)-Cit	Mn(VI), nitrate	60	Rod	Greene et al., 1997
Desulfuromonas acetexigens	Anoxic muds	Ac	Complete	PCIO	Mn(VI), S°, polysulfides, Fum, Mal	30	Rod	Coates et al., 1995
Desulfuromonas acetoxidans	Marine sediments	Ac, BtOH, EtOH Prop, Pyr	Complete	Fe(III)-Cit, Fe(III)-NTA	Mn(IV), Glut, Mal, Fum	30	Rod	Roden and Lovley, 1993
Desulfuromonas chloroethenica	Freshwater sediments	Ac, Pyr	ND	Fe(III)-NTA	PCE, TCE, Fum, S^{2-}	21–31	Rod	Krumholz, 1997
Desulfuromonas palmitatis	Marine sediments	Ac, Fum, Lac, Lau, Pal, Ste, Succ	Complete	PCIO, Fe(III)-Cit, Fe(III)-NTA, Fe(III)-P	Mn(IV), AQDS, S^0, Fum	40	Rod	Coates et al., 1995
Desulfuromusa bakii	Marine and freshwater muds	Ac	Complete	Fe(III)-NTA	S°, Mal, Fum	25	Rod	Lonergan et al., 1996
Desulfuromusa kysingii	Freshwater anoxic muds	Ac	Complete	Fe(III)-Cit, Fe(III)-NTA	S°, Mal, Fum, DMSO, nitrate	30	Rod	Liesack and Finster, 1994 Lonergan et al., 1996
Desulfuromusa succinoxidans	Marine sediments	Ac	Complete	Fe(III)-NTA	S°, Mal, Fum	30	Rod	Lonergan et al., 1996
Ferribacterium limneticum	Mine-impacted lake sediments	Ac	Complete	PCIO, Fe(III)-P	Nitrate, Fum	25	Rod	Cummings et al., 1999
Ferrimonas Balearica	Marine sediments	Lac	ND	PCIO, Fe(III)-Cit	Mn(IV), Nitrate	37	Rod	Rossello-Mora et al., 1995
"*Geobacter akaganeitreducens*"	Freshwater ditch	Ac, EtOH, For, Fum, H_2, Mal, Prop, PrOH, Pyr, Succ	ND	PCIO, Akaganeite	Mn(IV), S°, Fum, Mal	30	Rod	Straub et al., 1998
"*Geobacter arculus*"	Freshwater ditch	Ac, BtOH, Buty, Bzo, EtOH, For, Fum, H_2, Lac, Mal, Prop, PrOH, Pyr, Succ	ND	PCIO	Mn(IV), S^0, Fum, Mal	30	Rod	Straub et al., 1998
"*Geobacter chapellei*" (strain 172)	Deep subsurface	Ac, EtOH For, Lac	Complete	PCIO, Fe-NTA,	Mn(IV), AQDS, Fum	25	Rod	Coates et al., 1996
"*Geobacter grbicium*" (strain TACP-2)	Aquatic sediments	Ac, Buty, EtOH, For, Prop, Pyr	Complete	PCIO, Fe(III)-Cit	AQDS	30	Rod	Coates et al., 1996

(Continued)

Table 2. *Continued*

Organism	Source	Electron donors oxidized with Fe(III)[a]	Oxidation with Fe(III)[b]	Fe forms reduced[c]	Other electron acceptors[d]	Growth Temp (°C)	Morphology	Reference[e]
"*Geobacter humireducens*" (strain JW3)	Contaminated wetland	Ac, EtOH, For, H_2, Lac	Complete	PCIO, Fe(III)-Cit	Mn(IV), AQDS S°, nitrate, Fum,	30	Rod	Coates et al., 1998
"*Geobacter hydrogenophilus*" (strain H2)	Contaminated aquifer	Ac, Buty, Bzo, EtOH, For, H_2, Prop, Pyr, Suc	Complete	PCIO, Fe(III)-Cit	AQDS, Fum	30	Rod	Coates et al., 1996
Geobacter metallireducens	Aquatic sediments	Ac, Bz, BzOH, BtOH, Buty, Bzo, BzOH, p-CR, EtOH, p-HBz, p-HBzo, p-HBzOH, IsoB, IsoV, Ph, Prop, PrOH, Pyr, Tol, Valr	Complete	PCIO, Fe(III)-Cit	Mn(IV), Tc(VII)*, U(VI), AQDS, humics, Nitrate	30	Rod	Lovley and Philips, 1988; Lovley et al., 1993
Geobacter sulfurreducens	Contaminated ditch	Ac, For, H_2	Complete	PCIO, Fe(III)-Cit, Fe(III)-P	Tc(VII)*, Co(III), AQDS, S°, Fum, Mal	35	Rod	Cacavvo et al., 1994
Geothrix Fermentans	Contaminated aquifer	Ac, Lac	Complete	PCIO, Fe(III)-Cit, Fe-NTA	Mn(IV), AQDS, S°	30	Rod	Coates et al., 1999
Geovibrio Ferrireducens	Contaminated ditch	Ac, CAA, Fum, H_2, Lac, Pro, Prop, Pyr, Succ, YE	Complete	PCIO, Fe(III)-citrate, Fe(III)-P	Co(III), S°	35	Vibrio	Cacavvo et al., 1996
Pelobacter Carbinolicus	Marine sediments	EtOH, H_2	Incomplete	Fe(III)-NTA	S°	30	Rod	Lovley et al., 1995
Pelobacter Propionicus		Lac	Incomplete	Fe(III)-NTA	S°	30	Rod	Lonergan et al., 1996
Pelobacter Venetianus	Freshwater sediments	EtOH, For, H_2	Incomplete	Fe(III)-NTA	S°	30	Rod	Lonergan et al., 1996
"*Pseudomonas sp.*"	Swampy soil	H_2		PCIO	Nitrate, O_2		Rod	Balashova and Zavarzin, 1980
Pyrobaculum Islandicum	Geothermal water	H_2, Pept, YE	ND	PCIO, Fe(III)-cit	Mn(IV)*, U(VI)*, Co(III)*, Tc(VII)*, Cr(VI)*, Au(III)*, Cyst, Glut, S^0, SO_3^{2-}, $S_2O_3^{2-}$	100	Rod	Kashefi et al., 1999
Pyrobaculum Aerophilum	Marine hydrothermal waters	H_2, Pept, YE	ND	PCIO, Fe(III)-cit	Nitrate, nitrite, O_2	100	Rod	Kashefi et al., 1999
Shewanella alga	Estuarine Sediment	H_2, Lac	Incomplete	PCIO, Fe(III)-cit	Mn(VI), U(VI)*, $S_2O_3^{2-}$, AQDS, TMAO, Fum, O_2	30	Rod	Cacavvo et al., 1992

Shewanella Putrefaciens	Aquatic sediments and other diverse environments	For, H_2, Lac Pyr	Incomplete	PCIO, Fe(III)-cit	Mn(VI), U(VI), S°, $S_2O_3^{2-}$, AQDS, nitrate, Fum, O_2	30	Rod	Myers and Nealson, 1988; Lovley et al., 1989
"Shewanella putrefaciens CN32"	Subsurface	For, EtOH, H_2, Lac, Mal	Incomplete	PCIO Fe(III)-cit	Mn(IV), SO_3^{2-}, Nitrate, Fum, TmaO	30	Rod	D. Boone personal communication
Shewanella Saccharophilia	Aquatic Sediments	For, Glyc, H_2, Lac, Pyr, Suc, YE	Incomplete	PCIO, Fe(III)-cit, Fe-NTA, Fe(III)-P, Fe(III)-EDTA	Mn(IV), U(VI) *, S°, AQDS, $S_2O_3^{2-}$, nitrate, Mal, Fum, O_2	30	Rod	Coates et al., 1998
Sulfurospirillum Barnesii	Freshwater marsh	For, H_2, Lac	Incomplete	PCIO, Fe(III)-cit	Mn(IV), selenate, arsenate, $S_2O_3^{2-}$, S°, nitrite, nitrate, Fum. TMAO, O_2	30	Vibrio	Laverman et al., 1995
Thermotoga Maritima	Geothermally heated sea floor	H_2	ND	Fe(III)-Cit	S^0	80	Rod	Vargas et al., 1998
Thermoterrabact. Ferrireducens	Hot springs, Yellowstone	H_2, Glyc	Incomplete	PCIO, Fe(III)-Cit	AQDS, $S_2O_3^{2-}$, Fum	65	Rod	Slobodkin et al., 1997
Thermus strain SA-01	Deep gold-mine Groundwater	Lac	ND	Fe(III)-Cit, Fe(III)-NTA	Mn(VI), Co(III)*, Cr(VI)*, U(VI)*, S^0, AQDS, nitrate, O_2	65	Rod	Kieft et al., 1999
Thiobacillus Ferrooxidans	Mine drainage	S^0	ND	$Fe_2(SO_4)_3$	S°, O_2,	30	Rod	Das et al., 1992 Pronk et al., 1992

[a]Abbreviations for electron donors and acceptors: Acetate(Ac), Anthraquinone-2,6-disulfonic acid (AQDS), Alanine(Ala), Aspartate (Asp), Benzaldehyde (Bz), Benzoate(Bzo), Benzylalcohol (BzOH), 1,2 butanediol (1,2 Bu), Butanol (BtOH), Butyrate (Buty), Casamino acids (CAA), Casein (Cas), Cystine (Cyst), Dimethylsulfoxide (DMSO), Ethanol (EtOH), Formate (For), Fumarate (Fum), Gelatin (GE), Glucose (Glu), Glutamate (Glu), Glutathione, oxidized (Glut), Glycerol (Glyc), *p*-hydroxybenzoate (*p*-HB), *p*-hydroxybenzaldehyde (*p*-HBz), *p*-hydroxybenzylalcohol (*p*-HBzOH), *p*-cresol (*p*-Cr), Hydrogen (H_2), Inositol (Ino), Isobutyrate (IsoB), Isovalerate (IsoV), Lactate (Lac), Laurate (Lau), Malate (Mal), Maleate (Mle), Nitriloacetic acid (NTA), Oxaloacetate (OAA), Palmitate (Pal), Peptone (Pept), Phenol (Ph), Proline (Pro), Propanol (PrOH), Propionate (Prop), Pyruvate (Pyr), Ribose (Rib), Stearate (Ste), Succinate (Succ), Sucrose (Suc), Tetrachloroethylene (PCE), Trichloroethylene TCE), Toluene (Tol), Trimethylene oxide (To), Trimethylamine oxide (TMAO), Tryptone (Try), Valerate (Valr), Yeast extract (YE), Xylose (Xyl).

[b]Complete oxidation of multicarbon compounds to CO_2, or incomplete, typically to acetate.

[c]Fe(III) forms: Poorly crystalline iron oxide (PCIO), ferric citrate [Fe(III)-cit]; ferric nitriloacetic acid, [Fe(III)-NTA]; ferric pyrophosphate, [Fe(III)-P]; Fe(III) chloride, [Fe(III)-Cl_3]; Fe(III) ethylenediamine-tetraacetic acid.

[d]* Organism has the ability to reduce the metal but not determined whether energy to support growth is conserved from reduction of this metal.

[e]Reference in which the capacity to grow via Fe(III) reduction is described.

[f]ND = Not determined.

Superscript for electron acceptors if growth occurs.

Coates, J.D., Council, T., Ellis, D.J., 1999. Carbohydrate oxidation coupled to Fe(III) reduction—a novel form of anaerobic metabolism. *Anaerobe* 4 277–282.

Coates J. D., Bhupathivaju V., Achenbach L.A., McInerney M.J., Geobacter hydrogenophilus, Geobacter chapellii, Geobacter grbicium—three new strictly anaerobic dissimilatory Fe(III)-reducers IJSB submitted.

Coates Ellis Gaw Lovely. Geothrix fermentans gen. nov. sp. nov. a novel Fe(III) reducing bacterium from a hydrocarbon contaminated aquifer IJSB in press.

Patrick J. A., Achenbach L.A., and Coates J. D. 1999. Geobacter humireducens—Eight new humic-reducing bacteria from a diversity of environments. IJSB submitted.

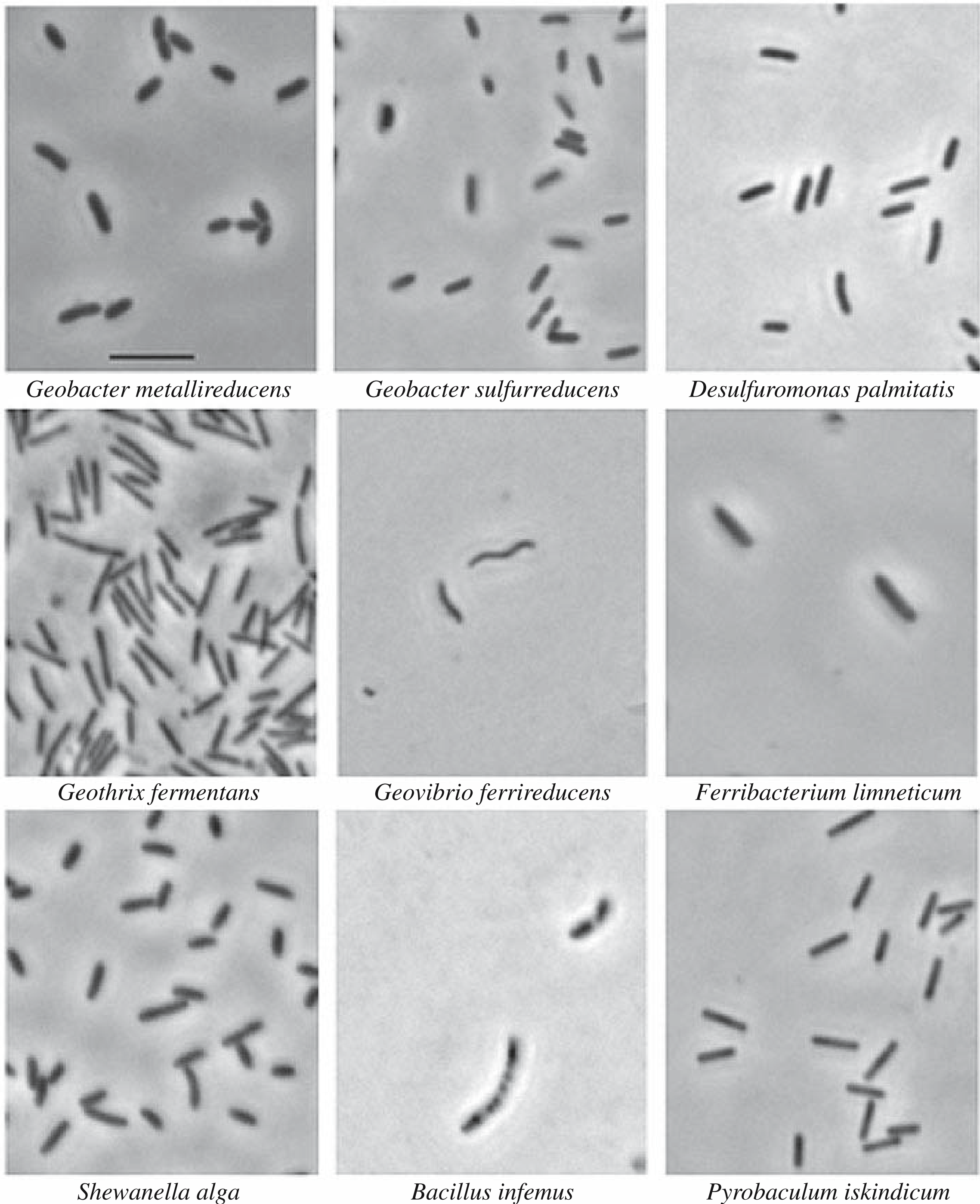

Fig. 3. Phase contrast micrographs of various organisms that conserve energy to support growth from Fe(III) reduction. Bar equals 5 μm, all micrographs at equivalent magnification.

Geobacteraceae are the Fe(III) reducers most commonly recovered from a variety of sedimentary environments when the culture media contains acetate as the electron donor and Fe(III) oxide or the humic acid analog, anthraquinone-2,6-disulfonate (AQDS) as the electron acceptor (Coates et al., 1996; Coates et al., 1998). Furthermore, analysis of 16S rDNA sequences in sandy aquifer sediments in which Fe(III) reduction was the predominant terminal electron accepting process indicated that *Geobacter* species were a major component of the microbial community

(Rooney-Varga et al., 1999; Synoeyenbos-West et al., 1999).

Geothrix *Geothrix fermentans* and closely related strains have been recovered from the Fe(III)-reducing zone of petroleum-contaminated aquifers (Anderson et al., 1998; Coates et al., 1999b). Like *Geobacter* species, *G. fermentans* can oxidize short-chain fatty acids to carbon dioxide with Fe(III) serving as the sole electron acceptor. It can also use long-chain fatty acids, as well hydrogen as an electron donor for Fe(III) reduction (Table 2) and can grow fermentatively on several organic acids. *G. fermentans*, along with *Holophaga foetida*, is part of a deeply branching group in the kingdom *Acidobacterium*. The 16S rDNA sequences from this kingdom are among the most common recovered from soil, but few organisms from this kingdom have been cultured (Barns et al., 1999). Studies in which Fe(III)-reducing microorganisms were recovered in culture media suggested that organisms closely related to *G. fermentans* might be as numerous as *Geobacter* species in the Fe(III) reduction zone of a petroleum-contaminated aquifer (Anderson et al., 1998). However, analyses of 16S rDNA sequences have indicated that *Geothrix* sp. are probably several orders of magnitude less numerous than *Geobacter* species in such environments (Rooney-Varga et al., 1999; Synoeyenbos-West et al., 1999).

Geovibrio ferrireducens and Deferribacter thermophilus Culturing from hydrocarbon-impacted soils and a petroleum reservoir have led to the recovery of the mesophile, *Geovibrio ferrireducens* (Caccavo et al., 1996) and the thermophile, *Deferribacter thermophilus* (Greene et al., 1997). These organisms are more closely related to each other than to any other known Fe(III)-reducing microorganisms and grow with similar electron donors for Fe(III)-reduction. *G. ferrireducens* has been shown to completely oxidize its carbon substrates to carbon dioxide and it is assumed that *D. thermophilus* can as well, but this has not been directly tested. An interesting feature of the metabolism of these organisms is the ability to use some amino acids as electron donors for Fe(III) reduction. The environmental distribution of these organisms has not been studied in detail.

Ferribacter limneticum *Ferribacter limneticum* (Cummings et al., 1999) is the only organism in the β-subclass of the *Proteobacteria* that is known to conserve energy to support growth from Fe(III) reduction. Unlike many Fe(III)-reducing microorganisms it does not utilize Mn(IV) as an electron acceptor. To date, this organism has only been recovered from mining-impacted lake sediments.

Shewanella–Ferrimonas–Aeromonas In contrast to the organisms discussed above, which only grow anaerobically, several genera within the γ-*Proteobacteria*, can grow aerobically, and under anaerobic conditions can use Fe(III), Mn(IV), or other electron acceptors (Table 2). These include species of *Shewanella*, *Ferrimonas*, and *Aeromonas*. Although many of these organisms can use a wide range of electron donors when oxygen is available as an electron acceptor, their range of electron donors with Fe(III) and Mn(IV) is generally restricted to hydrogen and small organic acids. An exception is *Shewanella saccharophila*, which also can use glucose as an electron donor for Fe(III) reduction. The *Shewanella* species, which have been studied in detail, incompletely oxidize multicarbon organic electron donors to acetate.

Another Fe(III)-reducing microorganism that may be related to this group is an unidentified microorganism referred to as a "pseudomonad," which was the first organism found to grow with hydrogen as the electron donor and Fe(III) as the electron acceptor (Balashova and Zavarzin, 1980). However, this organism does not appear to be available in culture collections for further study, and its true phylogenetic placement is unknown.

The FMR in the γ-*Proteobacteria* have been recovered from a variety of sedimentary environments including various aquatic sediments (Myers and Nealson, 1988; Caccavo et al., 1992; Coates et al., 1999a) and the subsurface (Pedersen et al., 1996; Fredrickson et al., 1998). However, in contrast to the organisms in the *Geobacteraceae* which are found to be numerous in both molecular and culturing analysis of widely diverse environments where Fe(III) reduction is important, the distribution of *Shewanella* is more variable. For example, *Shewanella* were found to account for ca. 2% of the microbial population in some surficial aquatic sediments, but could not be detected in other sediments (DiChristina and DeLong, 1993). *Shewanella* 16S rDNA sequences could not be recovered from aquifer sediments in which Fe(III) reduction was the predominant terminal electron-accepting process TEAP (Synoeyenbos-West et al., 1999). This was the case even when electron donors, such as lactate and formate, that are preferred by *Shewanella* species, were added to stimulate Fe(III) reduction.

Sulfurospirillum barnesii *Sulfurospirillum barnesii* which was initially isolated based on its ability to use selenate as an electron acceptor

(Oremland et al., 1994b), also can grow using the reduction of Fe(III) and the metalloid As(V) (Laverman et al., 1995). Although it has commonly been found that if one organism in a close phylogenetic group has the ability to reduce Fe(III) then others in the group also will be Fe(III) reducers (Roden and Lovley, 1993a; Lovley et al., 1995c; Lonergan et al., 1996; Kashefi and Lovley, 1999), *Sulfurospirillum arsenophilum* does not reduce iron [Fe(III); Stolz et al., 1999)]. *Wolinella succinogenes*, which is also in the ε-subclass of the *Proteobacteria*, also can reduce Fe(III) and metalloids (Lovley et al., 1997c; 1999b), but whether *W. succinogenes* conserves energy to support growth from metal reduction has not been determined.

Acidophilic Fe(III)-Reducing Microorganisms Although Fe(III) is highly insoluble at the circumneutral pH at which most Fe(III)-reducing microorganisms have been studied, Fe(III) is soluble at low pH. The redox potential of the Fe^{+3}/Fe^{+2} redox couple is significantly more positive than the Fe(III) oxide/Fe^{+2} redox couple and the oxidation of electron donors (such as S°) that might be unfavorable at circumneutral pH with Fe(III) oxides as the electron acceptors might be favorable in acidic pH where more Fe^{+3} is available. *Thiobacillus ferroxidans* can grow anaerobically with S° as the electron donor and Fe(III) as the electron acceptor (Das et al., 1992; Pronk et al., 1992). *Thiobacillus thiooxidans* also has been shown to reduce Fe(III) with S° as the electron donor (Brock and Gustafson, 1976), but the culture was grown aerobically and energy conservation from Fe(III) reduction was not demonstrated. This was also true of the thermophile, *Sulfolobus acidocaldarius* (Brock and Gustafson, 1976).

Acidophilic thermophiles that can reduce Fe(III) with glycerol or thiosulfate as the electron donor have been described (Bridge and Johnson, 1998), but the ability of these organisms to conserve energy to support growth from Fe(III) reduction has not been examined in detail. An acidophilic mesophile, designated strain SJH, exhibited Fe(III)-dependent growth in a complex organic medium containing glucose and tryptone (Johnson and McGinness, 1991), but further characterization of the electron donors for Fe(III) reduction and a detailed description of the organism were not provided.

Hyperthermophilic and Thermophilic Archaea and Bacteria In addition to *D. thermophilus* mentioned above, a number of other thermophiles and hyperthermophiles can conserve energy to support growth from Fe(III) reduction. The first thermophilic FMR reported was the deep subsurface isolate, *Bacillus infernus*, which has a temperature optimum of 60°C (Boone et al., 1995). It was also the first Gram-positive FMR identified. In contrast to all other members of the *Bacillus* genus, *B. infernus* is a strict anaerobe and can grow by fermentation when Fe(III) or other electron acceptors are not available. Other thermophilic FMR recovered from subsurface environments include *Thermoterrabacterium ferrireducens* (Slobodkin et al., 1997) and a *Thermus* species (Kieft et al., 1999).

As summarized in Tables 1 and 2, a wide phylogenetic diversity of hyperthermophilic microorganisms can transfer electrons to iron [Fe(III); Vargas et al., 1998)]. However, only three of these organisms, *Pyrobaculum islandicum*, *P. aerophilum*, and *Thermotoga maritima*, have been shown to conserve energy to support growth from Fe(III) reduction. *P. islandicum* and *T. maritima* grow with hydrogen as the electron donor and Fe(III) as the electron acceptor and *P. islandicum* and *P. aerophilum* also can grow with complex organic matter (peptone, yeast extract) as the electron donor and Fe(III) as the electron acceptor (Kashefi and Lovley, 1999).

Forms of Fe(III) and Mn(IV) That Can Serve as Electron Acceptors Unlike other types of respiration that use soluble electron acceptors, Fe(III) and Mn(IV) reduction require the reduction of insoluble electron acceptors in most environments. The insoluble Fe(III) and Mn(IV) oxides that are the most environmentally relevant forms of Fe(III) and Mn(IV) at circumneutral pH can be found in a wide diversity of forms (Dixon and Skinner, 1992; Schwertmann and Fitzpatrick, 1992). The nature of the oxides have a major impact on the rate and extent of Fe(III) and Mn(IV) reduction (Lovley, 1991a; Lovley, 1995a).

Pure cultures of Fe(III)-reducing microorganisms reduce a variety of insoluble Fe(III) and Mn(IV) forms (Lovley, 1991a), including the Fe(III) oxides naturally found in sedimentary environments (Lovley et al., 1990b; Coates et al., 1996). Early studies on Fe(III) reduction by fermentative microorganisms often employed highly crystalline Fe(III) oxides as the Fe(III) form (Table 1). However, studies on Fe(III) reduction in sediments suggested that the primary form of Fe(III) that FMR reduced in aquatic sediments was poorly crystalline Fe(III) oxides and that poorly crystalline Fe(III) oxides promoted the complete oxidation of organic compounds to carbon dioxide with Fe(III) serving as the electron acceptor (Lovley and Phillips, 1986a; Lovley and Phillips, 1986b; Phillips et al., 1993).

The use of poorly crystalline Fe(III)-oxide as the Fe(III) form permitted the first recovery of

a microorganism that could completely oxidize organic compounds to carbon dioxide with Fe(III) serving as the electron acceptor (Lovley et al., 1987c). Most subsequent studies that have enriched for Fe(III)-reducing microorganisms from the environment or that have evaluated mechanisms for Fe(III) oxide reduction by pure cultures of FMR have used poorly crystalline Fe(III) oxide as the electron acceptor.

FMR have been shown to reduce some of the more crystalline Fe(III) oxides, including hematite, goethite, akaganeite, and magnetite, under some conditions (Table 2; Lovley, 1991a; Kostka and Nealson, 1995; Roden and Zachara, 1996). However, the rates of reduction of the crystalline Fe(III) oxides are generally much slower than the reduction of poorly crystalline Fe(III) oxide. In most instances, sustained growth is difficult to maintain in consecutive transfer of pure cultures with crystalline Fe(III) oxides as the electron acceptor. In evaluating the potential for reduction of crystalline Fe(III) oxides, it is important to omit complex organic matter or organic acids, which chelate and solubilize Fe(III) from the Fe(III) oxides. The FMR reduction of crystalline Fe(III) oxides in soils and sediments has not been demonstrated conclusively.

An alternative, environmentally relevant, source of insoluble Fe(III) is structural Fe(III) in clays. Reduction of Fe(III) in clays is often observed in flooded soils and FMR have been shown to reduce this iron [Fe(III); Kostka et al., 1996; Lovley et al., 1998)].

Soluble Fe(III) forms are often used for culturing FMR. Although soluble Fe(III) may not represent an environmentally significant form of Fe(III), it provides an easy method for culturing FMR. Pure cultures generally reduce soluble Fe(III) forms faster than poorly crystalline Fe(III) oxide, and less insoluble precipitates are formed during reduction of soluble Fe(III). Furthermore, unlike poorly crystalline Fe(III) oxide, some soluble Fe(III) forms do not have to be synthesized because they are commercially available.

Fe(III)-citrate is the most commonly used form of soluble Fe(III) for the culture of FMR. It is highly soluble and can readily be provided at concentrations as high as 50 mM, even in media with a high salt content. However, Fe(III)-citrate may be toxic to some Fe(III)-reducing microorganisms (Lovley et al., 1990a; Lovley et al., 1993b; Roden and Lovley, 1993b). The Fe(III) chelated with nitrilotriacetic acid (Fe(III)-NTA) is a useful alternative. The limitations of Fe(III)-NTA are its frequent toxicity at concentrations above 10 mM and its tendency to precipitate as Fe(III) oxide when Fe(III)-NTA is added to media with high salt content or at temperatures of 60°C or above. Unlike Fe(III)-citrate, Fe(III)-NTA is not commercially available and must be synthesized, as described below. "Ferric pyrophosphate" has been successfully used for the culture of FMR (Caccavo et al., 1994; Caccavo et al., 1996). This is a somewhat undefined mixture that contains not only Fe(III) and phosphate, but also citrate and nitrilotriacetic acid which are likely to play an important role in maintaining the solubility of Fe(III) in this mixture.

The most commonly used form of Mn(IV) oxide in studies of Mn(IV) reduction by FMR is birsnessite, a readily synthesized Mn(IV) oxide (see method for synthesis below). However, there is a wide diversity of Mn(IV) oxides is found in the environment and rates of Mn(IV) reduction can be dependent upon the form of Mn(IV) oxide available (Burdige et al., 1992).

Products of Fe(III) and Mn(IV) Reduction

Products Fe(II) and Mn(II) are more soluble than Fe(III) and Mn(IV) and thus microbial Fe(III) and Mn(IV) reduction results in a marked increase in dissolved iron and manganese in anaerobic environments and in cultures of FMR. However, in both cultures and sediments, most of the Fe(II) and Mn(II) produced during microbial reduction of insoluble Fe(III) and Mn(IV) oxides often remains in solid forms (Lovley, 1991a; Lovley, 1995a; Schnell et al., 1998). In culture, microbial Fe(III) and Mn(IV) reduction has been shown to form such minerals as magnetite (Fe_3O_4) siderite ($FeCO_3$), vivianite ($Fe_3PO_4 \cdot 8H_2O$) and rhodochrosite ($MnCO_3$; Lovley, 1991a; Lovley, 1995b). The formation of such minerals in culture provides a model for the geologically significant deposition of iron and manganese minerals described above.

The fact that most of the Fe(II) and Mn(II) produced from microbial Fe(III) and Mn(IV) reduction is insoluble means that quantitative analysis of Fe(III) or Mn(IV) reduction either in cultures or environmental samples requires quantifying the amount of insoluble Fe(II) or Mn(II) produced. The Fe(II) may be solubilized in HCl (Lovley and Phillips, 1986a) or oxalate (Phillips and Lovley, 1987; Lovley and Phillips, 1988c) before measurement with Fe(II)-specific reagents such as ferrozine (Stookey, 1970) or ion chromatography (Schnell et al., 1998). Loss of Fe(III) in acid-solubilized samples also can be monitored (Lovley and Phillips, 1988b; Schnell et al., 1998).

Methods for quantitatively measuring Mn(IV) reduction are not as well established. Much of the Mn(II) produced during Mn(IV) reduction adsorbs onto the Mn(IV) oxide or forms insoluble Mn(II) minerals. Mn(II) can be solubilized in acid and soluble manganese measured with atomic absorption spectroscopy (Lovley and

Phillips, 1988c), but this is technically difficult because acid will also eventually dissolve the Mn(IV) oxide. A better strategy might be to solubilize all the manganese and specifically measure the Mn(II) produced with ion chromatography (Schnell et al., 1998).

Mechanisms for Electron Transfer to Fe(III) and Mn(IV) The mechanisms by which Fe(III)- and Mn(IV)-reducing microorganisms transfer electrons to insoluble Fe(III) and Mn(IV) are poorly understood. It is generally stated that Fe(III) and Mn(IV) reducers must directly reduce Fe(III) and Mn(IV) oxides by establishing contact with the oxides (Lovley, 1991a). Until recently, the primary evidence of the need for contact was the finding that Fe(III) and Mn(IV) were not reduced when Fe(III) or Mn(IV) oxides and Fe(III)- and Mn(IV)-reducing microorganisms were separated by semipermeable membranes, which should permit the passage of soluble substances. This result as well was considered evidence that Fe(III)- and Mn(IV)-reducing microorganisms do not produce chelators to solubilize Fe(III) or Mn(IV) and do not produce compounds that could serve as soluble electron-shuttles between Fe(III)- and Mn(IV)-reducing microorganisms and the insoluble oxides. However, recent studies have demonstrated that this approach is flawed because even when chelators or electron shuttles were added to cultures, Fe(III)-reducing microorganisms still did not significantly reduce Fe(III) oxide held within dialysis tubing (Nevin and Lovley, 1999a). Studies with strains of *Shewanella alga*, which were deficient in the ability to attach to Fe(III) oxides, continued to reduce Fe(III), suggesting that attachment to Fe(III) oxide was not necessary for Fe(III) oxide reduction (Caccavo et al., 1997). Thus, although studies have documented the association of Fe(III)-reducing microorganisms with Fe(III)-oxide particles, the current evidence is not definitive to clearly state that Fe(III)- and Mn(IV)-reducing microorganisms must attach to Fe(III) and Mn(IV) oxides in order to reduce them.

It was suggested that *Geobacter sulfurreducens* might reduce Fe(III) oxide in culture by releasing a low molecular weight (9.6 kDa) *c*-type cytochrome into the medium which could serve as a soluble electron shuttle between *G. sulfurreducens* and the Fe(III) oxide (Seeliger et al., 1998). However, further investigation has demonstrated that this c-type cytochrome is not an effective electron shuttle and that in healthy, actively growing cultures of *G. sulfurreducens*, little, if any, of the 9.6 kDa cytochrome is released into the growth medium (Lloyd et al., 1999). Therefore, the proposed shuttling mechanism is unlikely.

Iron [Fe(III)]-reducing microorganisms can use humics and other extracellular quinones as electron shuttles to promote Fe(III) oxide reduction (Lovley et al., 1996; Lovley et al., 1998; Lovley et al. 2000). As discussed below, humics and other extracellular quinones can serve as electron acceptors for Fe(III)-reducing microorganisms. The hydroquinone moieties that are generated as the result of the reduction of extracellular quinones can transfer electrons to Fe(III) oxides through a strictly abiotic reaction. This reduction of Fe(III) regenerates quinone moieties that can then again serve as electron acceptors for Fe(III)-reducing microorganisms. In this manner a small amount of extracellular quinone can promote a significant increase in the rate of reduction of poorly crystalline Fe(III) oxide. For example, studies with cultures and aquifer sediments have demonstrated that there is a significant potential for electron shuttling with as little as 100 nM AQDS (Lloyd et al., 1999; Nevin and Lovley, 1999b). Although electron shuttling to Mn(IV) oxides have not been studied in detail, a similar phenomenon is expected.

However, both the evidence that Fe(III)- and Mn(IV)-reducing microorganisms can reduce Fe(III) and Mn(IV) oxides in cultures without added electron shuttling compounds and chelators and the lack of evidence for release of electron shuttling or chelating compounds by the microorganisms (Nevin and Lovley, 1999a) suggests that FMR can directly transfer electrons to Fe(III) and Mn(IV) oxides. The Fe(III)-reductase activity is primarily localized in the membranes of Fe(III)- and Mn(IV)-reducing microorganisms such as *G. metallireducens* (Gorby and Lovley, 1991), *S. putrefaciens* (Myers and Myers, 1993), and *G. sulfurreducens* (Gaspard et al., 1998; Magnuson et al., 1999). The involvement of cytochromes of the *c*-type has been suggested to be involved in electron transport to Fe(III) in *G. metallireducens* (Lovley et al., 1993c) and *S. putrefaciens* (Myers and Myers, 1992; Myers and Myers, 1997; Beliaev and Saffarini, 1998). A NADH-dependent Fe(III) reductase complex was purified from *G. sulfurreducens* and a 90-kDa *c*-type cytochrome in the complex served as the Fe(III) reductase (Magnuson et al., 1999). However, no study has as yet definitively identified as yet the physiologically relevant Fe(III) or Mn(IV) reductase in any organism capable of conserving energy to support growth via Fe(III) or Mn(IV) reduction.

Other Respiratory Capabilities of FMR

Many FMR can reduce other electron acceptors well-known to support anaerobic respiration such as fumarate, nitrate, and S° (Table 2). Fumarate is reduced to succinate, and S° is

reduced to sulfide. In those documented instances of nitrate reduction, nitrite or ammonia has been found to be the product. It is interesting that nearly all microorganisms with the ability to reduce Fe(III) also can reduce S° to sulfide. In fact, screening of known S°-reducing microorganisms already available in culture has been a fruitful approach for discovering new FMR (Roden and Lovley, 1993a; Lonergan et al., 1996; Vargas et al., 1998).

Electron Transfer to Other Metals and Metalloids Many Fe(III)-reducing microorganisms can transfer electrons to metals other than iron or manganese [Fe(III) or Mn(IV); Table 2]. For example, *G. metallireducens* and *S. putrefaciens* can grow with U(VI) as the sole electron acceptor (Lovley et al., 1991b). Cell suspensions of other FMR have been found to transfer electrons to U(VI), but their ability to obtain energy to support growth from U(VI) reduction has not been evaluated. Many sulfate-reducing microorganisms, can effectively reduce U(VI), but attempts to grow these organisms with U(VI) as the sole electron acceptor have been unsuccessful (Lovley et al., 1993b).

U(VI), which is soluble in bicarbonate-based media is reduced to U(IV) that precipitates as the mineral uraninite (Gorby and Lovley, 1992; Lovley and Phillips, 1992). Visualization of microbial U(VI) reduction can be enhanced with the use a fluorescent light. The U(VI)-containing liquid cultures or agar plates fluoresce green, whereas the uraninite does not significantly fluoresce. Loss of U(VI) during U(VI) reduction can be monitored as loss of soluble uranium by monitoring total uranium concentrations in culture filtrates, but since U(IV) precipitation is not instantaneous (Gorby and Lovley, 1992), more quantitative estimates of U(VI) reduction can be more quantitatively estimated by monitoring loss of U(VI) with a kinetic phosphorescence analyzer (Lovley et al., 1991b) or by using ion chromatography.

Several Fe(III)-reducing microorganisms can reduce the oxidized form of the radioactive metal technetium, Tc(VII) to reduced forms (Table 2). Growth with Tc(VII) as the sole electron acceptor has not yet been documented as yet in any organism. Tc(VII) reduction can be monitored by following the formation of reduced technetium forms with paper chromatography and a phosphorimager (Lloyd and Macaskie, 1996).

FMR can reduce a variety of other metals and metalloids (Table 2). Several can reduce Cr(VI) to Cr(III), but growth with Cr(VI) as the sole electron acceptor has not been demonstrated (Lovley, 1995c). The FMR, *S. barnesii* can conserve energy from the reduction of Se(VI) to Se° and As(V) to As(III) (Laverman et al., 1995).

Electron Transfer to and from Humic Substances and Other Extracellular Quinones All FMR that have been evaluated to date, including the hyperthermophiles, have the ability to transfer electrons to humic substances (humics) or other extracellular quinones such as the humics analog, anthraquinone-2,6-disulfonate (AQDS) Lovley et al., 1996; Lovley et al., 1998; Lovley et al., 2000). In those organisms in which the potential for growth has been evaluated, energy to support growth is from electron transport to humics and this capability is conserved. Electron-spin resonance (ESR) studies have suggested that quinones are important electron-accepting groups in the humics (Scott et al., 1998). The ESR studies with AQDS as the sole electron acceptor have directly demonstrated that energy can be conserved from electron transfer to extracellular quinones has been directly demonstrated in studies with AQDS as the sole electron acceptor (Lovley et al., 1996; Coates et al., 1998; Lovley et al., 1998). Humics can chelate Fe(III) that is also available for microbial reduction (Benz et al., 1998; Lovley and Blunt-Harris, 1999a), but the concentration of microbially reducible Fe(III) in humics is a minor fraction of the total electron-accepting capacity (Lovley and Blunt-Harris, 1999a).

A wide diversity of humics can serve as electron acceptors for Fe(III)-reducing microorganisms (Lovley et al., 1996; Scott et al., 1998). Highly purified reference humics that have been extracted from diverse environments can be obtained from the International Humic Substances Society. Other commercially available humics are highly impure, differ from humics found in soils and sediments, and therefore should be avoided for definitive studies because commercially available humics are highly impure and their characteristics are unlike the humics found in soils and sediments (Malcolm and MacCarthy, 1986).

The expense and technical difficulty of conducting studies with humics makes it preferable to carry out some studies on microbial reduction of extracellular quinones with humics analogs, such as AQDS (Lovley et al., 1996; Lovley et al., 1998). The advantages of AQDS are its low cost, high solubility, and its easy detection [an orange color develops when AQDS is reduced to anthrahydroquinone-2,6-disulfonate (AHQDS)].

Several FMR have the ability to use reduced extracellular quinones as an electron donor for reduction of electron acceptors such as nitrate and fumarate (Lovley et al., 1999b). *Shewanella alga* and *Geobacter sulfurreducens* grew with

AHQDS as the electron donor. However, other FMR that could oxidize AHQDS in cell suspensions could not be grown with AHQDS as the sole electron acceptor. The ability of FMR to both reduce and oxidize extracellular quinones permits their use with other quinone-oxidizing and quinone-reducing microorganisms as an interspecies electron transfer system in which quinones serve as the electron shuttle between the microorganisms (Lovley et al., 1999b).

Proton Reduction in Syntrophic Association with Hydrogen-Consuming Microorganisms In the absence of Fe(III) or other suitable electron acceptors, some organisms in the *Geobacteraceae* can transfer electrons to protons to produce hydrogen gas. For hydrogen production to be thermodynamically favorable, a sink for hydrogen, such as a hydrogen-consuming microorganism, must keep hydrogen concentrations low enough. For example, several *Pelobacter* species can oxidize ethanol to acetate and carbon dioxide when grown in association with hydrogen-consuming microorganisms (Schink, 1992). *G. sulfurreducens* can oxidize acetate to carbon dioxide when cultured with *Wolinella succinogenes*, which oxidizes hydrogen with concomitant reduction of nitrate (Cord-Ruwisch et al., 1998).

Reductive Dechlorination Several Fe(III)-reducing microorganisms are capable of using chlorinated compounds as electron acceptors. *Desulfuromonas chlorethenica*, which was isolated as a tetrachloroethylene-respiring microorganism (Krumholz et al., 1996; Krumholz, 1997), was found to grow also with Fe(III) as the electron acceptor, as expected for microorganisms within the family *Geobacteraceae* (Lonergan et al., 1996). Other *Geobacteraceae* that were evaluated did not reduce tetrachloroethylene. *Desulfitobacterium dehalogenans* which can use chlorophenolic compounds as an electron acceptor (Utkin et al., 1994), also can grow with Fe(III) as the electron acceptor (Lovley et al., 1998). Another chlorophenol-respiring species in the same genus, *Desulfitobacterium hafniense*, was reported to reduce Fe(III), but it was not reported whether growth was conserved from Fe(III) reduction (Christiansen and Ahring, 1996).

Recovery of Fe(III)- and Mn(IV)-Reducing Microorganisms in Culture

Localizing Zones of Fe(III) and Mn(IV) Reduction Although FMR can be recovered from nearly any soil or sediment sample, it is generally of interest to study organisms from habitats in which Fe(III) and Mn(IV) are ongoing processes. Dissimilatory Fe(III) and Mn(IV) reduction are geochemically most significant in anaerobic environments such as freshwater and marine sediments; flooded soils or the anaerobic interior of soil aggregates; the deep terrestrial subsurface; and shallow aquifers contaminated with organic compounds. In aquatic sediments and the terrestrial subsurface Fe(III) and Mn(IV) reduction are most apparent in discrete anoxic sediment layers in which the endproducts of Fe(III) and Mn(IV) reduction, Fe(II) or Mn(II), are accumulating. In the typical zonation of respiratory processes found with depth in aquatic sediments or along the groundwater flow path in the subsurface, the zones of Fe(III) and Mn(IV) reduction are typically bounded on one side by the zone of nitrate reduction and on the other side by the zone of sulfate reduction (Lovley and Chapelle, 1995c). In addition to these larger discrete zones of Fe(III) reduction and Mn(IV) reduction in sedimentary environments, it is important to recognize that many soils and sediments that are predominately aerobic also may contain abundant anaerobic microzones in which Fe(III) and Mn(IV) reduction may be taking place.

Although accumulation of dissolved Fe(II) and Mn(II) in groundwater or porewater can be used to help identify the zones of Fe(III) and Mn(IV) reduction in subsurface or aquatic sediments, such standard geochemical measurements can often fail to accurately locate the metal reduction zones (Lovley et al., 1994b). A primary reason for this failure is that high concentrations of Fe(II) and Mn(II) may be found in sediments in which other TEAPs, such as methanogenesis, predominate.

In environments where conditions approach steady-state, such as aquatic sediments and aquifers, measurements of dissolved hydrogen can be used to identify zones in which Fe(III) reduction is the TEAP (Lovley and Goodwin, 1988a; Lovley et al., 1994c). This is because there is a unique range of dissolved hydrogen that is associated with Fe(III) reduction that is the predominant TEAP in steady-state environments. Hydrogen measurements have not been used to localize Mn(IV)-reducing zones because: 1) hydrogen concentrations under Mn(IV)-reducing conditions are very low and difficult to accurately measure accurately; 2) hydrogen concentrations for Mn(IV) and nitrate reduction are similar; and 3) the low concentrations of Mn(IV) in many soils means that the Mn(IV) reduction zone is not extensive.

An alternative method for determining the zone of Fe(III) reduction in soils and sediments is to use [2-^{14}C]-acetate (Lovley, 1997a). The reduction of Fe(III) can be considered to be the

TEAP if: 1) a tracer quantity of [2-^{14}C]-acetate added to the sediments is converted to $^{14}CO_2$ with no production of $^{14}CH_4$; 2) the production of $^{14}CO_2$ is not inhibited with the addition of molybdate; 3) the sediments are depleted of nitrate; and 4) the sediments contain some Fe(II). The reasoning for this is that: 1) lack of $^{14}CH_4$ production rules out methanogenesis as a TEAP; 2) molybdate inhibits acetate oxidation by sulfate reducers so the lack of inhibition with molybdate rules out sulfate reduction as the TEAP; 3) nitrate reduction can not be an important TEAP in the absence of nitrate; and 4) Mn(IV) reduction can not be the TEAP in the presence of Fe(II) because Fe(II) rapidly reacts with Mn(IV) (Lovley and Phillips, 1988b) and thus Fe(II) will only be found if reactive Mn(IV) has been depleted.

The rates of other TEAPs can often be quantified in sediments with the use of radiotracers. Unfortunately, attempts to measure rates of Fe(III) reduction in sediments with radioactively labeled Fe(III) were unsuccessful (Roden and Lovley, 1993b). This was because there was rapid isotope exchange between the radiolabelled Fe(III) and other iron pools, including Fe(II), was rapid. Thus, it was not possible to monitor rates of microbial Fe(III) reduction by measuring the production of radiolabeled Fe(II) from labeled Fe(III).

Rates of Fe(III) and Mn(IV) reduction in sediments can be estimated from anaerobic incubations of sediments by monitoring the accumulation of Fe(II) and Mn(II) are monitored over time. It is important that the solid phase Fe(II) and Mn(II) pools be measured after acidic extractions or some other technique because most of the Fe(II) and Mn(II) are not recovered in the dissolved phase (Lovley and Phillips, 1988c; Lovley, 1991a). Geochemical modeling has been used to estimate rates of Fe(III) and Mn(IV) reduction in some aquatic sediments and subsurface environments and potentially could be used to identify zones of Fe(III) and Mn(IV) reduction (Lovley, 1995a).

Isolation Procedures Although some FMR also can use oxygen as an electron acceptor or are tolerant of exposure to air, many are strict anaerobes. Therefore, unless the goal is to specifically select for facultative FMR, the use of strict anaerobic technique is preferable in initial enrichment and/or isolation procedures. To date, most FMR have been recovered using slight modifications of standard (Miller and Wolin, 1974; Balch et al., 1979) anaerobic techniques. This involves the use of culture tubes or bottles fitted with thick butyl rubber stoppers; removing traces of oxygen from gases by passing the gases through a column of heated copper filings; and carrying out transfers with syringes and needles or under a stream of anoxic gas.

Culture media can be prepared with the classical approach (Hungate, 1969) of boiling the media under a stream of anoxic gas to remove dissolved oxygen and then dispensing into tubes or bottles under anaerobic conditions. Alternatively, aerobic media may be dispensed into individual tubes or bottles and then the media can be vigorously bubbled with anoxic gas to strip dissolved oxygen from the media (Lovley and Phillips, 1988c). Both media preparation approaches appear to yield similar organisms. Reducing agents such as Fe(II)—typically supplied at 1–3 mM as ferrous chloride—cysteine (0.25–1 mM), or sulfide (0.25–1 mM) can be added to dispensed media from anoxic stocks just prior to inoculation. In addition to reacting with any trace oxygen in the media, cysteine and sulfide will reduce Fe(III) and Mn(IV) in the media, producing Fe(II) and Mn(II). Fe(II) rapidly reacts with traces of oxygen, forming Fe(III). Manganese [Mn(II)] will only slowly react abiotically with oxygen. Many FMR have been recovered without the addition of reducing agents to the media. Once Fe(III) reduction begins, the Fe(II) formed serves as protection against oxygen contamination. Reducing agents are rarely used in media designed for liquid-to-liquid transfer of Fe(III)-reducing cultures because the inoculum of the Fe(III)-reducing cultures typically contain millimolar quantities of dissolved Fe(II), which will scavenge traces of oxygen from the media to which the inoculum has been added.

A variety of media has been successfully employed for the enrichment and isolation of FMR, many of which are given in the references provided with each of the organisms in Table 2. An example of a freshwater and a marine medium are provided below. No definitive comparative studies of the efficacy of various media in recovering FMR have been carried out. However, it has been found that the freshwater medium described here can be used to recover *Geobacter* species with 16S rDNA sequences that are closely related to the 16S rDNA sequences that predominate in the Fe(III) reduction zone of sandy aquifers (Rooney-Varga et al., 1999; Synoeyenbos-West et al., 1999).

Most successful isolations of pure cultures of Fe(III)- and Mn(IV)-reducing microorganisms have used either organic acids, primarily acetate or lactate, or hydrogen as the electron donor. If an enrichment step is used in the initial stages of recovery of the organisms, then fermentable compounds such as glucose generally result in the enrichment of fermentative microorganisms. However, as summarized above, some Fe(III)- and Mn(IV)-reducing microorganisms can use sugars and amino acids as electron donors and

these electron donors potentially could be be used for direct isolation of FMR.

A variety of Fe(III) and Mn(IV) forms that were discussed above can be used as electron acceptors for enrichment or isolation. Iron added as Fe(III)-citrate and Fe(III) pyrophosphate is not ideal for enrichment cultures as the citrate is rapidly degraded by microorganisms other than Fe(III) reducers. Once the citrate is degraded, the Fe(III) from the Fe(III)-citrate precipitates as an insoluble Fe(III) oxide and thus defeats the purpose of adding the chelator. The compound Fe(III)-NTA is relatively resistant to anaerobic degradation and can be used as a soluble source of Fe(III) for enrichment of Fe(III) reducers. However, as noted above, it is not suitable for use in media with marine salinities or at high temperature. Both Fe(III)-citrate and Fe(III)-NTA are toxic to some Fe(III) reducers. Although solubilization of Mn(IV) with various chelators for use in recovery of Mn(IV)-reducing microorganisms may be possible, this approach has not been widely used.

As noted above, poorly crystalline Fe(III) oxide is typically the insoluble Fe(III) oxide of choice for culturing. A wide diversity of other Fe(III) oxides can be synthesized (Schwertmann and Cornell, 1991), if desired. If the media is dispensed aerobically into culture vessels, then a slurry of the Fe(III) or Mn(IV) oxide can be added to the vessels prior to addition of the media. An advantage of using poorly crystalline Fe(III) oxide as the electron acceptor is that most Fe(III)-reducing microorganisms convert the poorly crystalline Fe(III) oxide to the magnetic mineral magnetite during reduction. This is visually apparent as the reddish, non-magnetic Fe(III) oxide is transformed into a black, highly magnetic precipitate (Lovley et al., 1987c). Reduction of the Mn(IV) oxide is also visually apparent in bicarbonate-buffered media because reduction of the dark Mn(IV) oxide results in its dissolution and concomitant accumulation of rhodochrosite, a white Mn(II) carbonate mineral.

An alternative electron acceptor that can be used for the recovery of Fe(III)- and Mn(IV)-reducing microorganisms is the humics analog, AQDS, which is typically provided at 5 mM. All of the Fe(III)-reducing microorganisms that have been evaluated can reduce AQDS, whereas microorganisms that do not reduce Fe(III) can not reduce AQDS (Lovley et al., 1996; Lovley et al., 1998; Lovley et al., 2000). Recovery of AQDS-reducing microorganisms either through enrichment and isolation procedures or dilution-to-extinction approaches yield organisms that also can reduce iron [Fe(III); Coates et al., 1998)]. The reduction of AQDS to AHQDS is visually apparent as the conversion of the relatively colorless AQDS to the orange, AHQDS.

Fe(III)- and Mn(IV)-reducing microorganisms can be obtained in pure culture through standard anaerobic approaches of isolating colonies in tubes or on plates or through dilution-to-extinction in liquid media. Soluble Fe(III) forms or AQDS are often used for isolating colonies on agar-solidified media, but colonies also can be obtained by incorporating Fe(III) and Mn(IV) oxides into solidified media. The Fe(III)- and Mn(IV)-reducing microorganisms that have the ability to use other electron acceptors often can be successfully purified from Fe(III)- or Mn(IV)-reducing enrichment cultures with these alternative electron acceptors. Common alternative electron acceptors include nitrate, fumarate, sulfur, and oxygen.

Suggested Media for Enrichment and Culturing of FMR Freshwater and marine media suitable for culturing a diversity of mesophilic FMR are described below. A variety of other media have also been used which can be found in the references for the individual organisms. The media described here have a bicarbonate-carbon dioxide buffer system and the headspace gas typically contains 20% carbon dioxide to establish an initial pH of ca. 6.8.

Freshwater Medium

To 900 ml water add:

$NaHCO_3$	2.50 g
NH_4Cl	0.25 g
$NaH_2PO_4 \cdot H_2O$	0.60 g
KCl	0.10 g
Vitamin Solution	10.0 ml
Mineral Solution	10.0 ml

Bring solution to a final volume of 1 liter. Media is dispensed, sparged with an 80:20 mixture of N_2:CO_2 gas and then autoclaved.

Marine Medium

Medium contains, per liter:

NaCl	20.0 g
KCl	0.67 g
$NaHCO_3$	2.5.0 g
Vitamin solution	10.0 ml
Mineral solution	10.0 ml
RST minerals stock	20.0 ml
Salt stock*	50.0 ml

*Add salt solution aseptically and anaerobically after autoclaving.

RST Minerals Stock 50X

Stock contains, per 100 ml:

NH_4Cl	5.0 g
KCl	0.5 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	1.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g

Salt Stock

Stock contains, per 100 ml:

$MgCl_2 \cdot 6H_2O$	21.2 g
$CaCl_2 \cdot 2H_2O$	3.04 g

Vitamin Solution

Solution contains, per liter:

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine HCl	10.0 mg
Riboflavin	5.0 mg
Thiamine	5.0 mg
Nicotinic acid	5.0 mg
Pantothenic acid	5.0 mg
B-12	0.1 mg
p-Aminobenzoic acid	5.0 mg
Thioctic acid	5.0 mg

Mineral Solution

grams per liter

Trisodium nitrilotriacetic acid	1.5 g
$MgSO_4$	3.0 g
$MnSO_4 \cdot H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$ZnCl_2$	0.13 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$AlK(SO_4)_2 \cdot 12H_2O$	0.01 g
H_3BO_3	0.01 g
Na_2MoO_4	0.025 g
$NiCl_2 \cdot 6H_2O$	0.024 g
$Na_2WO_4 \cdot 2H_2O$	0.025 g

Preparation of Fe(III) and Mn(IV) Forms

Poorly Crystalline Fe(III) Oxide Dissolve $FeCl_36H_2O$ in water to provide final concentration of 0.4M. Stir continually while SLOWLY adjusting the pH to 7.0 dropwise with 10 M NaOH solution. It is extremely important not to let the pH rise above pH 7 even momentarily during the neutralization step because this will result in an Fe(III) oxide that is much less available for microbial reduction. Continue to stir for 30 minutes once pH 7 is reached and recheck pH to be sure it has stabilized at pH 7. To remove dissolved chloride, centrifuge the suspension at 5,000 rpm for 15 minutes. Discard the supernatant, resuspend the Fe(III) oxide in water, and centrifuge. Repeat six times. On the last wash, resuspend the Fe(III) oxide to a final volume of approximately 400 ml, and after determining iron content, adjust Fe(III) concentration to approximately 1 mole per liter. Typically, Fe(III) oxide is added to individual tubes of media to provide 100 mmol per liter.

Fe(III)-Citrate Prior to the addition of any of the media constituents, heat 800 ml of water on a stirring hot-plate to near boiling. Add Fe(III)-citrate [typically 13.7 g to provide a final concentration of ca. 50 mM Fe(III)]. Once the ferric citrate is dissolved quickly cool the medium to room temperature in an ice bath. Adjust pH to 6.0 using 10N NaOH. When the pH approaches 5.0, add the NaOH dropwise. Add medium constituents as outlined above. Bring to a final volume of 1 liter. Do not expose this media to direct sunlight to prevent photoreduction of the Fe(III).

Fe(III) Nitrilotriacetic Acid To make a stock of 100 mM Fe(III)-NTA, dissolve 1.64 g of $NaHCO_3$ in 80 ml water. Add 2.56 g $C6H6NO6Na_3$ (sodium nitrilotriacetic acid) and then 2.7 g $FeCl_36H_2O$. Bring the solution up to 100 ml. Sparge the solution with N_2 gas and filter sterilize into a sterile, anaerobic serum bottle. Do not autoclave. Typically, 100 mM Fe(III)-NTA stock is added to individual tubes of media to provide a final concentration of 5 or 10 mmol of Fe(III).

Goethite Prepare a 0.4M $FeCl_36H_2O$ solution. With continual stirring, adjust the pH to between 11 and 12 with 10 M NaOH solution. The suspension will become very thick. Ensure continual stirring and rinse the pH electrode frequently. The color of this suspension will turn to an ochre color as goethite is formed. One week at room temperature followed by 16 hours at 90°C is sufficient to convert the Fe(III) to goethite. The suspension should be washed to remove chloride, as described above for poorly crystalline Fe(III) oxide. The formation of goethite should be confirmed by X-ray diffraction analysis. The Fe(III) oxide also should be tested with extractants (Lovley and Phillips, 1987b; Phillips and Lovley, 1987) to ensure that it does not contain poorly crystalline Fe(III) oxide.

Hematite Hematite is readily available from chemical supply companies as "Ferric Oxide."

Manganese Oxide To 1 liter of a solution containing 80 mM NaOH and 20 mM $KMnO_4$ slowly add 1 liter of 30 mM $MnCl_2$ with mixing. Wash the manganese oxide precipitate, as described above for poorly crystalline Fe(III) oxide, to lower the dissolved chloride concentration.

Enumeration of Fe(III)- and Mn(IV)-Reducing Microorganisms The FMR in environments can be enumerated with standard most-probable-number (MPN) culturing techniques using variations of media described above. Enumerations typically use Fe(III) or AQDS as the electron acceptor with the understanding that the Fe(III)-reducing microorganisms recovered are likely to

have the ability to reduce Mn(IV) as well. Poorly crystalline Fe(III) oxide or Fe(III)-NTA is preferred over Fe(III)-citrate and Fe(III)-pyrophosphate, which promote the growth of fermentative microorganisms. One successful approach has been to add a combination of poorly crystalline Fe(III) oxide (100 mmol/liter) and 4 mM NTA to provide a supply of chelated Fe(III).

FMR also can be counted in plate counts in which Fe(III)-NTA or AQDS has been added as the electron acceptor. Clearing zones develop around FMR reducing Fe(III)-NTA, and growth with AQDS as the electron acceptor results in the formation or orange colonies or zones.

When possible, molecular enumeration rather than viable culturing enumeration techniques are the preferred methods because of the potential biases associated with the latter. The wide phylogenetic diversity of dissimilatory Fe(III) reducing microorganisms and the lack of an identified conserved gene associated with Fe(III) reduction make it impossible to enumerate Fe(III)-reducing microorganisms with one specific gene sequence (Lonergan et al., 1996). However, target 16S rRNA sequences that are selective for known groups of Fe(III)-reducing microorganisms have been identified and have been used to study the distribution of Fe(III)-reducing microorganisms in sedimentary environments (DiChristina and DeLong, 1993; Anderson et al., 1998; Rooney-Varga et al., 1999; Synoeyenbos-West et al., 1999).

Summary

Microbial reduction of Fe(III) and Mn(IV) is of environmental significance in a variety of aquatic sediments and the subsurface, influencing both the carbon cycle and the fate of many metals and metalloids, in both pristine and contaminated environments. Geological and microbiological evidence suggests that Fe(III) reduction was one of the earliest forms of respiration. A wide phylogenetic diversity of Fe(III)- and Mn(IV)-reducing microorganisms have been recovered in pure culture, but with the exception of the recently recognized importance of *Geobacter* in subsurface environments, little is known about the distribution or relative contributions of the various Fe(III)-reducing microorganisms. The study of the mechanisms of Fe(III) and Mn(IV) reduction are also in their infancy. However, now that methods for culturing these organisms are well developed, it seems likely that increased insight into the ecophysiology of Fe(III)- and Mn(IV)-reducing microorganisms is forthcoming.

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Prokaryotes (2006) 2:659–768
DOI: 10.1007/0-387-30742-7_22

CHAPTER 1.22

Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes

RALF RABUS, THEO A. HANSEN AND FRIEDRICH WIDDEL

Introduction

A unique characteristic in the prokaryotic world is the multiplicity of life strategies without any involvement of oxygen. Actually, life in anoxic habitats is prokaryotic to a large extent. Prokaryotes have evolved not only various fermentation pathways, but also the capacity to couple the oxidation of organic substrates to the reduction of inorganic compounds (other than O_2) to conserve energy for anaerobic growth. Electron acceptors reduced by prokaryotes under anoxic conditions are nitrate, manganese(IV), ferric iron, sulfate, elemental sulfur, other sulfur species (e.g., thiosulfate), carbon dioxide, protons and even oxidized forms of naturally less abundant elements such as arsenate(V), chromate(VI), selenate and uranium(VI). In several prokaryotes, even the electron donor may be inorganic, which results in purely inorganic (lithotrophic) redox reactions for energy conservation under anoxic conditions; notable examples are the oxidation of sulfur species with nitrate, or of molecular hydrogen with nitrate, iron(III), sulfate, sulfur or CO_2. Two organic compounds with some relationship to inorganic electron acceptors are dimethylsulfoxide (DMSO) and trimethylamine-N-oxide (TMAO). In these compounds, anaerobic microorganisms reduce the oxygenated sulfur or nitrogen moiety, respectively. In most cases, the electron transport to the inorganic electron acceptors is associated with a mode of energy conservation that may be regarded as an anaerobic analogue to respiration with O_2. This is particularly evident if the only electron donor is H_2. In such a process, ATP synthesis can be only explained by a chemiosmotic transmembrane process rather than by fermentative substrate-level phosphorylation. Because of this analogy to the known respiratory chain, growth by utilization of inorganic electron acceptors other than O_2 is usually termed "anaerobic respiration". In some microorganisms, inorganic compounds (as for instance ferric iron or sulfur) may be reduced in by-reactions without obvious connection to respiration-like chemiosmotic energy conservation. Such by-reactions may facilitate fermentation (disposal of reducing equivalents) but they should not be termed "anaerobic respirations". Interestingly, most types of anaerobic respirations have not been encountered so far in the eukaryotic domain. The only (thus far reported) case of anaerobic respiration in a eukaryote is nitrate-reduction by a flagellate (Finlay et al., 1983). The microbial reduction of inorganic compounds contributes significantly to the global cycling of elements and represents the counterpart to oxidative microbial processes, e.g., nitrification, iron oxidation and sulfur oxidation.

Among the anaerobic respirations, the reduction of sulfur species is most striking because it gives rise to a conspicuous end product, hydrogen sulfide (H_2S), which is commonly known as a toxic chemical with a characteristic smell. By its chemical reactivity (e.g., toward iron minerals and oxygen), H_2S has a pronounced impact on the chemistry of the environment. Despite of its toxicity, sulfide serves as electron donor for a great diversity of aerobic chemotrophic and anoxygenic phototrophic microorganisms that may form visible blooms in sulfidic habitats. The natural reduction and oxidation of sulfur species is known as the sulfur cycle. Because sulfate is the thermodynamically stable and most abundant form of sulfur in our oxic biosphere, sulfate reduction forms the basis of the biological sulfur cycle (Henrichs and Reeburgh, 1987; Jørgensen, 1987; Skyring, 1987; Widdel, 1988). A great diversity of sulfate-reducing microorganisms has been isolated from aquatic habitats.

The chemical and biological oxidation processes of sulfide do not always lead directly to sulfate, but often yield intermediate oxidation states such as elemental sulfur or thiosulfate. These may serve as electron acceptors for anaerobic microorganisms that cannot reduce sulfate. Among these, sulfur-reducing anaerobic microorganisms have been isolated most frequently, and their diversity is comparable to that of sulfate-reducing microorganisms.

The present chapter gives an overview of prokaryotes that reduce sulfate or elemental

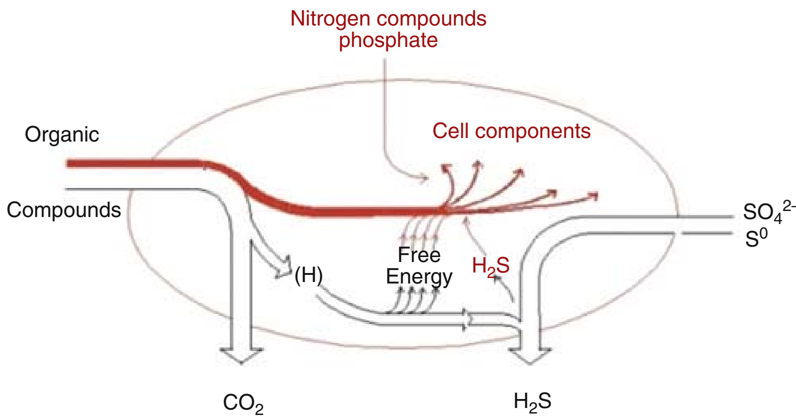


Fig. 1. Metabolic principle of sulfate-reducing bacteria. As in other anaerobic bacteria, the main part of the electron donor is oxidized for energy conservation, and only a minor fraction is assimilated into cell mass. Catabolism (energy metabolism) is shown in black; anabolism (cell synthesis) is shown in red.

sulfur in their energy metabolism (see Fig. 1). Growth by reduction of other sulfur species is also included. Such bacteria have also been summarized as sulfidogenic bacteria (sulfide-forming) bacteria (Zeikus, 1983; Lupton et al., 1984); however, strictly speaking, this term would also apply to putrefying bacteria that liberate sulfide from sulfur-containing organic molecules during their degradation.

Sulfate- or sulfur-reducing microorganisms are long-established functional groups, like denitrifying, sulfur-oxidizing, methylotrophic or phototrophic bacteria. They are not necessarily coherent from the viewpoint of modern molecular systematics such as grouping based on 16S rRNA sequences. Nevertheless, the treatment of such functional groups besides molecular systematic groups is still the most appropriate basis for an understanding and comparison of physiological, bioenergetic and enzymatic properties and the roles of microorganisms in their natural habitat. Hence, the present chapter is mostly organized according to functional aspects, but it will distinguish between the phylogenetic domains and treat bacterial and archaeal sulfate-reducers and sulfur reducers separately.

Historical Overview

Sulfate-Reducing Bacteria

Meyer (1864) and Cohn (1867) first recognized the production of striking concentrations of H_2S in aquatic habitats as a biologically mediated reduction of sulfate. Hoppe-Seyler (1886) demonstrated a complete oxidation of cellulose in anaerobic enrichment cultures with mud if gypsum ($CaSO_4$) was provided as a source of sulfate; the latter was reduced to sulfide. Beijerinck's (1895) investigations into microbial sulfide production resulted in the first isolation of a sulfate-reducing bacterium (named *Spirillum desulfuricans*), which was recognized as a strict anaerobe. The culture was isolated with malate and aspartate. Van Delden (1903a, 1903b) grew sulfate-reducing bacteria on lactate, which is often still used for cultivation. The first thermophilic sulfate reducer with an optimal growth temperature of 55°C was described by Elion (1925). Rubentschik (1928) observed a utilization of acetate and butyrate by sulfate reducers. In a comprehensive nutritional study, Baars (1930) demonstrated that *Vibrio desulfuricans* oxidized lactate or ethanol to acetate. Another type, *Vibrio rubentschikii*, was used in addition to acetate, propionate, butyrate and other compounds that were completely oxidized to CO_2; unfortunately, this species was not preserved. *Vibrio desulfuricans* was the former *Spirillum* which finally became *Desulfovibrio* (Kluyver and van Niel, 1936; Stephenson and Strickland, 1931) observed an oxidation of H_2 by sulfate reducers. The first described spore-forming sulfate-reducing bacteria were thermophiles named *Clostridium nigrificans* (Werkman and Weaver, 1927) and *Sporovibrio desulfuricans* (Starkey, 1938); they were later recognized as the same species (Campbell et al., 1957).

In the 1950s and 1960s, principal insights into the biochemistry of sulfate-reducing bacteria were achieved. *Desulfovibrio* was the first anaerobe in which a cytochrome was detected (Ishimoto et al., 1954b; Postgate, 1953). Earlier, this type of pigment was thought to be associated only with O_2 respiration. The type of cytochrome discovered in *Desulfovibrio* was termed c_3. Investigations into the biochemistry of dissimilatory sulfate-reduction revealed differences from the pathway of assimilatory sulfate reduction known at that time (Lipmann, 1958). In *Desulfovibrio*, adenosine-5′-phosphosulfate (APS) was not further phosphorylated to 3′-phosphoadenosine-5′-phosphosulfate (PAPS), as

in the assimilatory pathway, but rather directly reduced to sulfite and AMP (Peck, 1959; 1962; 1965). Furthermore, electron transfer was demonstrated to be coupled to phosphorylation (Peck, 1966). A green protein, desulfoviridin, was first described by Postgate (1956) and subsequently recognized as sulfite-reductase. The mechanism of sulfite-reduction to sulfide was less understood. In addition to the electron acceptor sulfate, the metabolic fate of selected organic substrates, such as pyruvate and cysteine, was studied in sulfate-reducing bacteria (Senez, 1954a; Senez and Leroux-Gilleron, 1954b). Cultures of sulfate-reducing bacteria existing at that time oxidized their substrates (such as lactate, ethanol or malate) incompletely to acetate. Sulfate reducers formerly grown on acetate or higher fatty acids (Rubentschik, 1928; Baars, 1930) had not been preserved.

In the 1960s, also a need for a proper classification of existing strains emerged. All sporeforming strains were classified or reclassified in the new genus *Desulfotomaculum* (Campbell and Postgate, 1965); the nonsporeforming, vibrio-shaped isolates were described as *Desulfovibrio* species (Postgate and Campbell, 1966). Later, nutritionally similar new mesophilic and thermophilic rod-shaped sulfate reducers were included in the genus *Desulfovibrio* (Rozanova and Khudyakova, 1974; Rozanova and Nazina, 1976); later, these sulfate reducers were reclassified as *Desulfomicrobium* and *Thermodesulfobacterium*, respectively.

In the 1970s major advances were achieved in the characterization of various electron carriers, e.g., the resolution of the crystal structure of cytochrome c_3 from *Desulfovibrio* (DerVartanian and LeGall, 1974). Furthermore, first evidence for a periplasmic location of hydrogenase emerged (Bell et al., 1974). In the field of biogeochemistry, new insights into the role of sulfate-reducing bacteria in natural habitats were rendered possible by the introduction of the radiotracer technique using $^{35}SO_4^{2-}$ (Sorokin, 1972). More than 50% of the organic carbon in marine sediments was shown to be mineralized via sulfate reduction (Jørgensen and Fenchel, 1974; Jørgensen, 1977; Jørgensen, 1982). This process could not be explained by the incomplete substrate oxidation to acetate in the sulfate-reducing bacteria (*Desulfovibrio* and *Desulfotomaculum* species) known at that time.

Anaerobic enrichment studies with various organic substrates lead to the recognition of diverse catabolic capacities including the degradation of aromatic organic acids in this group of microorganisms. Also, the capacity for acetate oxidation and complete mineralization of organic substrates, described in old reports (Hoppe-Seyler, 1886; Rubentschik, 1928; Baars, 1930), were confirmed to exist in sulfate-reducing bacteria and found in several novel types of this group. Some new species were facultatively autotrophic. The diversity of the isolates required the establishment of a new *Desulfotomaculum* species (Widdel and Pfennig, 1977) and new genera, such as *Desulfobacter*, *Desulfococcus*, *Desulfonema*, *Desulfobulbus* and *Desulfosarcina* (Widdel and Pfennig, 1977; Widdel, 1980; Widdel and Pfennig, 1981b; Pfennig et al., 1981c; Widdel and Pfennig, 1982; Widdel et al., 1983).

In the 1980s, main insights into enzymatic reactions and bioenergetics of entire metabolic pathways in sulfate-reducing bacteria were achieved and studies of functional genes began. Precise ATP balances of sulfate-reduction with H_2 were calculated from chemostat studies (Badziong and Thauer, 1978; Nethe-Jaenchen and Thauer, 1984). In carbon metabolism, two alternative pathways for complete oxidation of acetyl-CoA, the citric acid cycle (Brandis-Heep et al., 1983; Gebhardt et al., 1983) and the oxidative CO-dehydrogenase pathway (Schauder et al., 1986; Schauder et al., 1989; Spormann and Thauer, 1988) were shown to be operative in distinct groups of sulfate-reducing bacteria that oxidized their substrate completely to CO_2. In autotrophic sulfate-reducing bacteria (Widdel, 1980; Klemps et al., 1985; Brysch et al., 1987), the synthesis of acetyl-CoA from CO_2 was demonstrated to occur via the reductive citric-acid cycle (Schauder et al., 1987) or the reductive CO-dehydrogenase pathway (Jansen et al., 1984; 1985; Schauder et al., 1989). Investigations into the metal clusters and cellular localization of hydrogenases led to the recognition of three different types of this enzyme in *Desulfovibrio*, the [Fe], [NiFe] and [NiFeSe] hydrogenase (Huynh et al., 1984a; Rieder et al., 1984; Teixeira et al., 1986; for summary see Fauque et al., 1988). First investigations into the molecular biology and genetics of sulfate-reducing bacteria included the study of plasmids (Postgate et al., 1984c; Postgate et al., 1986; Postgate et al., 1988; Powell et al., 1989) and genes for nitrogenase (Postgate et al., 1986; Kent et al., 1989), hydrogenase (Voordouw and Brenner, 1985a; Voordouw et al., 1985b), cytochromes (van Rooijen et al., 1989; Pollock et al., 1991), other redox proteins (Krey et al., 1988; Curley and Voordouw, 1988; Brumlik and Voordouw, 1989) and genes for biosynthetic enzymes (Li et al., 1986; Fons et al., 1987) in *Desulfovibrio* species. Also, genetic exchange systems were established for *Desulfovibrio* strains (Rapp and Wall, 1987; van den Berg et al., 1989; Powell et al., 1989). Furthermore, basic insights into the energy-mode of sulfate transport in various genera of sulfate-reducing bacteria were obtained

(Cypionka, 1987; Cypionka, 1989; Warthmann and Cypionka, 1990).

Attempts to enrich acetate-oxidizing anaerobes with sulfur-oxo anions other than sulfate led to the discovery of growth by disproportionation of sulfite and thiosulfate (Bak and Pfennig, 1987). The fact that anaerobic bacteria in natural habitats may be confronted with oxic conditions led to studies on the relation of various species of sulfate-reducing bacteria to O_2 (Widdel, 1980; Cypionka et al., 1985; Dilling and Cypionka, 1990).

Until the early 1980s, sulfate reducers were traditionally classified by phenotypic characteristics, such as nutrition, morphology and chemical, or biochemical markers, or both (Pfennig et al., 1981c; Postgate, 1984a; Widdel and Pfennig, 1984). Examples for applied chemotaxonomic markers are desulfoviridin (Postgate, 1959), lipid fatty acids (Boon et al., 1977; Ueki and Suto, 1979; Taylor and Parkes, 1983; Dowling et al., 1986), or menaquinones (Collins and Widdel, 1986). As the application of 16S rRNA sequence analysis became more and more common for the elucidation of natural relationships among microorganisms, this approach became decisive in the systematics of sulfate-reducing bacteria. The first comparative analysis of 16S rRNA sequence of a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, revealed relationships to *Myxococcus* and phototrophic purple bacteria (Oyaizu and Woese, 1985). A following comprehensive study based on the 16S rRNA oligonucleotide catalogs included the spore-forming *Desulfotomaculum* species and various nonsporeforming sulfate-reducing bacteria (Fowler et al., 1986). *Desulfotomaculum* was shown to branch with Gram-positive bacteria, as already indicated by the electron microscopy of the cell wall structure (Sleytr et al., 1969; Nazina and Pivavora, 1979). All other sulfate reducers were found to affiliate with a branch of Gram-negative bacteria that also included the sulfur-reducing *Desulfuromonas* as well as *Myxococcus* and *Bdellovibrio* species. This branch of Gram-negative bacteria was termed the δ-subdivision of the purple bacteria and their nonphototrophic relatives (Woese, 1987), even though a phototroph belonging to this subdivision has not been discovered thus far. Later, this phylogenetic assemblage became known as δ-subclass of the Proteobacteria (Stackebrandt et al., 1988). Most described genera of sulfate-reducing bacteria affiliate with this subclass. Somewhat later, attempts were made to group the nutritionally diverse genera in meaningful higher taxa based on 16S rRNA sequences. First, two families were suggested within the sulfate-reducing bacteria of the δ-subclass, the *Desulfovibrionaceae* and the *Desulfobacteriaceae* (Devereux et al., 1990; Widdel and Bak, 1992). However, the number of new isolates of sulfate-reducing and other bacteria and recognizable phylogenetic lineages within the δ-subclass increased further. Today, a systematic structure of the δ-subclass needs the establishment of several families and even orders. A novel thermophilic sulfate-reducing bacterium, *Thermodesulfobacterium*, was isolated in 1983 (Zeikus et al., 1983). Metabolically it resembled *Desulfovibrio*, however the lipids were ether-linked (Langworthy et al., 1983). Later, this organism was recognized as a deeply branching line of decent within the eubacteria, distant from the δ-subclass of Proteobacteria (Henry et al., 1994). An earlier isolated thermophilic sulfate-reducing bacterium was recognized as a member of the same branch (Rozanova and Pivavora, 1988b). An overview of the major groups of sulfate-reducing bacteria and archaea (see following section) within the 16S rRNA-based tree of life is shown in Fig. 2.

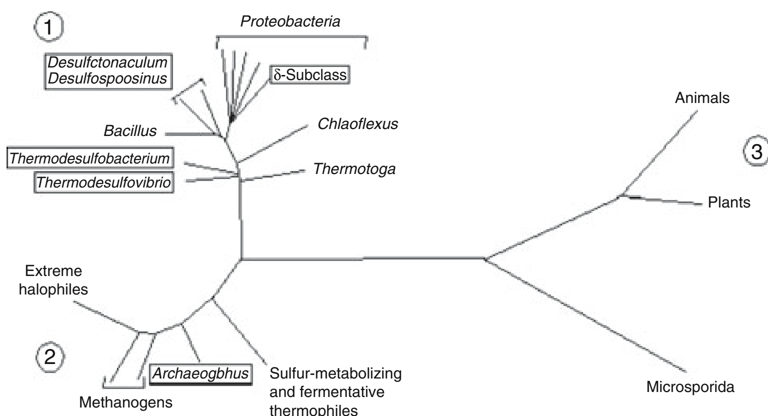


Fig. 2. Phylogenetic trees reflecting the relationships of groups of sulfate-reducing bacteria to other organisms on the basis of 16S rRNA sequences. (A) Overview showing the three domains of life: (1), Eubacteria; (2), Archaebacteria; (3), Eukaryotes. The tree was adapted from Achenbach-Richter et al. (1987) and Devereux et al. (1989). (B) More refined tree with genera. The tree was constructed using the ARB database and programs implemented therein (Ludwig et al., 1998). Scale bar represents 10 inferred nucleotide substitutions per 100 nucleotides.

Fig. 2. *Continued*

In the 1990s, many advances were achieved in the study of individual proteins, genes, degradative capacities and ecology of sulfate-reducing bacteria. Among the hydrogenases studied in various microorganisms, the first crystal structure was obtained from the enzyme in a sulfate-reducing bacterium, *Desulfovibrio gigas* (Volbeda et al., 1995). Structural and functional investigations into hydrogenases continued steadily (Volbeda et al., 1996; Higuchi et al., 1997; Nicolet et al., 1999) and included the recognition of cyanide and CO as ligands of the active-site iron atom. Crystal structures of cytochrome c_3 molecules from various *Desulfovibrio* sp. were also determined at high resolution (Matias et al., 1993; Czjzek et al., 1994; Fritz, 1999) and revealed a similar overall structure. Another contribution of general significance to biochemistry was the elucidation of the crystal structure of aldehyde oxidoreductase from *Desulfovibrio gigas* (Romão et al., 1995). The crystal structure was not only the first to be resolved within the xanthine oxidase family, but also provided the correct structure of a widespread class of co-factors, the molybdopterins. The first crystal structure of a dissimilatory nitrate reductase was again determined from a sulfate-reducing bacterium, *Desulfovibrio desulfuricans* (Dias et al., 1999).

Genetic studies were mainly carried out with *Desulfovibrio desulfuricans* and *D. vulgaris* because of the ease of cultivation and the applicability of antibiotics as selecting agents for mutants. Methods for the exchange of genetic material such as transduction, conjugation and transformation were further developed (for review see Voordouw and Wall, 1993b). Plasmids were constructed that can be applied as shuttle vectors for recombinant DNA (e.g., Rousset et al., 1998a). Transposons (Wall et al., 1996) and plasmids carrying the counterselectable marker *sacB* (Keon et al., 1997) were

applied to sulfate-reducing bacteria to create mutants.

In the 1990s, pure cultures of sulfate-reducing bacteria were isolated that could oxidize alkanes (Aeckersberg et al., 1991; Aeckersberg et al., 1998; So and Young, 1999a), toluene (Rabus et al., 1993; Beller et al., 1996), xylenes (Harms et al., 1999) or naphthalene (Galushko et al., 1999) completely to CO_2. Furthermore, it was demonstrated that sulfate-reducing bacteria could grow with crude oil as the sole source of organic substrates (Rueter et al., 1994; Rabus et al., 1996), an aspect that contributes to our understanding of sulfide production in oil reservoirs and oil production plants. Anaerobic degradation of hydrocarbons as chemically sluggish molecules requires a suite of unusual reactions (e.g., the fumarate-dependent activation of toluene to benzylsuccinate; Beller and Spormann, 1997b; Rabus and Heider, 1998) as first discovered in denitrifiers (Biegert et al., 1996). In addition to hydrocarbons, other organic molecules were newly recognized as organic substrates for sulfate-reducing bacteria. Glycolate can be oxidized completely to CO_2 by the novel sulfate reducer *Desulfocystis glycolicus* (Friedrich and Schink, 1995; Friedrich et al., 1996). Utilization of the sulfur compound dimethylsulfoniopropionate (DMSP) was demonstrated with several sulfate-reducing bacteria (van der Maarel et al., 1996a, b; Jansen and Hansen, 1998). Another type of novel sulfate-reducing bacterium was shown to oxidize a reduced inorganic phosphorous compound, phosphite (Schink and Friedrich, 2000).

The introduction of molecular methods, especially those based on 16S rRNA sequences, into microbial ecology was also fruitful for the study of natural populations of sulfate-reducing bacteria. After the first construction of 16S rRNA-targeted probes for *Desulfovibrio* species (Amann et al., 1990) and other groups of sulfate-reducing bacteria (Devereux et al., 1992), these and other probes were subsequently applied to biofilms (Ramsing et al., 1993; Santegoeds et al., 1999; Schramm et al., 1999), marine water columns (Ramsing et al., 1996; Teske et al., 1996), various sediments (Llobet-Brossa et al., 1998; Rooney-Varga et al., 1998; Sass et al., 1998; Sahm et al., 1999a), microbial mats (Fukui et al., 1999; Minz et al., 1999a) and an enrichment culture with crude oil (Rabus et al., 1996). Probe hybridization of rRNA after extraction or in whole cells, often in combination with counting series, confirmed the significance of sulfate-reducing bacteria in aquatic habitats, as shown in biogeochemical studies. Further approaches for the study of sulfate-reducing bacteria in habitats were based on reverse sample genome probing (Voordouw et al., 1991), hydrogenase genes (Wawer et al., 1997) or sulfite-reductase genes (Wagner et al., 1998; Minz et al., 1999b). Molecular methods in combination with cultivation and biogeochemical studies also provided basic insights into sulfate-reducing populations in cold sediments, which cover large areas of the ocean floor. Sulfate-reduction rates measured off Svalbard in the Arctic Ocean were comparable to those in marine sediments from temperate climate sites (Sagemann et al., 1998). Several previously unknown types of psychrophilic sulfate-reducing bacteria (e.g., *Desulfotalea*, *Desulfofaba*) could be isolated in pure cultures (Knoblauch et al., 1999a, b; Knoblauch and Jørgensen, 1999c) and shown to constitute a significant fraction of the natural cold-adapted population (Sahm et al., 1999b). The combination of pure-culture studies and molecular approaches also provided new insights into the ecology of gliding, filamentous sulfate-reducing bacteria, genus *Desulfonema* (Fukui et al., 1999).

Sulfate-Reducing Archaea

When, during the early 1980s, several breakthroughs occurred in the discovery of novel, extremely thermophilic Archaea (see for instance Stetter, 1985), the novel isolates initially comprised methanogenic, fermentative, sulfur-reducing and some aerobic microorganisms, but no sulfate reducers. Thermophilic sulfate-reducing microorganisms known at that time were bacteria with temperature optima below 75°C. In 1987, however, enrichment and isolation studies with samples from hydrothermal systems revealed the existence of archaeal sulfate reducers with a growth optimum of 83°C (Stetter et al., 1987). The new sulfate reducer named *Archaeoglobus fulgidus* (Stetter, 1988) contains the cofactor F_{420}, tetrahydromethanopterin (Stetter et al., 1987), and methanofuran (White, 1988; Gorris et al., 1991), which were known before only from methanogens. Furthermore, *Archaeoglobus* was shown to be phylogenetically more closely related to methanogens than to thermophilic archaeal sulfur-reducers or sulfur oxidizers (Achenbach-Richter et al., 1987). Further new species of the genus were *A. profundus* (Burggraf et al., 1990) and *A. lithotrophicus* (Stetter et al., 1993).

Because the existing biochemical knowledge about mesophilic sulfate-reducing bacteria and methanogens could be applied to the study of *Archaeoglobus*, progress in the understanding of its metabolic pathways, enzymes and underlying genes was rapid. In the carbon metabolism, the pathway for complete oxidation of lactate to CO_2 could be elucidated. It was recognized as an archaeal parallel of the CO dehydrogenase pathway in mesophilic sulfate-reducing bacteria

(Schauder et al., 1986; Schauder et al., 1989), with the involvement of the archaeal cofactors (Möller-Zinkhan et al., 1989; Möller-Zinkhan and Thauer, 1990; Schmitz et al., 1991; Klein et al., 1993; Schwörer et al., 1993). Also, enzymes in the transport of reducing equivalents were investigated (Kunow et al., 1994; Kunow et al., 1995). Autotrophic CO_2 fixation in *A. lithotrophicus* was recognized to occur via the reductive CO dehydrogenase pathway (Vorholt et al., 1995), again a parallel to CO_2 assimilation in sulfate-reducing bacteria (Jansen et al., 1984; 1985; Schauder et al., 1989). The reduction of sulfate was shown to involve the same enzymatic steps as in bacterial sulfate reducers. Enzymes of the sulfate-reduction pathway in *Archaeoglobus* were purified and compared to the analogous bacterial enzymes, especially on the gene level (Speich and Trüper, 1988; Dahl et al., 1990; 1993; 1994; 1999a; Speich et al., 1994; Sperling et al., 1998; Sperling et al., 1999).

In 1997, the complete genome sequence of *A. fulgidus* was published (Klenk et al., 1997). This was the first genome sequence of a sulfate-reducing prokaryote.

Sulfur-Reducing Bacteria

Biological reduction of sulfur to sulfide with endogenous or added organic electron donors has been reported several times since the end of the 19th century (Beijerinck, 1895; Starkey, 1937; Woolfolk, 1962; for overview see Roy and Trudinger, 1970). The reaction has been observed in bacteria, cell extracts, fungi, other plants, and in animal tissues. In several instances, the early observed processes of sulfur reduction appear to be by-reactions (incidental sulfur reduction) in an artificially created situation without bioenergetic or ecological significance.

First evidence for sulfur reduction as the sole source of energy for microbial growth was furnished by Pelsh (1936) who enriched novel vibrioid bacteria from mud using sulfur and H_2 as defined substrates. The first pure cultures definitely growing by sulfur reduction was *Desulfuromonas acetoxidans*, an obligately anaerobic mesophile using acetate as electron donor (Pfennig and Biebl, 1976). The bacterium was discovered as the chemotrophic partner in a deep-green phototrophic culture originally known as "*Chloropseudomonas ethylica*"; this culture was thought to be related to green sulfur bacteria, but differed from them by the ability to grow on acetate and even ethanol without addition of sulfide as electron donor. The actual process in this culture was elucidated as a sulfur-sulfide cycle involving a green phototrophic sulfur bacterium that oxidized sulfide to elemental sulfur, and *Desulfuromonas* that reduced sulfur with organic compounds (Pfennig and Biebl, 1976). *Desulfuromonas* was also the first pure culture of an obligate anaerobe shown to oxidize acetate and other organic substrates completely to CO_2 (Pfennig and Biebl, 1976); earlier, anaerobic acetate oxidation was only known in denitrifying bacteria. Subsequently, similar mesophilic bacteria including obligate sulfur reducers were isolated with organic compounds and sulfur (Pfennig, 1984; for overview see Widdel, 1988; for more recent classification see Finster et al., 1997b). Several of these sulfur reducers were shown to grow on acetate and fumarate.

The formerly observed growth by sulfur respiration with H_2 (Pelsh, 1936) was confirmed by isolation of a spirilloid bacterium (strain 5175) which in addition used formate (Wolfe and Pfennig, 1977). Fumarate was used as alternative electron acceptor. Subsequently, further morphologically similar, spirilloid bacteria with an anaerobic catabolism of fumarate (or aspartate) were recognized as facultative sulfur-reducing bacteria that oxidized H_2 or formate. These were a tentative *Campylobacter* species (Laanbroek et al., 1977; Laanbroek et al., 1978), a spirillum isolated on lactate and DMSO (Zinder and Brock, 1978a), and *Wolinella* (formerly *Vibrio*) *succinogenes* (Macy et al., 1986).

Neither *Desulfuromonas* nor the spirilloid sulfur-reducers were able to reduce sulfate. However, the capacity for growth by sulfur reduction was also detected in sulfate-reducing bacteria. Growth on lactate or ethanol in the presence of sulfur was observed with *Desulfovibrio gigas* (Biebl and Pfennig, 1977; Fauque et al., 1979), with an isolate tentatively named *Desulfovibrio multispirans* (He et al., 1986), and with nutritionally similar but rod-shaped, desulfoviridin-negative, sulfate reducers (Biebl and Pfennig, 1977) affiliating with the later proposed genus *Desulfomicrobium* (Rozanova and Nazina, 1976; Rozanova et al., 1988a). Later, anaerobes originally isolated as ferric iron-reducing bacteria were shown to be facultative sulfur reducers (Balashova, 1985; Myers and Nealson, 1988; Caccavo et al., 1994) and, vice versa, sulfur-reducing *Desulfuromonas* was shown to reduce ferric iron (Roden and Lovley, 1993). Furthermore, a *Pelobacter* species that had been originally isolated as a fermentative bacterium was recognized as facultative reducer of sulfur and ferric iron (Lovley et al., 1995c).

A novel moderately thermophilic type of sulfur-reducing, acetate-oxidizing anaerobe was designated *Desulfurella acetivorans* (Bonch-Osmolovskaya et al., 1990). Furthermore, the thermophiles Aquifex (Huber et al., 1992), *Ammonifex* (Huber et al., 1996) and *Desulfuro-*

bacterium (L'Haridon et al., 1998) were described as hydrogen-utilizing sulfur-reducing bacteria; *Ammonifex* was originally isolated as a nitrate-reducing bacterium.

Natural relationships of sulfur-reducing bacteria were first investigated by 16S rRNA oligonucleotide cataloguing of *Desulfuromonas* (Fowler et al., 1986); it affiliates with the δ-subclass of Proteobacteria and branches within completely oxidizing sulfate-reducing bacteria; the result was later confirmed by near-complete sequencing when similar species were classified as *Desulfuromusa* (Liesack and Finster, 1994). The phylogenetic branch that comprises spirilloid sulfur-reducing bacteria was termed the ε-subclass of Proteobacteria. The first isolate (strain 5175; Wolfe and Pfennig, 1977) was classified as *Sulfurospirillum deleyanum* (Schumacher et al., 1992). *Desulfurella* species were recognized as a distinct branch within the ε-subclass with no specific relationship to sulfate-reducing bacteria or *Desulfuromonas* (Rainey et al., 1993; Miroshnichenko et al., 1998).

The first biochemical studies of sulfur-reducing bacteria were devoted to certain redox proteins and metal centers (Probst et al., 1977; Bache et al., 1983) as well as to the metabolism of acetate (Gebhardt et al., 1985). Acetate oxidation was shown to occur via the citric acid cycle, either with initial activation by CoA transfer from succinyl-CoA as in *Desulfuromonas* (Gebhardt et al., 1985), or with ATP-dependent acetate activation as in *Desulfurella* (Schmitz et al., 1990).

For the investigation of the biochemistry and bioenergetics of sulfur respiration, *Wolinella* (formerly *Vibrio*) *succinogenes* was a highly suitable model organism. This bacterium had been originally isolated as a fumarate-respiring organism (Wolin et al., 1961). The experimental approaches and results from the detailed studies of the electron transport from formate (or H_2) to fumarate in *Wolinella* as a model of anaerobic respiration (see e.g., Kröger and Winkler, 1981; Graf et al., 1985; Hedderich et al., 1999) provided an important basis also for investigations into sulfur respiration by this bacterium. Evidence was provided that polysulfide and not elemental sulfur is the actual electron acceptor (Klimmek et al., 1991; Schauder and Kröger, 1993; Schauder and Müller, 1993; Fauque et al., 1994), and there was increasing support for a periplasmic rather than a cytoplasmic orientation of the active site of polysulfide reductase, as in the case of formate dehydrogenase and hydrogenase in *Wolinella* (Schröder et al., 1988; Krafft et al., 1992; Schauder and Kröger, 1993; Krafft et al., 1995). The three subunits of the polysulfide reductase were analyzed with respect to bound cofactors (e.g., molybdopterin, FeS centers) and the underlying genes (Krafft et al., 1992; Krafft et al., 1995). A protein that increased the efficacy (viz. decreased the K_M value) of polysulfide reduction was identified and termed Sud protein; it was suggested that Sud scavenges free polysulfide in the periplasm and transports it to the active site of the reductase (Kreis-Kleinschmidt et al., 1995; Klimmek et al., 1998).

Sulfur-Reducing Archaea

In the early 1970s, the first extremely thermoacidophilic microorganisms were reported (Brock et al., 1972; Brierley and Brierley, 1982). The organisms classified as *Sulfolobus* were aerobic sulfur oxidizers. Somewhat later, they were recognized as members of a new "kingdom" of life termed "Archaebacteria" (Woese and Fox, 1977; Woese et al., 1978). These findings stimulated (in the early 1980s) the search for further, novel thermophiles under alternative conditions for enrichment cultures. Anoxic media were used that contained complex organic substrates, H_2 as well as elemental sulfur, a potential electron acceptor known from mesophilic bacteria (see above). Indeed, novel extremely thermophilic archaea were detected that grew anaerobically and produced sulfide (Fischer et al., 1983; Stetter, 1982; 1983a; 1983b; Zillig et al., 1981; 1982; 1983), and the number of novel isolates increased steadily in subsequent years (for overview see e.g., Stetter et al., 1990; 1996). Several isolates seemed to reduce sulfur in a by-reaction or as mere electron sink to facilitate fermentation (Zillig et al., 1982; for more recent overview see Schönheit and Schäfer, 1995; Hedderich et al., 1999). Nevertheless, evidence for sulfur respiration as a mode of energy metabolism in archaea was clearly provided in cultures of *Thermoproteus* and *Pyrodictium* species that grew with H_2 as the only electron donor in the absence of organic compounds (Fischer et al., 1983; Stetter et al., 1983b). Further, newly isolated archaea that definitely grow by sulfur respiration, viz on H_2 and sulfur, were *Stygioglobus azoricus* (Segerer et al., 1991), *Pyrobaculum islandicum* (Huber et al., 1987) and *Stetteria hydrogenophila* (Jochimsen et al., 1997).

A unique versatility in sulfur metabolism was found in new lithoautotrophic thermophilic isolates, *Acidianus infernus* (Segerer et al., 1985; Segerer et al., 1986) and *Desulfurolobus* (originally *Sulfolobus*) *ambivalens* (Zillig et al., 1985; Zillig et al., 1986) that grew aerobically by sulfur oxidation as well as anaerobically by sulfur reduction with H_2.

In carbon assimilation during sulfur reduction with H_2, the reductive citric acid cycle and more recently the hydroxypropionate pathway were shown to be operative in *Thermoproteus neutro-*

philus (Schäfer et al., 1986) and *Acidianus* (Menendez et al., 1999), respectively. In the course of investigations into the sugar metabolism in several hyperthermophiles (for overview see Selig et al., 1997), pathways also were investigated in the sulfur-respiring, facultatively organotrophic *Thermoproteus tenax* (Siebers and Hensel, 1993). Evidence was provided for a non-phosphorylated Entner-Doudoroff pathway and a modified Embden-Meyerhof-pathway. Furthermore, complete oxidation of organic substrates via the citric acid cycle was demonstrated in the facultatively organotrophic sulfur-respiring species *Thermoproteus tenax* and *Pyrobaculum islandicum* (Selig and Schönheit, 1994). So far, these are the only extremely thermophilic sulfur-reducing microorganisms shown to couple sulfur reduction to complete mineralization of organic compounds, analogous to *Desulfuromonas* and *Desulfurella* (see above). The electron transport during sulfur reduction with H_2 was studied in *Pyrodictium brockii* (Phil et al., 1992; Maier, 1996) and *Pyrodictium abyssii* (Dirmeier et al., 1998); these species employ different transport chains.

Overview of Principal Properties

Sulfate-Reducing Bacteria and Archaea

Sulfate-reducing bacteria gain energy for cell synthesis and growth by coupling the oxidation of organic compounds or molecular hydrogen (H_2) to the reduction of sulfate (SO_4^{2-}) to sulfide (H_2S, HS^-), as schematically shown in Fig. 1. Hence, sulfate-reducing bacteria are easily recognized by the production of high sulfide concentrations (with non-limiting electron donor and sulfate, usually in the range of several millimolar) concomitantly with growth and the strict dependence of this process on the presence of free sulfate. This process is also termed "dissimilatory sulfate reduction," to allow clear differentiation from assimilatory sulfate reduction. Assimilatory sulfate reduction generates reduced sulfur for biosynthesis (e.g., of cysteine) and is a widespread biochemical capacity in prokaryotes and plants. Assimilatory sulfate reduction does not lead to the excretion of sulfide. Only upon decay (putrefaction) of the biomass is the assimilated reduced sulfur released as sulfide. The amounts of sulfide produced by dissimilatory sulfate reduction with a given amount of biomass is by orders of magnitude higher than the amount of sulfide liberated from the organic sulfur during putrefaction of the same amount of biomass. If the average formula of biomass is approximately written as that of a carbohydrate (CH_2O), an amount 1,000 g (33.3 mol) would yield 133 mol [H], and thus allow formation of 16.7 mol or 567 g H_2S by sulfate reduction (8 [H] needed per SO_4^{2-}). With the approximate natural content of 1% organic sulfur, the same amount of biomass would only yield 10 g of H_2S if degraded by merely putrefying bacteria.

The production of high concentrations of H_2S often indicates the activity and presence of sulfate-reducing microorganisms in natural habitats. The presence of H_2S is obvious by its characteristic smell, black precipitation of ferrous sulfide when iron minerals are present, and white patches of elemental sulfur as an oxidation product formed in contact with air. Such signs for the activity of sulfate reducers are often encountered if organic substances accumulate in the presence of sulfate under anoxic conditions. Growth conditions for sulfate-reducing microorganisms prevail in sediments of virtually all aquatic habitats, which may be cold, moderate or geothermally heated up to ca. 105°C. But also flooded soils such as rice paddies and technical aqueous systems (as for instance sludge digestors, oil tanks or vats in the paper-making industry) may offer suitable growth conditions for sulfate-reducing microorganisms.

From such habitats, in particular marine sediments, a great variety of sulfate-reducing microorganisms has been isolated. The classification of the major groups of sulfate-reducing microorganisms is today based on 16S rRNA sequence analysis. This method is usually relevant for the definition of the more refined taxa, viz. genera and sometimes species; nevertheless, phenotypic features such as nutritional capacities or chemotaxonomic properties may be decisive as well on the genus level, and in combination with DNA-DNA hybridization, in particular on the species level. Bacterial sulfate reducers fall into three major branches, the δ-subclass of Proteobacteria with more than twenty-five genera, the Gram-positive bacteria with the genera *Desulfotomaculum* and *Desulfosporosinus*, and branches formed by *Thermodesulfobacterium* and *Thermodesulfovibrio* (Fig. 2). Sulfate reducers in the latter branch are thermophilic, whereas the two other branches comprise psychrophilic, mesophilic as well as thermophilic species. Currently recognized genera of sulfate-reducing bacteria and archaea are summarized in Table 1.

Sulfate-reducing bacteria are morphologically diverse; cell forms include cocci, rods, curved (vibrioid) types, cell aggregates (sarcina-like) and multicellular gliding filaments. Sulfate-reducing microorganisms are strict anaerobes, even though certain species may tolerate and reduce oxygen for a limited period of time. Many sulfate-reducing microorganisms can grow by

Table 1. Morphological and physiological properties of the genera of sulfate-reducing bacteria and archaea.

Genus	Morphology	Optimum temperature (°C)	Desulfoviridin[a]	Electron acceptors for growth (other than SO_4^{2-})	Oxidation of organic electron donors[b]	Electron donors[c]									
						H_2	Acetate	Propionate	Higher fatty acids	Ethanol	Lactate	Succinate, fumarate, and/or malate	Fructose, and/or glucose	Phenyl-substituted organic acids	Others utilized by some species
Bacteria[d]															
Desulfovibrio	Vibrio	30–38	+	SO_3^{2-}, $S_2O_3^{2-}$, Fumarate	i	+	–	–	–	+	+	±	±	–	Methanol, glycerol, glycine, alanine, choline, furfural
Desulfomicrobium	Oval or rod	28–37	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	–	–	±	+	+	–	–	–
Desulfobulbus	Oval	28–39	–	SO_3^{2-}, $S_2O_3^{2-}$, NO_3^-	i	+	–	+	–	+	+	–	–	–	–
Desulfobacter	Oval or vibrio	28–32	–	SO_3^{2-}, $S_2O_3^{2-}$	CAC	±	+	–	–	±	–[e]	–	–	–	–
Desulfobacterium	Oval	20–35	–	$S_2O_3^{2-}$	CO	±	(+)	(±)	±	±	±	±	–	±	Methanol, glutarate, glutamate, phenol, aniline, nicotinate, indole
Desulfococcus	Sphere	28–36	+	SO_3^{2-}, $S_2O_3^{2-}$	CO	–	(+)	+	+	+	+	–	–	+	Acetone
Desulfosarcina	Oval (forms aggregates)	33	–	SO_3^{2-}, $S_2O_3^{2-}$	CO	+	(+)	+	+	+	+	±	–	+	–
Desulfomonile	Rod	37	+	$S_2O_3^{2-}$, 3-Cl-benzoate	c	+	–[f]	ND	ND	–	–	–	–	+	3- or 4-Anisate
Desulfonema	Multicellular filaments	30–32	±	SO_3^{2-}, $S_2O_3^{2-}$	c	±	(+)	+	+	–	±	+	–	±	–
Desulfobotulus	Vibrio	34	–	SO_3^{2-}	i	–	–	–	+	–	+	–	–	–	–
Desulfoarculus	Vibrio	35–39	–	SO_3^{2-}, $S_2O_3^{2-}$	CO	–	(+)	(+)	+	–	–	–	–	–	–
Desulfotomaculum	Straight or curved rod, sporulates	30–38 50–65[h]	–	$S_2O_3^{2-}$ Fumarate	i or CO	±	±	±	±	+	±	±	±	±	Methanol, alanine

Desulfosporosinus	Straight or curved rod, sporulates	30–37	–	$S_2O_3^{2-}$	i	+	–	–	ND	+	+	ND	–	+	3,4,5-Trimethoxybenzoate
Thermodesulfovibrio	Vibrio	65	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	–	–	–	+	–	–	–	–
Thermodesulfobacterium	Rod	65–70	–	$S_2O_3^{2-}$	i	+	–	–	–	–	+	–	–	ND	–
Thermodesulforhabdus	Rod	60	–	SO_3^{2-}	c	–	+	–	+	+	+	+	ND	–	–
Desulfacinum	Oval	60	–	SO_3^{2-}, $S_2O_3^{2-}$	ND	+	+	+	+	+	+	+	–	–	Hexanol
Desulforhopalus	Oval	18–19	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	+	ND	+	+	–	–	–	–
Desulforhabdus	Rod	37	–	SO_3^{2-}, $S_2O_3^{2-}$	c	+	+	+	ND	+	+	–	–	–	–
Desulfonatronovibrio	Vibrio	37	–	SO_3^{2-}, $S_2O_3^{2-}$	ND	+	–	–	ND	–	–	–	–	ND	–
Desulfonatronum	Vibrio	37–40	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	–	ND	+	–	–	–	ND	–
Desulfohalobium	Rod	37–40	–	SO_3^{2-}, $S_2O_3^{2-}$, S^0	i	+	–	–	ND	+	+	–	–	ND	–
Desulfofustis	Rod	28	–	SO_3^{2-}, S^0	i	+	+	+	+	+	+	+	+	+	Glycolate, betaine, choline, triethanolamine, indole
Desulfocella	Vibrio	34	–	–	i	–	–	–	+	–	–	–	–	–	l-Alanine, 2-methylbutyrate
Desulfocapsa	Rod	20–30	–	–	i	–	–	–	ND	+	–	–	–	–	–
Desulfobacca	Oval	37	–	SO_3^{2-}, $S_2O_3^{2-}$	c	–	+	–	–	–	–	–	–	–	–
Desulfuromusa	Rod	30	ND	S^0, NO_3^- Fumarate DMSO Fe(III)-citrate	c	ND	+	+	±	–	+	+	–	ND	–
Desulfospira	Curved rod	26–30	–	SO_3^{2-}, $S_2O_3^{2-}$, S^0	c	+	–	–	+	–	+	+	–	±	Betaine, proline
Desulfobacula	Rod	28	ND	ND	c	–	+	–	–	+	–	+	ND	+	Toluene, *p*-cresol, benzaldehyde, benzoate, phenylacetate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate
Desulfofrigus	Rod	10	–	SO_3^{2-}, $S_2O_3^{2-}$ Fe(III)-citrate	c	±	+	–	+	+	+	+	–	–	–

(Continued)

Table 1. *Continued*

Genus	Morphology	Optimum temperature (°C)	Desulfoviridin[a]	Electron acceptors for growth (other than SO_4^{2-})	Oxidation of organic electron donors[b]	Electron donors[c]									
						H_2	Acetate	Propionate	Higher fatty acids	Ethanol	Lactate	Succinate, fumarate, and/or malate	Fructose, and/or glucose	Phenyl-substituted organic acids	Others utilized by some species
Desulfofaba	Rod	7	–	SO_3^{2-}, $S_2O_3^{2-}$	i	–	–	+	–	+	+	+	–	–	–
Desulfotalea	Rod	10	–	SO_3^{2-}, $S_2O_3^{2-}$ Fe(III)-citrate	i	+	–	–	–	+	+	+	–	–	–
Archaea[d]															
Archaeoglobus	Sphere	82–83	–		CO	+	–[g]	ND	ND	ND	+	ND	+	ND	Starch, peptides

[a]Symbols: +, present; ±, present or absent; –, absent.

[b]Symbols: c, complete to CO_2 via unknown pathway; CAC, complete oxidation via citric acid cycle; CO, complete oxidation via carbon monoxide dehydrogenase/C_1 pathway; i, incomplete oxidation to acetate as an end product.

[c]Symbols: +, utilized; (+), poorly utilized; ± , utilized or not utilized; (±) poorly of not utilized; –, not utilized; ND, not determined or not reported.

[d]References: *Desulfovibrio* (Postgate, 1984b), *Desulfomicrobium* (Rozanova et al., 1988), *Desulfobulbus* (Widdel and Pfennig, 1982), *Desulfobacter* (Widdel and Pfennig, 1981b; Widdel, 1987), *Desulfobacterium* (Brysch et al., 1987), *Desulfococcus* (Widdel, 1980), *Desulfosarcina* (Widdel, 1980), *Desulfomonile* (DeWeerd et al., 1990), *Desulfonema* (Widdel et al., 1983), *Desulfobotulus* (Widdel, 1980), *Desulfoarculus* (Widdel, 1980), *Desulfotomaculum* (Widdel and Pfennig, 1977; Widdel and Pfennig, 1981b), *Desulfosporosinus* (Stackebrandt et al., 1997; Campbell and Postgate, 1965; Klemps et al., 1985), *Thermodesulfovibrio* (Henry et al., 1994), *Thermodesulfobacterium* (Zeikus et al., 1983), *Archaeoglobus* (Burggraf et al., 1990; Stetter et al., 1987; Stetter, 1988), *Thermodesulforhabdus* (Beederet al., 1995), *Desulfacinum* (Rees et al., 1995), *Desulforhopalus* (Isaksen and Teske, 1996), *Desulforhabdus* (Oude Elferink et al., 1995), *Desulfonatronovibrio* (Zhilina et al., 1997), *Desulfonatronum* (Pikuta et al., 1998), *Desulfohalobium* (Ollivier et al., 1991), *Desulfofustis* (Friedrich etal., 1996), *Desulfocella* (Brandt etal., 1999), *Desulfocapsa* (Janssen et al., 1996), *Desulfobacca* (Oude Elferink et al., 1999), *Desulfuromusa* (Liesack and Finster, 1994), *Desulfospira* (Finster etal., 1997a), *Desulfobacula* (Rabus et al., 1993), *Desulfofrigus* (Knoblauch et al., 1999b), *Desulfofaba* (Knoblauch et al., 1999b), and *Desulfotalea* (Knoblauch et al., 1999b).

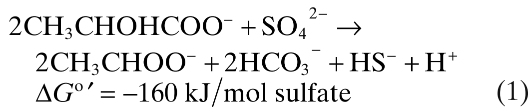
[e]Utilized by a few unnamed strains but not by the validly published species.

[f]May be utilized with thiosulfate as electron acceptor.

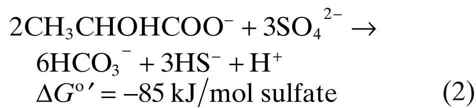
[g]For further description see Daumas et al., 1988; Min and Zinder, 1990; Nazina et al., 1988; and Widdel, 1988.

[h]Thermophilic species.

utilizing sulfite or thiosulfate as alternative electron acceptors, which are also reduced to sulfide. Fewer species have been described to utilize elemental sulfur or nitrate as electron acceptors (for growth), which are reduced to sulfide or ammonia, respectively. The involvement of an external electron acceptor in the energy metabolism allows anaerobic growth even on highly reduced compounds that cannot be utilized by purely fermentative bacteria. Indeed, the electron donors of sulfate-reducing microorganisms include end products of fermentative bacteria. Bacterial sulfate reducers are known to utilize a great variety of low-molecular mass organic compounds, including mono- and dicarboxylic aliphatic acids, alcohols, polar aromatic compounds and even hydrocarbons. Growth with polymers, such as polysaccharides, as in the case of archaeal sulfate reducers, has not been observed. Oxidation of organic compounds may be incomplete, leading to acetate (often simultaneously with CO_2) as an end product, or complete, leading entirely to CO_2. In the case of lactate, a relatively common substrate, the two possibilities for its metabolism are as follows:



$$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3CHOO^- + 2HCO_3^- + HS^- + H^+ \quad \Delta G^{o\prime} = -160\ \text{kJ/mol sulfate} \quad (1)$$



$$2CH_3CHOHCOO^- + 3SO_4^{2-} \rightarrow 6HCO_3^- + 3HS^- + H^+ \quad \Delta G^{o\prime} = -85\ \text{kJ/mol sulfate} \quad (2)$$

Incomplete oxidation of organic substrates is due to the lack of a mechanism for the terminal oxidation of acetyl-CoA. Because of this fundamental catabolic difference, it is common to distinguish between two physiological groups, the incomplete and complete oxidizers. However, these are purely physiological or functional groups that overlap only partly with molecular systematic groups.

The energy gain from dissimilatory sulfate-reduction is relatively low in comparison to aerobic respiration. For instance, the free energy change ($\Delta G°$) of the complete oxidation of acetate or lactate with sulfate as electron acceptor is –48 or –128 kJ, respectively, whereas acetate or lactate oxidation with O_2 provides –844 or –1323 kJ, respectively (here caluclated per mol of the organic substrate). Accordingly, by far the greater part of the organic substrate (or of H_2) consumed by sulfate-reducing bacteria is oxidized in the energy metabolism (Fig. 1), as is obvious from relatively low growth yields. Examples of measured dissimilatory growth yields ($Y_{Sulfate}$, cell dry mass formed per mol sulfate reduced) are as follows: *Desulfovibrio vulgaris*, H_2 (with acetate and CO_2 as carbon source), 8.3 g (Badziong and Thauer, 1978); *Desulfobacter postgatei*, acetate, 4.8 g (Widdel and Pfennig, 1981b); *Desulfovibrio inopinatus*, lactate (incompletely oxidized), 17.8 g (Reichenbecher and Schink, 1997); *Desulfococcus multivorans*, benzoate (completely oxidized), 6.2 g (Widdel, 1980); strain NaphS2, naphthalene (completely oxidized), 6.4 g (average; Galushko et al., 1999). The portions of the organic electron donors and carbon sources assimilated into cell material were ca. 9% (acetate) and 11% (lactate, benzoate, naphthalene). However, growth yields are not constants. They may be influenced by substrate limitation and resulting growth rate (Badziong and Thauer, 1978), the sulfide concentration (Widdel and Pfennig, 1977), and temperature (Isaksen and Jørgensen, 1996a; Sass et al., 1998b; Knoblauch and Jørgensen, 1999c). Variable growth yields of the same bacterial species (growing on one type of substrate) may be interpreted as a variable efficacy of coupling between electron transport and energy conservation, or a variable portion of the conserved energy (viz. ATP) that is needed for maintenance and hence does not contribute to net cell growth.

As in other bacteria, there is no strict, causal connection between free energy changes and highest growth rates (μ_{max}) that can be reached under optimum conditions. Still, the tendency has been often observed that electron donors which allow high free energy changes and involve simple, common enzyme mechanisms (e.g. H_2, formate, ethanol, lactate, malate) allow, in principle, faster growth than electron donors that provide less energy and require more complicated, "unusual" enzyme mechanisms (e.g. aromatic compounds, alkanes). But there are exceptions. Some specialized species may utilize the former type of substrates (if used at all) more slowly than one of the latter. Growth rates observed with sulfate-reducing bacteria under optimal conditions (in synthetic media in the laboratory, with saturating or almost saturating substrate concentrations) cover a wide range, as illustrated with a few examples: *Desulfovibrio vulgaris*, H_2, 0.15 h^{-1} (doubling time, 4.6 h; Badziong and Thauer, 1978); *Desulfobacter* species, acetate, 0.035–0.039 h^{-1} (doubling time 20–18 h; Widdel and Pfennig, 1981b; Widdel, 1987); strain NaphS1, naphthalene, ca. 0.004 h^{-1} (doubling time, 1 week; Galushko et al., 1999). The resulting highest specific sulfate reduction rates ($V_{max} = \mu_{max}/Y_{Sulfate}$) with H_2, acetate and naphthalene were 18, 7.3–8.1, and 0.64 mmol sulfate per g cell dry mass and hour, respectively.

Most sulfate-reducing bacteria tolerate more than 10 mM sulfide, as repeatedly shown during

characterization of various species (for references see Table 1). Sulfate-reducing bacteria utilizing aromatic hydrocarbons formed as much as 20–25 mM sulfide before growth ceased (Harms et al., 1999; Rueter et al., 1994). In contrast, some *Desulfotomaculum* species appear to be more sensitive to sulfide, which affects their growth at concentrations of 4–7 mM (Klemps et al., 1985; Widdel and Pfennig, 1977).

In comparison to bacterial sulfate reducers, archaeal sulfate reducers have been detected relatively recently and fewer species are known. As thermophilic microorganisms with optimal growth at temperatures around 80°C or higher, archaeal sulfate reducers are less ubiquituous than their bacterial counterparts. Rather, archaeal sulfate reducers appear to be restricted to habitats like hydrothermal vents, hot springs and deep, warm oil reservoirs. So far, fewer substrates are known for archaeal than for bacterial sulfate reducers. However, archaeal sulfate reducers were shown to utilize the polymers, starch and peptides. Oxidation of organic compounds is always complete, in the case of lactate according to equation (2).

Sulfur-Reducing Bacteria and Archaea

In addition to sulfate-reducing microorganisms, a variety of prokaryotes exists that reduce elemental sulfur (or other, lower oxidation states of this element) but not sulfate. Among the lower oxidation states, the element sulfur (often written as S^0, S_8) is probably the most widespread sulfur species in sediments and geological deposits. Many chemical and biological oxidation processes of H_2S do not directly lead to sulfate (the highest oxidation state) but rather to elemental sulfur, which therefore may accumulate. Prokaryotes that reduce sulfur do not form phylogenetically coherent groups of bacteria or archaea.

Many prokaryotes have been directly enriched and isolated with sulfur as an electron acceptor (e.g., Pfennig and Biebl, 1976; Wolfe and Pfennig, 1977; Bonch-Osmolovskaya et al., 1990; Stetter, 1985). Furthermore, the capacity for growth with sulfur as electron acceptor has been documented for bacteria that were originally isolated on the basis of growth with other electron acceptors such as manganese (IV) (Myers and Nealson, 1988) or iron (III) (Caccavo et al., 1994). Conversely, microorganisms isolated with sulfur are often able to reduce other electron acceptors such as nitrate, iron(III), or thiosulfate. In contrast to dissimilatory sulfate reduction, the capacity for sulfur reduction also has been observed in bacteria that grow definitely with O_2 and which are, therefore, facultative anaerobes. However, many sulfur-reducing microorganisms are strictly anaerobic.

Among the sulfate-reducing bacteria, only a few species can grow with elemental sulfur (Biebl and Pfennig, 1977; Table 1). Other sulfate-reducing bacteria may produce some H_2S in a by-reaction not leading to growth when transferred from sulfate-grown cultures to media with crystalline (rhombic) or colloidal sulfur. Growth of many species of sulfate reducers is even inhibited by sulfur (e.g., Widdel and Pfennig, 1981b; Widdel et al., 1983; Bak and Widdel, 1986a; 1986b; Burggraf et al., 1990), probably because elemental sulfur as an oxidant shifts the potential of redox couples in the medium and cells to unfavorable, positive values.

Analogous to capacities in sulfate-reducing bacteria, the oxidation of organic substrates in sulfur-reducing bacteria may be incomplete and lead to acetate as an end product (as for instance in *Sulfos pirillum*, *Wolinella*, *Shewanella* and *Pseudomonas mendocina*), or complete and lead to CO_2 as the final product (as for instance in *Desulfofuromonas* or *Desulfurella*).

Whereas bacterial sulfur reducers may be mesophilic or moderately thermophilic, archaeal sulfur reducers are all extremely thermophilic. Typical habitats of the hyperthermophilic sulfur reducers are solfataric fields, hot springs and hydrothermal systems in the deep sea, whereas mesophilic bacterial sulfur reducers can be isolated from almost every freshwater or marine sediment, or even from wet soil.

Unlike sulfate reduction, the reduction of the lower oxidation states of sulfur is not always a respiratory process. The compounds may only serve as hydrogen sinks for a "facilitated fermentation," or they may even be reduced in by-reactions without an obvious bioenergetic benefit. These processes vary, forming a spectrum ranging between true sulfur respiration and sulfur reduction as a mere by-reaction. A freshwater Beggiatoa was found to reduce stored sulfur under anoxic conditions with added acetate (Nelson and Castenholz, 1981). A certain increase in cell mass indicated that the process allowed a limited energy conservation. A Chromatium species and the cyanobacterium *Oscillatoria limnetica* was found to reduce photosynthetically formed intracellular or extracellular sulfur, respectively, in the dark under anaerobic conditions, using storage carbohydrate (van Gemerden, 1968; Oren and Shilo, 1979); growth did not occur. The reactions probably sustained a maintenance metabolism. However, it is not quite clear whether energy was gained only by substrate-level phosphorylation during sugar degradation or in addition by sulfur respiration.

We propose to apply the term "sulfur-reducing bacteria" to those bacteria in which sulfur reduction is associated with a respiratory type of energy conservation (sulfur respiration). An overview of the morphological and physiological properties of bacteria and archaea definitely capable of S^0-respiration is provided in Table 2. Additional microorganisms that can reduce S^0 to H_2S, even though a respiratory function remains unclear, have been summarized by Hedderich et al. (1999).

Physiology, Biochemistry and Molecular Biology

Sulfate-Reducing Bacteria

Much of the research on sulfate-reducing microorganisms has been devoted to their unique metabolism in which five major aspects may be distinguished: 1) Sulfate reduction to sulfide, which is biochemically more complicated than O_2 reduction in aerobic organisms, requires an array of enzymes. Like carbon and nitrogen, sulfur may occur in eight different oxidation states. In biochemistry, sulfur may form bonds to hydrogen, carbon and oxygen, but also chains with S-S-bonds. Oxidation states lower than +VI (sulfate) are rather reactive and may undergo interconversions or autoxidation even at room temperature. This reactivity complicates analyses of intermediates in sulfur metabolism, but also confronts research with interesting questions. 2) Sulfate-reducing bacteria utilize a wide variety of organic compounds. Even though these are of low molecular mass and relatively simple in their structure, their oxidation under anoxic conditions often involves biochemically intriguing reactions. 3) The flow of reducing equivalents ([H], electrons) from the electron donors to the electron acceptor is associated with the respiratory energy conservation, and a great variety of electron carriers seem to be involved. 4) Synthesis of cell material from most organic substrates is expected to proceed via pathways commonly known from other bacteria and therefore has not been a major field of research. However, the capacity of a number of sulfate-reducing bacteria for cell synthesis solely from CO_2 (and mineral salts) during growth on H_2 and SO_4^{2-} as sole energy source has attracted particular attention. 5) A fifth main aspect, metabolic regulation, is widely unexplored in sulfate-reducing bacteria. In the study of all these aspects, molecular and genetic analyses are of increasing importance.

Reduction of Sulfate to Sulfide Reduction of sulfate to sulfide is an eight-electron step process that occurs via a number of intermediates. However, unlike many nitrate-reducing bacteria, sulfate-reducing bacteria usually do not excrete the intermediate oxidation states, but only the final product sulfide. Only in two cases, excretion by *Desulfovibrio desulfuricans* of minor concentrations of sulfite or thiosulfate has been reported (Vainshtein et al., 1980; Fitz and Cypionka, 1990); this does not necessarily indicate that thiosulfate is a direct intermediate.

Sulfate Transport Because all enzymatic steps leading from sulfate to sulfide occur in the cytoplasm or in association with the inner side of the cytoplasmic membrane, sulfate has to be transported into the cell. Sulfate uptake in sulfate-reducing bacteria is driven by an ion-gradient, as demonstrated in studies with *Desulfovibrio species*, *Desulfobulbus propionicus* and *Desulfococcus multivorans* (Cypionka, 1987; Cypionka, 1989; Cypionka, 1994; Cypionka, 1995; Warthmann and Cypionka, 1990). In the freshwater species (*Desulfovibrio desulfuricans*, *Desulfobulbus propionicus*), sulfate is transported simultaneously with protons, as revealed by instantaneous pH shifts in active cell suspensions upon addition of sulfate. In contrast, sulfate uptake in moderately salt-dependent species (*Desulfovibrio salexigens*, *Desulfococcus multivorans*) is driven by sodium ions (Warthmann and Cypionka, 1990; Stahlmann et al., 1991; Kreke and Cypionka, 1992). Cells grown at very limiting (e. g., micromolar) sulfate concentrations as in a chemostat (Cypionka and Pfennig, 1986) most likely transported sulfate with three protons or sodium ions, which allowed sulfate to accumulate by factors of 10^3 to 10^4 (Stahlmann et al., 1991). If the efflux of a neutral end product, H_2S, is taken into account, sulfate transport is electrogenic under these conditions. The driving force for sulfate transport was mainly the electric component of the electrochemical potential and to a lesser extent the cation concentration gradient. There is evidence for an H^+/Na^+ antiporter which creates a sodium gradient across the cytoplasmic membrane of sulfate-reducing bacteria (Varma et al., 1983; Kreke and Cypionka, 1992). With increasing sulfate concentration in the growth medium, the high-accumulating sulfate-transport system was no longer detectable. Instead, cells obviously produced a low-accumulating system causing sulfate concentration inside the cell by a factor not higher than 10^2, thus avoiding the buildup of deleterious sulfate concentrations. The latter system probably transported sulfate with two H^+ or Na^+ ions. At very high (28 mM) sulfate concentration as in seawater or most laboratory cultures, another regulation system seemed to attenuate

Table 2. Morphological and physiological properties of Bacteria and Archaea capable of respiratory reduction of elemental sulfur.

Species	Morphology	Optimum temperature (°C)	Oxidation of organic electron donors[a]	Electron donors				Electron acceptors						
				H_2	Acetate	Lactate and/or pyruvate	Others utilized by some species	Sulfur	Thiosulfate	Sulfite	Sulfate	Fumarate	Nitrate	O_2
Bacteria[a]														
Desulfuromonas acetoxidans	Rod	30	c	–	+	±	Ethanol, propionate, succinate, glutamate	+	–	–	–	±	±	–
Desulfurella acetivorans	Rod	52–57	c	–	+	–	–	+	–	–	–	–	–	–
Desulfovibrio gigas	Vibrio	30–36	i	ND[b]	–	+	–	+	+	+	+	+	–	–
Desulfomicrobium species	Rod	28–37	i	ND	–	+	–	+	+	+	+	–	–	–
Dethiosulfovibrio peptidovorans	Vibrio	42	i	–	–	–	Peptides, amino acids	+	+	–	–	–	–	–
Desulfitobacterium chlororespirans[c]	Rod	37	i	+	–	+	Butyrate	+	+	+	–	–	–	–
Sulfurospirillum deleyianum	Curved spiral	25–30	ND	+	–	+	Succinate, fumarate, malate aspartate, oxaloacetate[d]	+	+	+	–	+	+	+
Wolinella succinogenes and similar spirilloid types	Spirillum or vibrio	30–37	i	+	–	+	Formate	+	+	+	–	+	+	+[e]
Pseudomonas mendocina subsp.	Rod	35–36	ND	+	ND	ND	Succinate, malate, glutamate	+	+	ND	–	ND	+	+
Geobacter sulfurreducens	Rod	35	ND	+	+	+	Ethanol, butyrate, succinate	+	–	–	–	+	–	–
Pelobacter carbinolicus	Rod	35	i	–	–	–	2,3-butandiol, acetoin,ethylene glycol	+	–	–	–	–	–	–
Sulfurospirillum arcachonense	Curved rods	26	i	+	+	+	glutarate, glutamate	+	–	–	–	–	–	+

Hippea maritima	Rod	52–54	c	+	+	–	Ethanol, stearate, palmitate	+	–	–	–	–	–	–
Desulfurobacterium thermolithotrophum	Rod	70	–[f]	+	–	ND		+	+	+	–	ND	–	–
Aquifex pyrophilus	Rod	85	–	+	–	–	(With O_2: sulfur, thiosulfate)	+	ND	ND	ND	ND	+	+
Ammonifex degensii	Rod	70	ND	+	–	+	formate	ND	–	–	+	–	+	–
Archaea[a]														
Acidianus infernus	Lobed coccus	90	–[f]	+	–	–	(With O_2: sulfur)	+	–	–	–	ND	–	+
Sulfolobus ambivalens	Lobed coccus	88	–[f]	+	–	–	(With O_2: sulfur)	+	–	–	–	ND	–	+
Pyrobaculum islandicum	Long rod	100	ND	+	–	–	Yeast extract	+	+	+	–	ND	–	–
Pyrodictium occultum	Disc with fibers	105	–[f]	+	–	–	–	+	–	–	–	ND	–	–
Thermoproteus tenax	Rod	80	c	+	–	–	Yeast extract	+	+	+	–	ND	–	–
Stygiolobus azoricus	Lobed	80	–[f]	+	–	ND		+	ND	–	–	ND	ND	–
Stetteria hydrogenophila	Coccus (irregular)	95	–[f]	+	–	–	Yeast extract	+	+	–	ND	–	–	ND
Thermodiscus maritimus	Disk	85	–[f]	+				+						

Symbols: c, complete oxidation (under anoxic conditions); i, incomplete oxidation; +, utilized; ±, utilized or not utilized; –, not utilized; ND, not determined or not reported.

[a]For further description and literature: *Desulfuromonas acetoxidans*, *Desulfovibrio gigas* and *Desulfomicrobium* species (Pfennig and Biebl 1976, 1981; Biebl and Pfennig, 1977; and Widdel, 1988; some data were personal communication from R. Bache and N. Pfennig); *Desulfurella acetivorans* (Bonch-Osmolovskaya etal., 1990; Schmitz et al., 1990); *Dethiosulfovibrio peptidovorans* (Magot et al., 1997); *Desulfitobacterium chlororespirans* (Sanford et al., 1996); *Sulfurospirillum deleyianum* (Wolfe and Pfennig, 1977; Schumacher et al., 1992); *Wolinella succinogenes* (Wolin et al., 1961; Macy et al., 1986); *Pseudomonas mendocina* subsp. (Balashova, 1985); *Geobacter sulfurreducens* (Caccavo et al., 1994); *Pelobacter carbinolicus* (Schink, 1984; Lovley et al., 1995); *Sulfurospirillum arcachonense* (Finster et al., 1997b; Stolz et al., 1999); *Hippea maritima* (Miroshnichenko et al., 1999); *Desulfurobacterium thermolithotrophum* (L'Haridon et al., 1998); *Aquifex pyrophilus* (Huber et al., 1992); *Ammonifex degensii* (Huber et al., 1996); *Acidianus infernus* (Segerer et al., 1985, 1986; Stetter et al., 1990); *Sulfolobus ambivalens* (Zillig et al., 1985, 1986 [the 1986 paper is about *Desulfurolobus ambivalens*]); *Pyrobaculum islandicum* (Huber et al., 1987; Selig and Schönheit, 1994); *Pyrodictium occultum* (Fischer et al., 1983; Stetter et al., 1983); *Thermoproteus tenax* (Zillig et al., 1981; Fischer et al., 1983; Schäfer et al., 1986; Stetter et al., 1990; Selig and Schönheit, 1994); *Stygiolobus azoricus* (Segerer et al., 1991); *Stetteria hydrogenophila* (Jochimsen et al., 1997); *Thermodiscus maritimus* (Fischer et al., 1993).

[b]Not tested, but likely to be utilized.

[c]*Desulfitobacterium chlororespirans* can also grow on lactate coupled to reductive dehalogenation of 3-chloro-4-hydroxybenzoate.

[d]Utilized during fementative metabolism.

[e]Low partial pressure (microaerobic conditions).

[f]Obligate lithoautotrophs that do not oxidize organic compounds.

even the low-accumulating system to prevent excess buildup of sulfate. Most likely, the sulfate transport systems operate near equilibrium (Cypionka, 1994; Cypionka, 1995). This means that the free energy from the gradient of the cotransported cations is not completely dispersed, but rather conserved more or less in the resulting sulfate gradient, rendering subsequent reactions of sulfate energetically more favorable than they would be at the lower ambient sulfate concentration. Hence, the consumption of $^1/_4$–$^1/_3$ ATP equivalent per sulfate (assuming consumption of one electrogenically produced H^+ ion, and a 3–4 H^+/ATP stoichiometry of ATP synthase; Thauer and Morris, 1984; Stock et al., 1999) for sulfate transport at very low concentration must be regarded as energetically highly economic (Cypionka, 1995). The need for such reversible, energy-conserving transport processes in the catabolism is understandable in view of the relatively low ATP gain per mol sulfate.

Sulfate uptake solely for biosynthesis (assimilatory sulfate reduction) differs completely from that in dissimilatory sulfate reduction. Sulfate transport in *Escherichia coli* for assimilation was shown to occur via an ABC transporter involving a periplasmic binding protein (Hryniewicz et al., 1990; Sirko et al., 1990). Such a mechanism for sulfate uptake is also likely in the cyanobacterium, Anacystis (Jeanjean and Broda, 1977). Sulfate uptake via ABC transporters for anabolic (assimilatory) purposes is irreversible (1 ATP/SO_4^{2-}); however, this dissipation of energy is negligible in view of the relatively low portion of reduced sulfur needed for cell synthesis (around 1% of dry mass).

Activation of Sulfate The free sulfate dianion (SO_4^{2-}) with its oxygen atoms in a tetrahedral arrangement is chemically sluggish and not easily reduced. The redox potential of the free anion pair SO_4^{2-}/ SO_3^{2-} is lower (E_0' = –0.516 V) than redox potentials of most catabolic redox couples (Fig. 3). Before being reduced, sulfate is activated by ATP sulfurylase (Peck, 1959; Peck, 1962); the product is adenosine-5′-phosphosulfate (APS), which is also termed adenylylsulfate. The ATP sulfurylase has been studied in several sulfate-reducing bacteria belonging to the genera *Desulfovibrio* and *Desulfotomaculum* (Fauque et al., 1991). Sulfate assimilation in nonsulfate-reducing bacteria and plants is also initiated by ATP sulfurylase; in the assimilatory pathways, APS either undergoes direct reduction, as in dissimilatory sulfate reduction, or phosphorylation to 3′-phospho-adenosine-5′-phosphosulfate (PAPS) before reduction (Trudinger and Loughlin, 1981; Fischer, 1988; Peck and Lissolo, 1988).

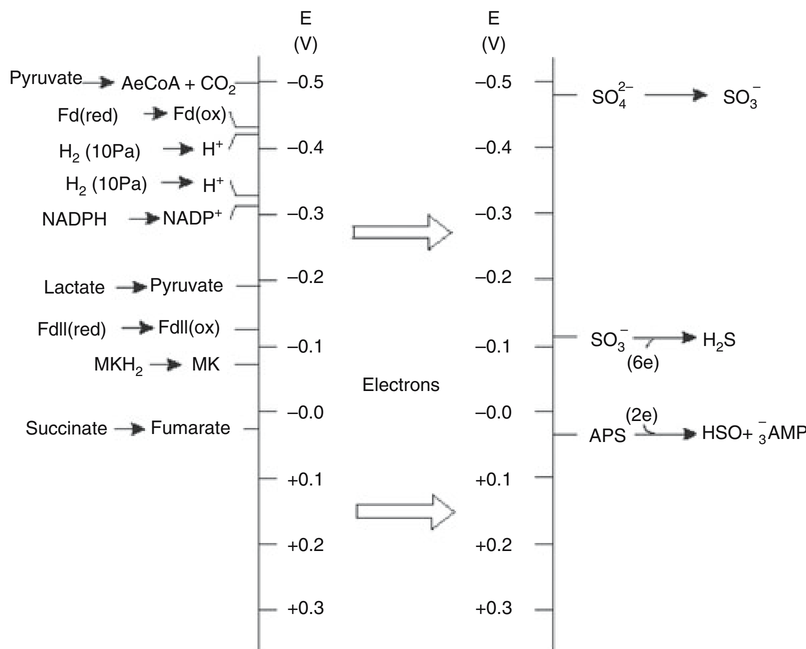
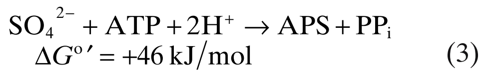
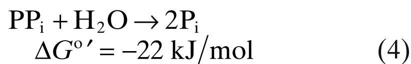


Fig. 3. Comparison of redox potentials of some important electron-donating and electron-accepting reactions in sulfate-reducing bacteria. As mechanism of sulfite reduction to sulfide, a direct reduction with six electrons (6 e^-) is assumed. For ferredoxin, an average of the E_0' (–0.440 V) given by Fauque et al. (1991) and the E_0' (–0.400 V) given by Thauer (1988) and Thauer et al. (1989b) is indicated. Abbreviations: Fd, ferredoxin; MK, menaquinone.

The equilibrium of the ATP sulfurylase reaction is far on the side of the reactants (K_{eq} around 10^{-8}; Akagi and Campbell, 1962), as has also been observed for the reaction in yeast (Robbins and Lipmann, 1958; Wilson and Bandurski, 1958). The hydrolysis of formed pyrophosphate (PP_i) by a pyrophosphatase pulls the ATP sulfurylase reaction and thus favors APS formation (Wilson and Bandurski, 1958), according to the following reactions:

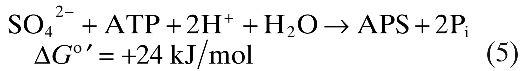


$$SO_4^{2-} + ATP + 2H^+ \rightarrow APS + PP_i \quad \Delta G^{o\prime} = +46\ kJ/mol \tag{3}$$



$$PP_i + H_2O \rightarrow 2P_i \quad \Delta G^{o\prime} = -22\ kJ/mol \tag{4}$$

Sum reaction:



$$SO_4^{2-} + ATP + 2H^+ + H_2O \rightarrow APS + 2P_i \quad \Delta G^{o\prime} = +24\ kJ/mol \tag{5}$$

High pyrophosphatase activities were found in *Desulfovibrio* (Fauque et al., 1991), *Desulfobulbus* (Kremer and Hansen, 1988a), *Desulfosporosinus orientis* (Thebrath et al., 1989). Lower activities were observed in other *Desulfotomaculum* strains. However, earlier claims that PP_i in this genus is used for an indirect phosphorylation of ADP via PP_i:acetate kinase and acetate kinase (Liu and Peck, 1981a) have been questioned and are not supported by more recent experimental data (Thebrath et al., 1989). Still, use of PP_i instead of ATP for certain phosphorylations during cell synthesis cannot be ruled out (Thauer, 1989a). Also, the possibility of energy conservation from PP_i hydrolysis by using this reaction for proton translocation has been considered (Thebrath et al., 1989; Cypionka, 1995). On the other hand, any energy-conserving reaction that makes use of PP_i has a certain reversible character and would diminish the pulling effect needed in reaction (4). Even with PP_i hydrolysis, the thermodynamic equilibrium of the net reaction is still in favor of the reactants. With an assumed approximate concentration of sulfate, ATP and phosphate of a few millimolar (Thauer et al., 1977; Cypionka, 1995), the concentration of APS would have to be less than 0.1 μM to allow a net reaction according to equation (5). This indicates the need for effective scavenging of APS by reduction. One possibility to achieve this would be a close association of enzymes or enzyme complexes, in which molecules can be channelled between reaction centers and are not released into a cytoplasmic pool until the final product, sulfide, has been formed. However, such assumptions are presently speculative in view of experimental data.

Also in the activation of sulfate, the assimilatory and dissimilatory processes differ. Recent studies of assimilatory sulfate-reduction in *E. coli* K12 have revealed a novel mechanism for overcoming the unfavorable energetics of APS formation. In *E. coli* the intracellular concentration of PP_i may be too high (ca. 0.5 μM; Kukko-Kalske et al., 1989) to allow formation of a substantial APS concentration. However, ATP-sulfurylase in this organism was found to catalyze GTP hydrolysis in addition to APS formation. ATP-sulfurylase is a tetramer built of two heterodimers; each dimer consists of a CysN (53 kDa) subunit, which carries the GTPase activity, and a CysD (23 kDa) subunit, which carries the APS-synthesizing activity (Leyh et al., 1988; Liu et al., 1998). The presence of saturating concentrations of GTP stimulates APS formation by more than 100 fold (Leyh and Suo, 1992). The stoichiometry of GTP hydrolysis and APS formation was found to be 1 : 1 (Liu et al., 1998). The energy from GTP hydrolysis is transferred via conformational change to the formation of APS (Wei and Leyh, 1998; Wei and Leyh, 1999). The ATP-sulfurylase in *E. coli*, therefore, has been termed the "ATP sulfurylase-GTPase system". The assimilatory ATP sulfurylase from *E. coli* and the dissimilatory enzyme from *Desulfovibrio* species also differ markedly on the structural level. A recent study on the composition of ATP-sulfurylase from two sulfate-reducing bacteria, *Desulfovibrio desulfuricans* and *Desulfovibrio gigas*, demonstrated that here the ATP-sulfurylase is a homotrimer and contains the metals cobalt and zinc (Gavel et al., 1998).

Reduction of APS APS is the actual electron acceptor, which is converted to sulfite or bisulfite and AMP. The $E^{o\prime}$ of the APS/SO_3^- + AMP couple is –0.060 V. The actual redox potential may be more negative because of the expected low APS concentration (see above). APS reduction is catalyzed by a reductase that has been purified from *Desulfovibrio* strains (Bramlett and Peck, 1975; Lampreia et al., 1987), *Desulfobulbus propionicus* (Stille and Trüper, 1984), and *Thermodesulfobacterium mobile* (formerly *Desulfovibrio thermophilus*; Fauque et al., 1986). Presence of APS reductase was also demonstrated in *Desulfobacter*, *Desulfococcus* and *Desulfosarcina* (Stille and Trüper, 1984). Moreover, a type of this enzyme is found in some of the lithotrophic phototrophic purple and green bacteria and a few thiobacilli (Kelly, 1988; Fischer, 1988; Brune, 1989; Trüper, 1989). In these bacteria, APS reductase catalyzes the inverse reaction. All APS reductases are nonheme iron-sulfur flavoproteins. Purification of APS reductase from *Desulfovibrio desulfuricans* and *Desulfovibrio*

vulgaris under strictly anoxic conditions yielded highly active enzymes. The purified enzyme has a heterodimeric structure (αβ), the total molecular mass being 95 kDa. Based on a characteristic motif in the primary structures, the α-subunit is proposed to carry one flavin adenine dinucleotide (FAD) molecule and the β-subunit to contain two [4Fe-4S] centers (Fritz, 1999).

Two possible mechanisms for the reduction of APS to sulfite by APS reductase have been discussed. In the first proposed mechanism, the FAD group in the α-subunit is the active site. APS reacts with reduced flavin, $FADH_2$, by a nucleophilic attack of the N^5-atom. AMP is released and an $FADH_2$-sulfite adduct is formed. Dissociation of sulfite with the binding electron pair then yields the oxidized FAD and protons (Peck and Bramlett, 1982a; Fig. 4). A formation of an $FADH_2$-sulfite adduct as a possible intermediate during APS reduction was already suggested by Michaelis et al. (1970) and inferred from studies with artificial sulfite-flavin adducts (Müller and Massey, 1969). More recent studies furnished increasing evidence for this mechanism. For instance, binding of APS, AMP and $FADH_2$ could be demonstrated by spectroscopic measurements (Fritz, 1999). In the second proposed mechanism, a thiolate anion ($R\text{-}S^-$) at the active site carries out a nucleophilic attack on the sulfur atom of APS. Such a reaction would result in the cleavage of the S-O-P bond, the release of AMP and the formation of a thiosulfonate group ($R\text{-}SSO_3^-$). Subsequent reduction of the thiosulfonate group with two electrons releases sulfite and restores the thiolate group of the active site (Fig. 5). The assumption that a thiolate group serves as active site was based on the finding that thiol-blocking agents inhibited the APS reductase from *Desulfovibrio desulfuricans* (Peck et al., 1965). However the recent experimental evidence that blockage of the thiol groups did not abolish but only reduce the activity rendered this mechanism unlikely (Fritz, 1999). In principle, the thiolate mechanism would resemble that in

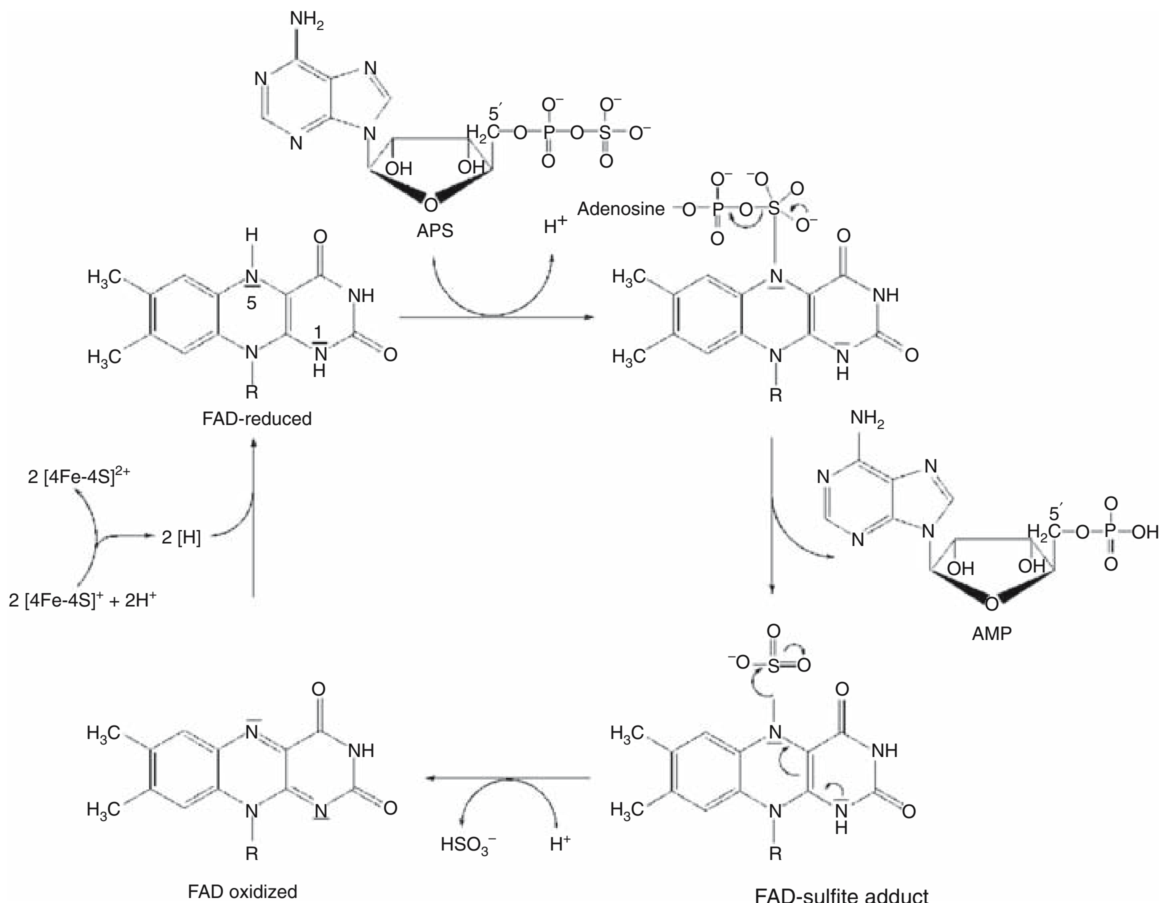


Fig. 4. Proposed mechanism of APS reduction to sulfite with FAD as the catalytically active component. FAD is bound via a residue (R) to the enzyme. Electrons for the reduction of FAD are delivered by the [4Fe-4S] centers, the oxidation of which is indicated by the change of charge. Nucleophilic attack of N-5 results in binding of APS sulfur to reduced FAD. The FAD-sulfite adduct is formed upon release of AMP. Separation of sulfite from the enzyme yields oxidized FAD, which can reenter the reaction cycle. Abbreviations: APS, adenosine-5′-phosphosulfate; FAD, flavin adenine dinucleotide.

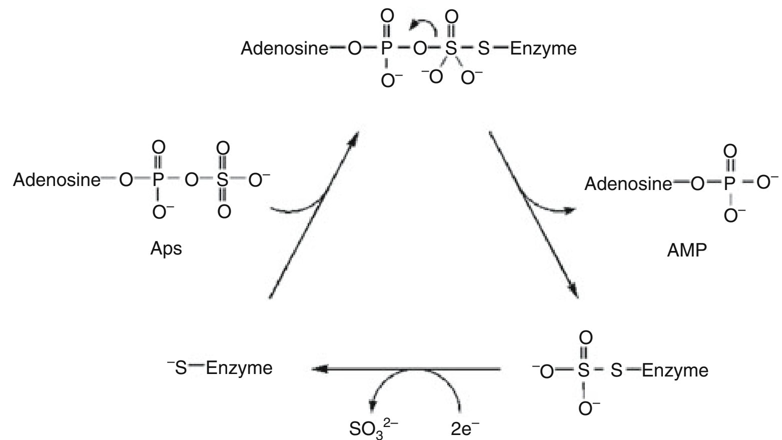
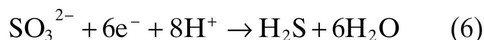


Fig. 5. Proposed mechanism of adenosine-5′-phosphosulfate (APS) reduction to sulfite with a thiolate group as the catalytically active component. Nucleophilic attack of the enzyme-bound thiolate group leads to binding of APS. Upon release of AMP, a sulfite-enzyme adduct is formed. Reduction by two electrons (2 e^-) allows separation of sulfite and regeneration of free thiolate.

one of the assimilatory pathways. In the known assimilatory pathways, the sulfonate moiety from APS or PAPS is also transferred to a thiol, which can be glutathione or thioredoxin, to yield an organic thiosulfonate; this is either reduced to the corresponding organic persulfide (RSS^-) or reductively cleaved with formation of sulfite, respectively (Trudinger and Loughlin, 1981; Imhoff, 1982; Fischer, 1988; Peck and Lissolo, 1988).

In general, it is not known what electron donor is used in the cell to reduce APS. However, from *Desulfovibrio vulgaris* (strain Hildenborough), Chen et al. (1994d) isolated a flavin mononucleotide containing protein which not only catalyzed the oxidation of NADH by O_2 with a concomitant formation of hydrogen peroxide (H_2O_2), but also fully reduced APS reductase with NADH as electron donor.

Reduction of Sulfite Sulfite ($:SO_3^{2-}$) or the protonated form bisulfite (tautomeric forms, $[:SO_2O\text{-}H]^-$ and $[H\text{-}SO_2O:]^-$), which are approximately equally abundant at pH 7.0 ($pK_{a2} = 6.99$), are pyramidal molecules with free electron pairs at the sulfur and much more reactive than sulfate. Their metabolism needs no further activation by ATP. Early reports have suggested that bisulfite rather than sulfite is the actual substrate in the reduction to sulfide (Suh and Akagi, 1969; Drake and Akagi, 1977), and subsequently sulfite reductase has often been referred to as bisulfite reductase (Hatchikian, 1994). The reduction of sulfite (+IV) to sulfide (–II) by sulfite reductase involves the transfer of six electrons (equation 6).



$$SO_3^{2-} + 6e^- + 8H^+ \rightarrow H_2S + 6H_2O \qquad (6)$$

The active centers of dissimilatory and assimilatory sulfite reductases (and nitrite reductases) are characterized by two metallo-cofactors, a reduced porphyrin of the isobacteriochlorin class, the siroheme (Murphy and Siegel, 1973a; Murphy et al., 1973b; Murphy et al., 1974; Scott et al., 1978; Cole, 1988) and an iron-sulfur cluster ([FeS]). These metallo-cofactors function in the transfer of the electrons to the substrate, as indicated schematically in Fig. 6. Siroheme-containing reductases have been isolated from a wide range of organisms. Siroheme was identified in assimilatory sulfite reductase from *Escherichia coli* (Murphy et al., 1973b), dissimilatory sulfite reductase from *Desulfovibrio* species (Murphy et al., 1973c), the dissimilatory "reverse" sulfite reductase of thiobacilli (Schedel et al., 1975; Trüper, 1994) and Chromatium (Schedel et al., 1979), and in the ammonium-producing dissimilatory nitrite reductase from *Escherichia coli* (Jackson et al., 1981; Lin and Kuritzkes, 1987), higher plants (Hucklesby et al., 1976; Vega and Kamin, 1977), algae (Zumft, 1972) and fungi (Vega and Garret, 1975).

Four major types of dissimilatory sulfite reductases are distinguished in sulfate-reducing bacteria, according to ultraviolet/visible absorption spectra and other molecular characteristics, the green protein desulfoviridin, the reddish brown colored desulforubidin and desulfofuscidin and P582 (Table 3; Fauque et al., 1991). Dissimilatory sulfite reductases generally have an $\alpha_2\beta_2$ tetrameric subunit composition (Crane and Getzoff, 1996). However a third type of subunit (γ) has been observed in a desulfoviridin-type of dissimilatory sulfite reductase in *Desulfovibrio vulgaris* (Pierik et al., 1992a) and *Desulfovibrio desulfuricans* strain Essex (Steuber et al., 1995), suggesting a hexameric structure ($\alpha_2\beta_2\gamma_2$). The γ-subunit is not encoded in the same operon as the α- and β-subunits and is not expressed coordinately with the α- and β-subunits (Karkhoff-Schweizer et al., 1993). The molecular mass of

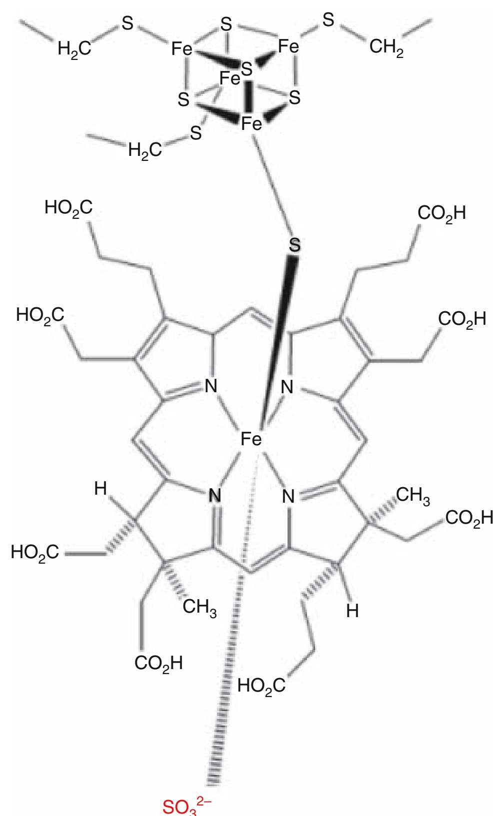


Fig. 6. Prosthetic group of sulfite reductase. Two metallo-cofactors, the [FeS] cluster and siroheme, are covalently coupled via a sulfur bridge. Sulfite is ligated to the iron atom of siroheme from the opposite direction on the non-bridging side.

dissimilatory sulfite reductases ranges between 145 and 225 kDa. *Desulfoviridin* has been identified in virtually all *Desulfovibrio species* and has since been regarded as a taxonomic marker for this genus (Lee and Peck, 1971; Lee et al., 1973a; Postgate, 1984b). However, desulfoviridin also has been detected in *Desulfococcus multivorans* (Widdel, 1980) and most *Desulfonema* species (Fukui et al., 1999), which are unrelated to *Desulfovibrio*. *Desulfoviridin* is unique among the dissimilatory sulfite reductases in that it does not react with CO and contains siroheme (two per $\alpha_2\beta_2$ holoenzyme) that is partly iron-free (viz. partly present as sirohydrochlorin). Siroheme and sirohydrochlorine are relatively easily released. The release of sirohydrochlorin is responsible for the red fluorescence in UV light of cells or extracts treated with dilute alkali (Postgate, 1956; Postgate, 1959). Siroheme prepared from desulfoviridin was found to catalyze the reduction of sulfite to sulfide and thiosulfate in the presence of artifical electron donors (Seki and Ishimoto, 1979). Analysis of the total iron content and spectroscopic investigations led to architectural models of the siroheme and the [FeS] clusters in desulfoviridin. Hagen and coworkers reported that each molecule of desulfoviridin from *Desulfovibrio vulgaris* contains 20 iron ions and a demetallated siroheme. EPR and Mössbauer spectroscopy revealed an unusually high cluster spin of S = 9/2 of a putative $[Fe_6S_6]$ prismane supercluster. Based on this finding, a superspin cluster was suggested with similarity to an $[Fe_6S_6]$ prismane cluster observed in another redox protein from *Desulfovibrio vulgaris* (Marritt and Hagen, 1996). In the latter protein, four iron atoms probably form a core that is flanked on opposite sites by two iron atoms of more ionic character; the latter couple ferromagnetically through the core (Pierik et al., 1992b; Pierik et al., 1992c). Such a cluster should be able to accept more than one electron. However, other analyses of the crystal structure of the protein revealed the presence of only four Fe ions in a novel [4Fe-3S-2O] cluster structure (Arendsen et al., 1998). Furthermore, EPR spectra of dissimilatory sulfite reductase purified under strict exclusion of O_2 yielded only weak signals, which also contradict the presence of a prismane-type super cluster (Fritz, 1999). Based on these findings, it is supposed that resonance signals previously thought to originate from a super cluster may actually result from oxidative damage of the [FeS] cluster of dissimilatory sulfite reductase. *Desulfoviridin* (containing 80% demetallated siroheme) from *Desulfovibrio desulfuricans* was reported to contain a total of 24 Fe ions (Steuber et al., 1995). Other reports on desulfoviridin from *Desulfovibrio vulgaris* furnished evidence for a total content of 10 Fe ions, and the presence of rhombic $[Fe_4S_4]$ clusters (Moura et al., 1988; Wolfe et al., 1994). *Desulforubidin* was identified in a *Desulfomicrobium* strain (formerly regarded as a *Desulfovibrio desulfuricans* strain), which lacks desulfoviridin (Lee et al., 1973b), and in *Desulfosarcina variablis* (Arendsen et al., 1993). The *Desulfomicrobium* desulforubidin has been reported to possess an $\alpha_2\beta_2$ structure (Moura et al., 1988; DerVartanian, 1994), whereas the corresponding enzyme from *Desulfosarcina* was demonstrated to have an $\alpha_2\beta_2\gamma_2$ structure (Arendsen et al., 1993). Reports from the same authors on the total iron content and structure of the [FeS] cluster also suggest differences from the aforementioned results. *Desulfofuscidin* was purified and characterized from thermophilic sulfate-reducing bacteria, *Thermodesulfobacterium commune* (Hatchikian and Zeikus, 1983; Hatchikian, 1994) and *Thermodesulfobacterium mobile* (Fauque et al., 1990). In both *Thermodesulfobacterium* species, the structure of desulfofuscidin was of the $\alpha_2\beta_2$ type. In contrast to the two aforementioned dissimilatory sulfite reductases (des-

Table 3. Biochemical characteristics of sulfite reductases.

Properties	Type of sulfite reductase							
	Desulfoviridin	Desulforubidin	Desulfofuscidin	P582	alSiR[a]	Archaeal SiR	Reverse SiR	aSiR
Organism	*Desulfovibrio*	*Desulfovibrio* *Desulfosarcina*	*Thermodesulfo-bacterium*	*Desulfotomaculum*	*Desulfovibrio*	*Archaeoglobus*	*Thiobacillus* *Chromatium*	*Escherichia*
Absorption maxima (nm)	628, 580, 408, 390, 279[1]	720, 580, 545, 392[1]	693, 576, 389, 279[1]	700, 582, 392, 280[1]	590, 545, 400[1]	593, 545, 394, 281[1]	700[3], 594, 393, 274[1]	714, 587, 386, 280[1]
Subunit structure	$\alpha_2\beta_2$ (%)[2,3] (n = 1–3)	$\alpha_2\beta_2\alpha_2\beta_2$ (%)[2,3]	$\alpha_2\beta_2$[1]		monomer	$\alpha_2\beta_2$[1]	$\alpha_2\beta_2$[1]$\alpha_4\beta_4$ [3]	$\alpha_2\beta_2$[1]
Molecular weight (kDa)	226[1]	225[1]	167[1] 190[2]	145[1]	27[2]	178.2[1]	160[1] 280[3]	685
Number of sirohemes	2[4]	2[2]	4[1,2]		1[1]	2[1]		4[1/β]
Total iron	10[4], 22[3]	15[3], 21[2]	21[1], 32[2]	54 µatoms per g protein	5[1]	22–24[1]	24[1], 51[3]	
Acid-labile Sulfur	10[4], 18[3]	19[3]	16–17[1]	15 µatoms per g protein	5[1]	20[2]	20[1], 47[3]	
[FeS] clusters	$[Fe_4S_4]$[4] $[Fe_6S_6]$[5]	$[Fe_4S_4]$[2] $[Fe_6S_6]$[3]	$[Fe_4S_4]$		$[Fe_4S_4]$[1]	$[Fe_4S_4]$	$[Fe_4S_4]$[1]	$[Fe_4S_4]$
Reaction with CO	–[1]	+	+[1]	+[1]	+			
Known substrate(s)	SO_3^{2-}[1], NO_2^-[4] NH_2OH[4]	SO_3^{2-}[1,3]	SO_3^{2-}, NO_2^- NH_2OH[2]	SO_3^{2-}, NO_2^- NH_2OH[1]	SO_3^{2-}	SO_3^{2-}	SO_3^{2-}, NO_2^-[1,2]	SO_3^{2-}, NO_2^- NH_2OH[1]
Major products	S^{2-}[1], NH_4^+[4]	S^{2-}, $S_3O_6^{2-}$[1]	$S_3O_6^{2-}$, NH_4^+[1,2]	S^{2-}[1,2]	S^{2-}		S^{2-}[3]	S^{2-}, NH_4^+
Minor products	$S_3O_6^{2-}$, $S_2O_3^{2-}$[6,7]		S^{2-}, $S_2O_3^{2-}$[1]	$S_3O_6^{2-}$, $S_2O_3^{2-}$[2]	–		$S_2O_3^{2-}$, $S_2O_3^-$[3]	–
Electron donor (in vitro)	methylviologen[1]	methylviologen[1]	methyl- or benzylviologen	methylviologen[1]	methylviologen	methylviologen	methyl- or benzylviologen	NADPH methylviologen
References	[1]Lee et al., 1973a [2]Pierik et al., 1992a [3]Steuber et al., 1995 [4]Wolfe et al., 1994 [5]Pierik et al., 1992b,c [6]Akagi, 1983 [7]Lee and Peck, 1971	[1]Lee et al., 1973b [2]Moura et al., 1988 [3]Arendsen et al., 1993	[1]Hatchikian and Zeikus, 1983	[1]Trudinger, 1970 [2]Akagi and Adams, 1973	[1]Moura and Lino, 1994	[1]Dahl et al., 1993 [2]Dahl et al., 1994	[1]Triper, 1994 [2]Schedel et al., 1975 [3]Schedel et al., 1979	[1]Siegel and Davis, 1974 [2]Siegel et al., 1982

[a]aSir, sulfite reductase.

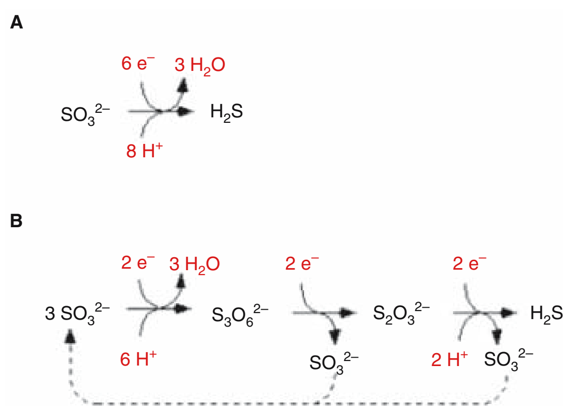
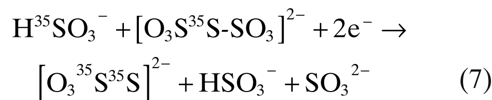


Fig. 7. Possible pathways of sulfite reduction to sulfide. (A) Direct reduction with six electrons without the formation of intermediates. (B) Trithionate pathway. The reduction occurs via three consecutive two-electron steps with the formation of tetrathionate and trithionate as intermediates.

ulfoviridin and desulforubidin), four instead of two siroheme cofactors per enzyme were found in desulfofuscidin. P582 was identified in the spore-forming *Desulfotomaculum nigrificans* (Trudinger, 1970; Akagi and Adams, 1973).

Two different pathways for the reduction of sulfite to sulfide are discussed (Fig. 7): a sequential reduction in three two-electron steps with the formation trithionate and thiosulfate as intermediates, and a direct six-electron reduction without the formation of the aforementioned intermediates. Evidence for the first pathway, termed trithionate pathway (Fig. 7b) is mostly based on in vitro studies (Kobayashi et al., 1972; Akagi, 1983). In the in vitro experiments methyl- or benzylviologen were used as artificial electron donors; they were in a coupled system generated by reduction with hydrogen/hydrogenase. Under these conditions, trithionate and thiosulfate were identified in addition to sulfide as products of sulfite reduction. Under certain assay conditions, trithionate and thiosulfate were formed at concentrations similar to those of sulfide (Kobayashi et al., 1974). Also the enzymes in support of the proposed trithionate pathway, viz. trithionate reductase and thiosulfate reductase, were identified (Akagi et al., 1994). The purified desulfoviridin from *Desulfovibrio gigas* reduced sulfite with reduced methylviologen exclusively to trithionate (Lee and Peck, 1971). A "thiosulfate-forming" enzyme was isolated from *Desulfovibrio vulgaris* which formed thiosulfate from bisulfite and trithionate. Labeling experiments with ^{35}S demonstrated that the sulfur of formed thiosulfate originated from bisulfite and the inner S atom of trithionate, according to the following equation (Drake and Akagi, 1977).



$$H^{35}SO_3^- + [O_3S^{35}S\text{-}SO_3]^{2-} + 2e^- \rightarrow [O_3^{35}S^{35}S]^{2-} + HSO_3^- + SO_3^{2-} \qquad (7)$$

Also purified from *Desulfovibrio vulgaris* was a "trithionate-reducing system", which could form thiosulfate from trithionate and sulfite with flavodoxin (reduced by hydrogenase) serving as electron donor. In this system, a second protein was acting in close association with desulfoviridin and was required for trithionate formation (Kim and Akagi, 1985). A thiosulfate reductase that stoichiometrically reduced thiosulfate to sulfite and sulfide was purified from *Desulfotomaculum nigrificans* (Nakatsukasa and Akagi, 1969), *Desulfovibrio gigas* (Hatchikian, 1975) and *D. vulgaris* (Badziong and Thauer, 1980; Aketagawa et al., 1985). In summary, the stoichiometric formation of sulfite during the reduction of trithionate to thiosulfate and the reduction of thiosulfate to sulfite would add two loops to the pathway of sulfite reduction, as proposed by Kobayashi et al. (1974; Fig. 7b). Fitz and Cypionka (1990) reported the formation of trithionate and thiosulfate during reduction of sulfite with deenergized cells of *Desulfovibrio desulfuricans*. The occurrence of a trithionate pathway would be understandable from certain viewpoints of bioenergetics. The formation of trithionate would provide a relatively strong oxidant ($E_0' = +0.225$ V) and thus a favorable acceptor even for high potential electron donors, as for instance from dehydrogenation of succinate (E_0' = +0.030 V). The nature of the natural electron donor of the three two-electron reduction steps of the trithionate pathway has not been been resolved unequivocally (Peck and Lissolo, 1988). Furthermore, there are also arguments against a trithionate pathway (Chambers and Trudinger, 1975; Trudinger and Loughlin, 1981). The formation of trithionate and thiosulfate may be regarded as by-reactions. These may become dominant under in vitro conditions, for instance due to the relatively high concentrations of added bisulfite. Excess bisulfite or sulfite could react with intermediates bound to siroheme (Trudinger and Loughlin, 1981). Also, a reaction of bisulfite with formed H_2S seems possible. Bisulfite and sulfide are known to react chemically to thiosulfate and thionates, especially at low pH (Heunisch, 1976). If the side-activities of certain proteins facilitated such a reaction under in vitro conditions, the produced sulfide would not accumulate but rather be scavenged to give rise to the observed oxo-anions. Trithionate and thiosulfate reductases may serve for utilization of their substrates from the environment or for scavenging them as byproducts of the bisulfite reductase reaction. Low concentrations (5–100 μM) of thiosulfate formed in deenergized

cells from added sulfite (Fitz and Cypionka, 1990) or in cells growing on sulfate (Vainshtein et al., 1980) also may be interpreted as byproducts resulting from a reversely operating thiosulfate reductase with sulfide and sulfite as reactants; the electrons from this low potential reaction ($E_0' = -0.402$ V) could be easily consumed by other reductive processes. Evidence for the six-electron reduction of bisulfite to sulfide was achieved in a reconstitution assay with membrane-bound desulfoviridin, cytochrome c_3 and hydrogenase, all from *Desulfovibrio desulfuricans* (Steuber et al., 1994); thiosulfate and trithionate were only detected in small amounts. This experiment also indicated that cytochrome c_3 can act as electron donor for desulfoviridin, an observation that is topologically not yet understandable. The view of a six-electron reduction without the formation of free trithionate or thiosulfate as intermediates is favored if one compares sulfite reductases in dissimilatory and assimilatory sulfate metabolism and assumes that these enzymes, which are both siroheme proteins, employ in principle the same mechanism.

In assimilatory sulfur metabolism, the assimilatory sulfite reductase generates sulfide for the synthesis of the sulfur-containing amino acid cysteine. Methionine and cofactors (like coenzyme A) derive their sulfur from cysteine. In contrast to dissimilatory sulfite reductase, none of the known assimilatory sulfite reductases (aSiRs) forms detectable amounts of trithionate or thiosulfate in vitro (Lee et al., 1973a; Peck and Lissolo, 1988). Thus aSiRs reduce sulfite with high fidelity directly via a six-electron reduction to sulfide. A sulfite reductase isolated from the sulfate-reducing bacterium *Desulfovibrio vulgaris* shared the high fidelity reduction of sulfite to sulfide with the aSiR from *Escherichia coli.* Therefore it was termed "assimilatory-like sulfite reductase" (Lee et al., 1973a). The enzyme has been studied in much detail. The aSiR from *Desulfovibrio vulgaris* was also functionally expressed in other *Desulfovibrio* hosts (Tan et al., 1994). The aSiRs from sulfate-reducing bacteria differ from the aSiR from *Escherichia coli* and the dissimilatory sulfite reductases from sulfate-reducing bacteria. The former are composed of only one polypeptide and do not form multimeric proteins; they have low-spin iron instead of high-spin iron and only one siroheme and [FeS]-cluster per molecule (Huynh et al., 1984a; Huynh et al., 1984b; Moura and Lino, 1994). Tan and Cowan (1991) proposed a mechanism for the the six-electron reduction catalyzed by aSiR, which may also serve as a working hypothesis to understand other sulfite reductases. The sulfur atom of sulfite binds the Fe^{2+} ion of the siroheme from the nonbridging face. A two-electron reduction prepares the O-atom of the S-O bond for protonation so that a hydroxyl anion can be eliminated. Through repeated reduction by two electrons and subsequent protonation, the oxygen atoms are stepwise removed from the sulfur resulting in the formation of sulfide (Fig. 8). According to the model presented by Tan and Cowan (1991), the electrons for the reduction steps are "pushed" from the electron loaded [FeS]-clusters via the siroheme into the sulfite. In addition, the local environment in the sulfite-binding pocket may participate in the reduction reaction by providing protons from amino acid side chains to the O-atoms of sulfite. Such a mechanism would correspond to the "push and pull" paradigm, which has also been used to describe the cleavage of O-O bonds of peroxides by heme-containing oxygenases (Dawson, 1988; Poulos, 1988). Lui and Cowan (1994) have also proposed a six-electron reduction via a push-and-pull mechanism for dissimilatory sulfite reductase from *Desulfovibrio vulgaris.* In intact desulfoviridin, sulfite can only bind to reduced siroheme, whereas sulfite can bind to free siroheme in its oxidized state. These observations suggested a gating mechanism of dissimilatory sulfite reductase where a redox-linked structural transformation is required for substrate binding (Lui and Cowan, 1994).

Insight into the mechanism of sulfite reduction and the structure of sulfite reductase have also benefited to a great extent from studies of the aSiR from *Escherichia coli.* This enzyme consists of eight flavoprotein subunits (α-subunits), which accept electrons from NADPH, and four hemoprotein subunits (β-subunits), which accept the electrons from the flavoprotein subunits and catalyze the six-electron reduction of sulfite to sulfide. Thus aSiR from *E. coli* has an overall $\alpha_8\beta_4$-structure. Each hemoprotein-subunit carries one siroheme and one $[Fe_4S_4]$-cluster (Siegel et al., 1974; Siegel and Davis, 1974; Siegel et al., 1982). A chemical link between the siroheme and the $[Fe_4S_4]$-cluster was indicated by electronic exchange coupling observed by spectroscopic studies (Christner et al., 1984). The analysis of the crystallographic structure of the hemoprotein at a resolution of 3 Å suggested that a sulfur anion of a cysteine (Sγ) covalently links the central iron in siroheme with one of the Fe ions in the cluster (McRee et al., 1986). A more recent analysis of the crystallographic structure of the hemoprotein at a resolution as high as 1.6 Å (Crane et al., 1995; Crane and Getzoff, 1996) demonstrated that the Sγ is provided by Cys^{483}. This bridge was found to be maintained in all reduction states of the enzyme studied so far on a structural level. The 1.6 Å structure also allowed recognition of further refined details of the structure. The hemoprotein consists of three

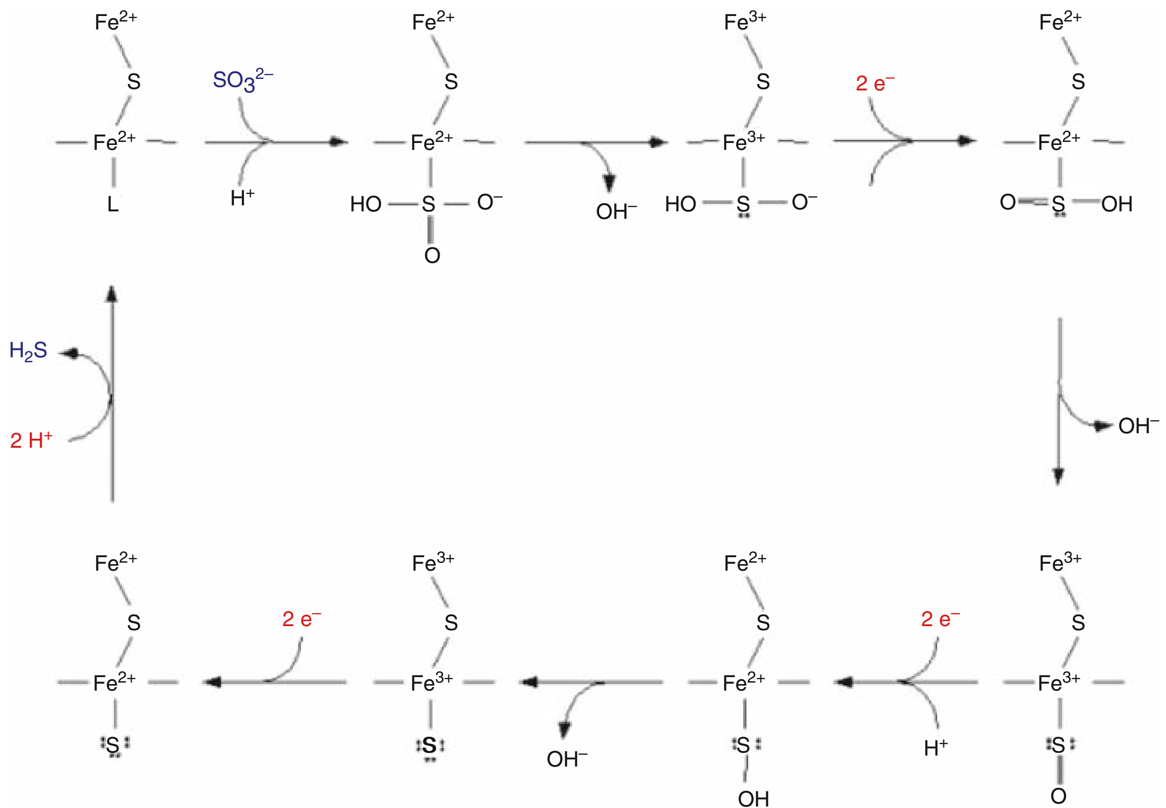
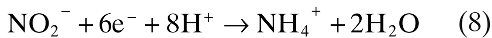


Fig. 8. Suggested mechanism for the reduction of sulfite to sulfide by subsequent two-electron steps. The [4Fe-4S] cluster that is coupled to the Fe atom of siroheme via a sulfur bridge is represented by only one Fe-ion. The L represents the protein ligand that coordinates the Fe ion of siroheme. During catalysis, L is substituted by sulfite. Modified from Moura and Lino (1994).

domains that ligate the two metallo-cofactors at their interface. This interface is predominantly formed by β-sheets which are flanked at the outside by solvent-exposed α-helices. Domain 1 and 1′ form a novel architecture reminiscent of a parachute and project harness hairpins into the interface-cofactor area. Domain 2 contributes the residues for the siroheme binding and positively charged residues that form the binding pocket for the anion substrate at the distal face of the siroheme. Domain 3 provides four cysteine residues (including the bridging ligand Cys^{483}) to ligate the $[Fe_4S_4]$-cluster at the proximal side of the siroheme. The anion-binding pocket facing the distal side of the siroheme is remarkably rich in positively charged side chains, for instance Arg and Lys residues. Thus a strongly polarizing and proton-rich environment is established which may "pull" electrons of the S-O bond into the direction of the O-atom. Also water molecules could be positioned to interact directly with the anion substrate. Thus the structural details of the active site support the earlier model of a "push-and-pull" mechanism of the six-electron reduction of sulfite to sulfide. The structure of the hemoprotein from *E. coli* is characterized by a vertical pseudo-twofold axis that relates an N-terminal sequence repeat (domain 1 and 2) to a C-terminal sequence repeat (domain 1′ and 3); this suggests that the hemoprotein arose by gene duplication. Furthermore, analysis revealed the presence of five homologous regions in the sequence of the hemoprotein. Three of them (homology regions 1–3) encompass regions essential for the active center and for stabilization of the protein structure. Such homology regions have also been observed in dissimilatory sulfite reductases and therefore support the idea that dissimilatory sulfite reductases exhibit similar structure and also catalyze a six-electron reduction without formation of intermediates (Crane et al., 1995; Crane and Getzoff, 1996).

There is a striking similarity between sulfite reductase and another enzyme with the capacity for a six-electron reduction, the ammonifying nitrite reductase (not to be confused with NO-forming nitrite reductase in denitrifiers), which catalyzes the dissimilatory reduction of nitrite to ammonia (equation 8). Such types of nitrite

reductases also contain siroheme in the active center.

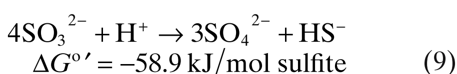


$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \quad (8)$$

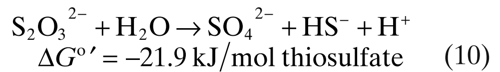
Cytochrome *c* nitrite reductase from Sulfurospirillum deleyianum not only catalyzes the six-electron reduction of nitrite to ammonia, but also that of sulfite to sulfide. Interestingly, the analysis of the crystal structure of this enzyme (Einsle et al., 1999) revealed marked structural differences from the aSiR from *E. coli*. The former enzyme is a homodimer that is predominately composed of α-helices and contains 10 closely arranged hemes. Apparently, different structures and probably also varied mechanisms have evolved to accomplish a six-electron reduction. A membrane-bound cytochrome *c*-containing nitrite reductase (also isolated from *Desulfovibrio desulfuricans*) catalyzes the six-electron reduction of nitrite to ammonia as well as that of sulfite to sulfide (Liu et al., 1994; Pereira et al., 1996).

Sequence analysis of the genes (*dsr*) encoding dissimilatory sulfite reductases from *Desulfovibrio vulgaris*, *Archaeglobus fulgidus* and *Chromatium vinosum* demonstrated that the three proteins are true homologues (Dahl et al., 1993; Hipp et al., 1997; Karkhoff-Schweizer et al., 1995). A more detailed study by Wagner et al. (1998) revealed that the evolutionary relationships derived from *dsr* sequences of sulfate-reducing microorganisms were nearly identical to relationships inferred from the 16S rRNA sequences. The authors concluded that bacterial and archaeal dissimilatory sulfite reductases originated from a common ancestor.

Dismutation of Sulfur Species A unique metabolic capacity of certain sulfate-reducing bacteria is growth by dismutation (disproportionation) of sulfite or thiosulfate, a process which may be formally described as an inorganic fermentation (Bak and Pfennig, 1987). The reactions are carried out by *Desulfovibrio sulfodismutans*, *Desulfobacter curvatus* and a so far unnamed species, strain NTA3, that grew significantly better by dismutation than by sulfate reduction. Growth by disproportionation of thiosulfate was also reported for an anaerobic bacterium, designated strain DCB-1 (Mohn and Tiedje, 1990a). Disproportionation of thiosulfate was demonstrated by radiotracer experiments in marine sediments and recognized as an important part of the sulfur cycle (Jørgensen, 1990; Jørgensen and Bak, 1991).

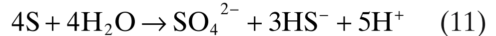


$$4SO_3^{2-} + H^+ \rightarrow 3SO_4^{2-} + HS^- \quad \Delta G^{o\prime} = -58.9\ kJ/mol\ sulfite \quad (9)$$



$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+ \quad \Delta G^{o\prime} = -21.9\ kJ/mol\ thiosulfate \quad (10)$$

A dismutation of elemental sulfur with standard activities of the products is thermodynamically unfavorable. However, because the activity of the insoluble, elemental sulfur is always equal to 1, the free energy of the reaction is strongly influenced by the concentrations of the products and the pH:



$$4S + 4H_2O \rightarrow SO_4^{2-} + 3HS^- + 5H^+ \quad (11)$$

Standard concentrations; pH = 7: $\Delta G^{o\prime} = +10.2$ kJ/mol sulfur

SO_4^{2-} and HS^-, 0.001 M; pH = 7: $\Delta G^{o\prime} = +6.9$ kJ/mol sulfur

SO_4^{2-} and HS^-, 0.001 M; pH = 8: $\Delta G = -11.3$ kJ/mol sulfur

A purely chemical dismutation of sulfur to H_2S or polysulfide and oxygen-containing sulfur compounds was favored by elevated temperature and pH values above 7 (Belkin et al., 1985). If bacteria dismutated the formed sulfur-oxygen compounds to sulfide and sulfate, reactions (9) and (10) would result. Evidence for a microbial disproportionation of sulfur to sulfate and sulfide was provided by Thamdrup et al. (1993), who demonstrated this process in marine-enrichment cultures. The disproportionation of sulfur by these enrichment cultures was accompanied by a fractionation of the sulfur isotopes; sulfate was enriched in ^{34}S and sulfide depleted in ^{34}S (Canfield and Thamdrup, 1994). The sulfate reducer *Desulfobulbus propionicus* was the first microorganism shown to disproportionate sulfur in pure culture, even though growth under these conditions was very slow (Lovley and Phillips, 1994b; Fuseler and Cypionka, 1995). Two species of the new genus *Desulfocapsa*, *D. thiozymogenes* and *D. sulfoexigens*, grew well by disproportionation of sulfur. Both species required the presence of a sulfide scavenger (e.g., ferrihydrite) for growth with sulfur as sole source of energy and also can grow by disproportionation of thiosulfate and sulfite. *Desulfocapsa thiozymogenes*, but not *Desulfocapsa sulfoexigens*, can grow by reduction of sulfate to sulfide as the mode of energy conservation (Janssen et al., 1996; Finster et al., 1998).

Evidence has been furnished that the disproportionation of sulfite or thiosulfate to sulfate and sulfide proceeds via a reversal of the reactions of dissimilatory sulfate reduction (Krämer and Cypionka, 1989). Thus, ATP sulfurylase and not ADP sulfurylase, which is found in many lithotrophic purple phototrophs (Brune, 1989; Fischer, 1988; ; 1989) and some thiobacilli (Kelly,

1988; Kelly, 1989), is involved in the formation of sulfate from APS and allows "inorganic" substrate-level phosphorylation; it has not yet been established how stoichiometric amounts of PP_i are formed for the conversion of APS to sulfate and ATP. Reducing equivalents are derived from conversion of bisulfite to APS, which has a relative positive potential ($E_0' = -0.060$ V; see also Fig. 3). For the reductive part leading to H_2S, shifting of these reducing equivalents by reversed electron transport seems to be necessary; this was indeed indicated by the sensitivity of the dismutation to uncouplers (Krämer and Cypionka, 1989).

Electron Acceptors Other Than Sulfate

Inorganic Sulfur Species Most sulfate-reducing bacteria can use thiosulfate and sulfite as electron acceptors in addition to sulfate (Table 1). *Desulfotomaculum acetoxidans* (Widdel and Pfennig, 1981b), *Desulfonema magnum* (Widdel et al., 1983), *Desulfocella halophila* (Brandt et al., 1999), and some sulfate reducers originally assigned to *Desulfobacterium* did not reduce sulfite in growth tests. Inasmuch as sulfite is assumed to be generally a free intermediate in dissimilatory sulfate reduction, the failure of sulfate-reducing bacteria to grow with sulfite at nontoxic concentrations may be due to the lack of a specific transport system.

Oxoanions (other than sulfite and thiosulfate) have scarcely been tested in cultures of sulfate reducers. *Desulfovibrio* strains have been reported to reduce trithionate ($S_3O_6^{2-}$), tetrathionate ($S_4O_6^{2-}$), and dithionite ($S_2O_4^{2-}$) (Postgate, 1951; Ishimoto et al., 1954a; Fitz and Cypionka, 1990).

Among the sulfate-reducing bacteria, some species such as of the genera *Desulfohalobium*, *Desulfofustis*, *Desulfuromusa* and *Desulfospira* can grow with elemental sulfur (see Sulfur-reducing bacteria). Other sulfate reducers may produce some H_2S in a by-reaction without growth after transfer of sulfate-grown cells to media with crystalline (rhombic) or colloidal sulfur. Growth of many species of sulfate reducers is even inhibited by sulfur (e.g.,Widdel and Pfennig, 1981b; Widdel et al., 1983; Bak and Widdel, 1986b), probably because sulfur as an oxidant shifts the potential of redox couples in the medium and cells into an unfavorable range. In sulfate-reducing bacteria able to grow with sulfur, its reduction is probably directly catalyzed by the tetraheme cytochrome c_3 (Fauque et al., 1979; Fauque et al., 1980; Cammack et al., 1984).

Sulfonates, DMSO Reduction of sulfonates by sulfate-reducing bacteria was first described by Lie et al. (1996). These authors demonstrated utilization of cysteate, isethionate (2-hydroxyethanesulfonate), and acetaldehyde-2-sulfonate by *Desulfovibrio desulfuricans* strain IC1. Isethionate was converted to sulfide and acetate. Cysteate was also used as an electron acceptor by strains of *Desulfomicrobium baculatum* DSM 1741 and *Desulfobacterium autotrophicum*. The former strain and *Desulfovibrio desulfuricans* ATCC 29577 also used isethionate. *Desulfovibrio strain* RZACYSA can use taurine (aminoethanesulfonate), cysteate, isethionate and aminoethanesulfonate as electron acceptors (Laue et al., 1997b). Cysteate and taurine also can be fermented by some sulfate-reducing bacteria (see below).

Utilization of dimethylsulfoxide (DMSO) as an electron acceptor for growth of sulfate-reducing bacteria resulting in the production of dimethylsulfide was first reported by Jonkers et al. (1996). Out of eight strains of sulfate reducers isolated from a marine or high-salt environment, five were shown to use DMSO; most of them were *Desulfovibrio* strains. In addition, one strain of the barophilic *Desulfovibrio profundus* was also shown to use DMSO by Bale et al. (1997); the same study also demonstrated DMSO reduction by the type strain of *Desulfovibrio salexigens*, which was reported by Jonkers et al. (1996) not to reduce this electron acceptor. Sulfate and DMSO were reduced simultaneously.

Nitrate, Nitrite Nitrate is reduced by a few *Desulfovibrio* species (Seitz and Cypionka, 1986; Keith and Herbert, 1983; McCready et al., 1983; Mitchell et al., 1986), *Desulfobulbus propionicus* (Widdel and Pfennig, 1982) and *Desulfobacterium catecholicum* (Szewzyk and Pfennig, 1987; Moura et al., 1997). Nitrate may be preferred over sulfate (Seitz and Cypionka, 1986), or vice versa (Widdel and Pfennig, 1982). Dalsgaard and Bak (1994) showed that in an isolate from rice paddy soil, *Desulfovibrio desulfuricans* strain C4S, nitrate reduction was strongly inhibited by sulfide; at 0.46 mM sulfide, the specific growth rate was less than 10% of the maximum value, and no growth occurred at 0.75 mM sulfide. As the authors suggested, this implies that some negative results from growth tests of sulfate reducers with nitrate may be questioned because of the inclusion of 0.5 mM sulfide as a reducing agent in the media.

In sulfate and sulfur reducers, the end product of nitrate reduction, which occurs via nitrite, is ammonia and not N_2 as in denitrifying bacteria. The nitrate reductase of *Desulfovibrio desulfuricans* was purified and shown to be a monomeric

74-kDa protein with a [4Fe-4S] center and a molybopterin guanine dinucleotide cofactor (Moura et al., 1997). The crystal structure of this periplasmic enzyme has been determined (Dias et al., 1999); this is the first resolution of the three-dimensional structure of a nitrate reductase. Although bisulfite reductases also show activity toward nitrite, specific nitrite reductases appear to be involved in the subsequent reduction of formed nitrite to ammonium. A hexaheme cytochrome c_3 acting as nitrite reductase and consisting of 62-kDa and 19-kDa subunits has been isolated from *Desulfovibrio desulfuricans* (Liu and Peck, 1981b; Moura et al., 1997).

Iron (III) Whereas several non-sulfate-reducing members of the δ-subclass of Proteobacteria, including sulfur-reducing bacteria, can grow with iron(III) compounds as electron acceptors, this capacity has only been occasionally observed in sulfate-reducing bacteria. Reduction of chelated iron(III) was demonstrated in enzymatic tests with several *Desulfovibrio* species, *Desulfobacterium autotrophicum* and *Desulfobulbus propionicus* (Lovley et al., 1993b), and in growth tests with *Desulfovibrio profundus* (Bale et al., 1997) and several psychrophilic species (Knoblauch et al., 1999b). Among the latter, *Desulfotalea psychrophila* also reduced insoluble (nonchelated) inorganic ferric iron (ferrihydrite); however, growth was not observed.

Oxygen The study of the influence of O_2 on bacteria with an anaerobic metabolism is an ecologically relevant and biochemically interesting topic. Exposure of anaerobic bacteria to O_2 is a frequent, natural event in environments with fluctuating O_2 penetration and at the anoxic/oxic interface. Furthermore, if oxic environments such as soils or oligotrophic sediments turn anoxic due to flooding or eutrophication, respectively, communities of anaerobic bacteria gradually become established; this is most likely to occur via passage of "inocula" through the oxic environment, as for instance oxic water.

Studies of the effects of O_2 on anaerobic bacteria include several aspects; these are, for instance, anaerobe tolerance of O_2 and survival under oxic conditions, the possibility that O_2 at low concentrations may even serve as electron acceptor and allow energy conservation, and the protection of cells against harmful effects.

Pure cultures of sulfate-reducing bacteria in aerated media in laboratory experiments died off at different rates, depending on the species (Hardy and Hamilton, 1981; Cypionka et al., 1985; Abdollahi and Wimpenny, 1990). Simultaneous presence of sulfide sometimes increased the detrimental effect of O_2 (Cypionka et al., 1985). This sensitivity to long-term oxic conditions suggest that permanently oxic waters or soil usually do not harbor nonsporeforming sulfate-reducing bacteria. Only endospores of *Desulfotomaculum* species may be present in such environments at significant numbers (Widdel, 1988). However, in dense aquatic microbial populations, nonsporeforming sulfate-reducing bacteria were also observed in oxic zones. Studies on the natural distribution of sulfate-reducing bacteria revealed high numbers in zones that are exposed to rapid changes of the O_2 concentration or that are even oxic over prolonged periods, e.g., in biofilms (Ramsing et al., 1993) and microbial mats (Canfield and Des Marais, 1991; Krekeler et al., 1997; Teske et al., 1998). The existence of anoxic microniches (Jørgensen, 1977) in such zones, which might explain the occurrence of active sulfate-reducing bacteria in oxic environments, is questionable; in microbial aggregates, the O_2-reducing activity with the available electron donors was not sufficient to cope with O_2 penetration (Plough et al., 1997).

The effect of O_2 on pure cultures of sulfate-reducing bacteria in horizontal oxic/anoxic transition zones was studied in sulfidic agar with an organic electron donor under an oxic head space. In opposed O_2-sulfide gradients, several species of sulfate-reducing bacteria exhibited growth, even though sulfate was absent (Widdel, 1980; Cypionka et al., 1985). However, there was evidence that the sulfate reducers used a chemical oxidation product of sulfide, most probably thiosulfate, as electron acceptor, without getting into direct contact with O_2. The oxidation product was again reduced to sulfide. The resulting sulfur cycle mediated between the sulfate-reducing bacteria and the otherwise harmful O_2 that served indirectly as final electron acceptor. Such a mediating cycle also may occur in natural habitats as long as electron donors are available.

However, there is also evidence that sulfate-reducing bacteria are able to utilize O_2 directly. In experiments with cell suspensions of *Desulfovibrio*, O_2 was shown to serve directly as electron acceptor for H_2 oxidation, and to enable significant proton translocation (Dilling and Cypionka, 1990; Dannenberg et al., 1992). Growth due to O_2 utilization has not been observed in these experiments. Nevertheless, owing to the high rates of O_2 consumption, which were even higher than in aerobic bacteria (Krekeler et al., 1997; Kuhnigk et al., 1996), the respiratory activity of *Desulfovibrio* may be of

considerable ecological relevance for the scavenging of O_2 and an ATP gain for survival, if the habitat turns transiently oxic. The underlying mechanism of the buildup of a proton gradient with O_2 as electron acceptor is not understood. It is true that cytochrome c_3 can directly react with O_2; however, as a periplasmic enzyme and electron acceptor of hydrogenase, cytochrome c_3 reacting with O_2 would not allow the generation of a proton gradient. Hence, O_2 is expected to react with one or some of the redox proteins of the electron transport chain so that a proton gradient can be formed. From the viewpoint of thermodynamics, O_2 is the most favorable of all electron acceptors and could replace any of the intermediates from the pathway of sulfate reduction. The problem lies in a controlled reaction of O_2 that avoids instantaneous damage of proteins and redox centers by reactive oxygen species (e.g., superoxide or peroxide).

In other experiments, O_2 at concentrations as low as 0.24 to 0.48 μM were observed to support growth of *Desulfovibrio vulgaris* strain Hildenborough in lactate medium with a strongly limiting sulfate concentration (Johnson et al., 1997). However, it cannot be completely excluded that O_2 was only indirectly reduced via a mediating sulfur cycle as suggested before (Cypionka et al., 1985). At concentrations above approximately 1 μM, O_2 arrested growth of *D. vulgaris* (Johnson et al., 1997). In other experiments using four different strains of sulfate-reducing bacteria, the rate of sulfate reduction was strongly affected by an O_2 concentration of 15 μM (Marshall et al., 1993).

Reduction of O_2 by sulfate-reducing bacteria may occur not only with electron acceptors directly utilized from the medium, but also with storage compounds. *Desulfovibrio gigas* and *Desulfovibrio salexigens* both can accumulate massive amounts of polyglucose during anaerobic growth with lactate and sulfate (Stams et al, 1983; van Niel et al., 1996; van Niel et al., 1998). Polyglucose utilization was shown to be involved in the survival under oxic conditions. In *Desulfovibrio gigas*, NADH produced during the breakdown of polyglucose was reoxidized by NADH:rubredoxin oxidoreductase, a dimeric flavoprotein consisting of a 27- and a 32-kDa subunit, and containing two molecules of each, FAD and FMN per enzyme molecule (LeGall and Xavier, 1996). The rubredoxin is oxidized with O_2 at a flavoheme protein, yielding water as the end product (Gomes et al., 1997).

A further argument for the assumption that O_2 is not only a harmful agent, but at low concentrations, also a potential electron acceptor for respiratory energy conservation in sulfate-reducing bacteria comes from the observation of aerotaxis in *Desulfovibrio* species. In medium with lactate and without (or limiting) sulfate in capillary tubes, *Desulfovibrio* species positioned themselves in bands at low O_2 concentration (Johnson et al., 1997; Eschemann et al., 1999). *Desulfovibrio oxyclinae* formed ring-shaped bands around O_2 bubbles (Krekeler et al., 1998). Band formation was dependent on the presence of an electron donor. Measurements of the O_2 gradient with microelectrodes revealed that the side of the bands facing the bubbles was exposed to O_2 concentrations of up to 50 μM, whereas the other side of the band was anoxic. This indicates an intensive O_2 respiration within the band. Thus aerotactic band formation and O_2 respiration can be regarded as a means to decrease the O_2 concentration completely and restore anoxic conditions within a narrow zone (Eschemann et al., 1999). The attraction of sulfate-reducing bacteria by O_2 at low concentrations is so far unique among anaerobic bacteria; they are usually assumed to be repelled by O_2 (Armitage, 1997).

A molecular key element of bacterial chemotactic response is the presence of methyl-accepting chemotaxis proteins (MCPs), which receive and transmit the attracting or repelling signal. MCPs consist of a periplasmic N-terminal domain which binds the attractant or repellent, a transmembrane spanning segment, and a cytoplasmic C-terminal domain which functions as signal transducer. These proteins have been well studied in *Escherichia coli* and *Salmonella typhimurium* (Stock and Surette, 1996). A 73-kDa protein discovered in *Desulfovibrio vulgaris* (and named DcrA) shows in its C-terminal domain similarities to that of MCPs in *E. coli*; the C-terminal domain in the latter is the site of methylation. There is also evidence for a cytoplasmic location of the C-terminal domain of DcrA, in accordance with that of MCPs in *E. coli* (Dolla et al., 1992; Deckers and Voordouw, 1994b). In contrast, the N-terminal domain of DcrA did not exhibit significant sequence homology with known MCPs. The N-terminus of DcrA was found to harbor a *c*-type heme. Addition of O_2 or the reducing agent dithionite resulted in a decrease or increase, respectively, in the methylation of DcrA. DcrA, a *c*-type cytochrome that was unknown before, may function in sensing O_2 or the redox potential of the medium (Fu et al., 1994). To further elucidate the role of DcrA in chemotaxis, a knock-out mutant of the coding gene, *dcrA*, was constructed. However, phenotypic analysis of the mutant did not reveal a deficiency in aerotaxis (Fu and Voordouw, 1997). Subsequent analysis of a genome library of *D. vulgaris* strain Hildenborough revealed the presence of at least 11 additional *dcr* genes (*dcrB* to *dcrL*; Deckers and Voordouw, 1994a). Phylogenetic analysis suggested that the *dcr* family is

distinct from the *mcp* families in other eubacteria and arose early in evolution (Deckers and Voordouw, 1996).

Also, proteins that might be involved in the detoxification of damaging oxygen species have been identified in sulfate-reducing bacteria. Superoxide dismutase and catalase activity have been detected in *Desulfovibrio* species (Bruschi et al., 1977; Hatchikian et al., 1977). *Desulfoferredoxin* (Moura et al., 1990) and neelaredoxin (Chen et al., 1994c) are mononuclear non-heme iron proteins that have been purified from *Desulfovibrio* species found to catalyze removal of superoxide (Romão et al., 1999; Silva et al., 1999b). Neelaredoxin is encoded in an operon with two additional open reading frames (ORFs) which putatively encode two chemotaxis proteins (Silva et al., 1999b). Interestingly, significant sequence similarities between desulfoferredoxin and neelaredoxin from *Desulfovibrio* and neelaredoxin and superoxide oxidoreductase from *Pyrococcus furiosus* were reported; the latter does not dismutate, but rather catalyzes a net reduction to H_2O_2 (Jenney et al., 1999). This observation indicates a mechanism of superoxide detoxification in sulfate-reducing bacteria that is different from the mechanism of superoxide dismutase. The genes *rub* and *rbo*, which code for rubredoxin and a putative rubredoxin oxidoreductase, respectively, were identified in *Desulfovibrio vulgaris* (Hildenborough) as one transcriptional unit (Brumlik et al., 1989). The *rub-rob* genes from *Desulfoarculus baarsii* complemented an *Escherichia coli* mutant that was deficient in superoxide dismutase (Pianzzola et al., 1996). The gene product Rob is suggested to scavenge superoxide not via dismutation as superoxide dismutase, but via a reductive mechanism using electron donors such as NAD(P)H (Liochev and Fridovich, 1997), possibly comparable to the abovementioned superoxide oxidoreductase.

Definite aerobic growth of sulfate-reducing bacteria (viz. for an infinite number of generations in oxic media) has not been observed so far, despite their capacity to couple O_2 reduction with energy conservation, their chemotaxis toward microaerobic zones, and their detoxification mechanisms. From the viewpoint of biochemistry, there is no obvious reason to assume that the capacity for dissimilatory sulfate reduction and aerobic growth in the same bacterium are mutually exclusive.

Fumarate Some *Desulfovibrio* species ferment fumarate or malate. In the presence of an additional electron donor (e.g., H_2 or formate), fumarate and malate are quantitatively reduced to succinate (Grossmann and Postgate, 1955; Miller and Wakerley, 1966; Barton et al., 1970; Wolfe and Pfennig, 1977), which represents a purely respiratory type of energy conservation (fumarate respiration; Graf et al., 1985; Kröger, 1987). *Desulfovibrio desulfuricans* reduced fumarate even prior to sulfate.

Acrylate Reduction of acrylate as an alternative electron acceptor by sulfate-reducing bacteria was discovered by van der Maarel et al. (1996c). Acrylate can be formed in marine sediments by the cleavage of dimethylsulfoniopropionate (DMSP), an osmolyte of many marine algae; such a cleavage can be carried out by the acrylate-reducing sulfate reducer *Desulfovibrio acrylicus*. The DMSP lyase from this organism has been purified (van der Maarel et al., 1996b). Acrylate reduction to propionate also occurs in the presence of sulfate.

Reductive Dehalogenation Reductive dehalogenation coupled to anaerobic bacterial growth was first demonstrated with 3-chlorobenzoate in a mixed culture (Dolfing and Tiedje, 1987). The organism responsible for the reaction was identified as a new type of sulfate-reducing bacterium named *Desulfomonile tiedje* (DeWeerd et al., 1990). 3-Chlorobenzoate and 3,5-dichlorobenzoate were used as electron acceptors for growth with formate as electron donor (Dolfing, 1990; Mohn and Tiedje, 1990b). *Desulfovibrio strain* TBP-1 can grow by coupling the oxidation of lactate to the reductive dehalogenation of 2,4,6-tribromophenol to phenol (Boyle et al., 1999); other halogenated compounds that are used as alternative electron acceptors include 2-, 4-, 2,4- and 2,6-bromophenol.

Arsenate, Chromate and Uranium Anaerobic reduction of arsenate coupled to the oxidation of acetate was originally demonstrated with *Chrysiogenes arsenatis*. This strictly anaerobic bacterium cannot reduce sulfate (Macy et al., 1996). *Desulfotomaculum auripigmentum* is the first example of a sulfate-reducing bacterium that can grow with arsenate as a terminal electron acceptor (Newman et al., 1997b). This bacterium reduced arsenate to arsenite and preferred arsenate to sulfate when both were included in the medium; under such conditions, precipitation of As_2S_3 took place both intra- and extracellularly (Newman et al., 1997a). Two sulfate-reducing bacteria, *Desulfomicrobium* strain Ben-RB and *Desulfovibrio* strain Ben-RA, can reduce sulfate and arsenate concomitantly (Macy et al., 2000). Studies on bacterial utilization of arsenate as electron acceptor have been summarized by Stolz and Oremland (1999b).

Reduction of chromate(VI) with H_2 as electron donor was observed with whole cells of *Desulfovibrio vulgaris*; reduction in cell-free extracts depended on cytochrome c_3 (Lovley and Phillips, 1994a). Cytochrome c_3 from *D. vulgaris* is also capable of uranium(VI) reduction (Lovley et al., 1993a).

Electron Carriers and Possible Functions The reduction of one molecule sulfate to sulfide consumes eight electrons that are ultimately provided by the electron-donor substrate. Unlike the situation in aerobic respiration in mitochondria and bacteria, there is not one terminal-oxidase analogue in sulfate reducers. These bacteria possess at least two simultaneously operating enzymes that are functionally analogous to a terminal oxidase, namely APS reductase and bisulfite reductase; two other enzymes, trithionate reductase and thiosulfate reductase, could have such a function in the case of stepwise sulfite reduction or with trithionate or thiosulfate as external electron acceptors. Unlike oxidases in aerobic respiration, the reductases of the sulfate-reducing bacteria were in most cases not found to be associated with the cytoplasmic membrane. In immunoelectron microscopy, the bisulfite reductases of *Desulfovibrio vulgaris*, *D. gigas* and *Thermodesulfobacterium mobile* (formerly *D. thermophilus*) and the APS reductases of *D. vulgaris* and *D. gigas* appeared to be cytoplasmic enzymes; only APS reductase of *T. mobile* was mainly membrane-associated (Kremer et al., 1988c). In the construction of electron flow models for chemiosmotic energy conservation by dissimilatory sulfate reduction, the frequent finding of bisulfite reductases and APS reductases in the cytoplasm and the possible involvement of two other reductases (trithionate reductase and thiosulfate reductase) are complicating factors. There is evidence that certain redox carriers have highly specific roles in the electron flow by transporting reducing equivalents to particular acceptors only (LeGall and Fauque, 1988; Peck and Lissolo, 1988; Fauque et al., 1991).

Suggested mechanisms of energy conservation are discussed in connection with particular electron donors (see Energy Conservation in this Chapter). In the following subsections, a few characteristics of major redox proteins are presented. More detailed information is given by Fauque et al. (1990) and LeGall and Fauque (1988).

Cytochromes Several different types of cytochromes, which differ in molecular mass, subunit composition and heme content, have been identified in sulfate-reducing bacteria (Widdel, 1988; Fauque et al., 1991; LeGall and Fauque, 1988). The physiological function of cytochromes with respect to their position in electron transfer is not yet completely understood. Principal types of cytochromes that have been recognized are the tetraheme cytochrome c_3, the hexadecaheme high molecular mass cytochrome (Hmc), and the small cytochrome c_{553}.

The type of cytochrome that was named c_3 has been identified in all *Desulfovibrio* species (Postgate, 1984a; LeGall and Fauque, 1988; Fauque et al., 1991), *Desulfobulbus elongatus* (Samain et al., 1986a) and both *Thermodesulfobacterium* species (Hatchikian et al., 1984; LeGall and Fauque, 1988; Fauque et al., 1991). Cytochrome c_3 (M_r of ca. 13,000) consists of one polypeptide chain and contains four hemes with midpoint potentials ranging from –0.125 to –0.325 V; it is also termed tetraheme cytochrome c_3. The ligands of each iron atom are two histidine molecules. The observed occurrence of cytochrome c_3 in the periplasm (Badziong and Thauer, 1980; LeGall and Fauque, 1988; Fauque et al., 1991) has been confirmed by the signal sequence in the gene (Voordouw and Brenner, 1986). In cell-free systems, tetraheme cytochrome c_3 is required for the reduction of ferredoxin, flavodoxin and rubredoxin by hydrogenase and apparently plays a key role in H_2 metabolism (Fauque et al., 1991; LeGall and Fauque, 1988). Still, the mode of electron transfer by cytochrome c_3 in vivo is unsatisfactorily understood. Cytochrome c_3 may interact with the transmembrane spanning Hmc complex to channel electrons through the membrane into the cytoplasm (Voordouw, 1995). The crystal structures of cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki (Higuchi et al., 1984), *D. vulgaris* Hildenborough (Matias et al., 1993), *D. desulfuricans* (Norway; Czjzek et al., 1994) and *D. desulfuricans* (Essex; Fritz, 1999) were resolved at resolutions lower than 2 Å. The three cytochromes had a similar overall structure with an extended α-helix and a short β-strand as the prominent secondary structure elements. The four heme groups are all solvent exposed and arranged in pairs (termed heme I/II and heme III/IV pairs). Conserved lysine residues surrounding heme IV are proposed to be essential for the contact between cytochrome c_3 and the electron-delivering hydrogenase. *D. africanus* contains two different types of tetraheme cytochrome c_3, one being acidic and another being basic. In contrast to the basic c_3, the acidic c_3 showed only poor reactivity towards either [Fe] or [NiFe] hydrogenase (Pieulle et al., 1996; Magro et al., 1997).

An octaheme cytochrome c_3 that is found in most *Desulfovibiro* species is structurally rather different from tetraheme cytochrome c_3, but also can react with hydrogenase (Fauque et al., 1991;

LeGall and Fauque, 1988). Studies with mutants have indicated that heme IV is most likely the interactive heme in the cytochrome-hydrogenase complex, and that Tyr73 has an important structural function (Aubert et al., 1997; Aubert et al., 1998a). Octaheme cytochrome c_3 may be involved in the supposed thiosulfate reduction of the trithionate pathway.

A high molecular mass cytochrome *c* was isolated from *Desulfovibrio vulgaris* (Hildenborough); it had an estimated mass of 70 kDa and contained 16 heme groups (Higuchi et al., 1987). From the same organism, a dimeric 26-kDa cytochrome c_3 (also referred to as cytochrome cc_3) was isolated that possessed four identical heme groups in each subunit (Loutfi et al., 1989). A DNA probe designed from a partial amino acid sequence of cytochrome cc_3 led to the identification of the *hmc* gene, coding for the hexadecaheme cytochrome Hmc in *Desulfovibrio vulgaris* (Hildenborough). In addition, the amino acid composition of the two cytochromes proved to be highly similar, thus suggesting that cytochrome c_3 and Hmc are identical (Pollock et al., 1991). The *hmc* gene from *D. vulgaris* (Hildenborough) was overexpressed in *D. desulfuricans* G200 and the recombinant Hmc protein was purified. Studies on the arrangement of the heme-binding sites of this Hmc revealed that the protein contained three complete cytochrome c_3-like and one incomplete c_3-like domain, suggesting that Hmc arose via gene duplication (Bruschi et al., 1992). The *hmc* gene of *D. vulgaris* (Hildenborough) is part of an operon containing eight open reading frames, Orf1 to Orf6 (also termed *hmcA* to *hmcF*), Rrf1 and Rrf2. The open reading frame Orf1 represents the *hmc* gene. Based on sequence homologies, putative functions and cellular locations were suggested for the other open reading frames: Orf2 is a putative transmembrane protein containing four [FeS] clusters, Orf3 to Orf5 are membrane integral proteins, and Orf6 is a cytoplasmic protein containing two [FeS] clusters. It is proposed that Hmc and Orf2 to Orf6 are assembled in one transmembrane protein complex that functions in transferring electrons from the periplasm to the cytoplasm (Rossi et al., 1993). The two genes *rrf1* and *rrf2* code for regulatory proteins. Deletion of genes *rrf1* and *rrf2* resulted in an overexpression of the *hmc* operon and a more rapid growth on H_2 and sulfate. From these results, it was concluded that the Hmc-complex mediates the electron transfer between periplasmic hydrogenase and the cytoplasmic enzymes involved in sulfate reduction (Keon et al., 1997). Even though Hmc from *D. vulgaris* (Hildenborough) can in principle accept electrons directly from [NiFe] hydrogenase, the rates of electron transfer are increased by the presence of cytochrome c_3, suggesting that this cytochrome acts as a mediator between hydrogenase and Hmc (Pereira et al., 1998). However, an Hmc isolated from *Desulfovibrio gigas* could accept electrons directly from hydrogenase (Chen et al., 1994a).

In the case of a transmembrane hexaheme cytochrome *c* from *Desulfovibrio desulfuricans*, a function of the protein as nitrite reductase could be demonstrated (Liu and Peck, 1981b).

A "split-Soret" cytochrome, which is a dimer with two identical 26 kDa subunits and two heme groups per subunit, was isolated from *Desulfovibrio desulfuricans* (Liu et al., 1988). The complete amino acid sequence of this cytochrome *c* revealed that the C-terminal part contained the heme-binding site, similar to that in cytochrome c_3, and an additional domain that could harbor a putative non-heme iron-containing cluster (Devreese et al., 1997).

During investigations on the natural electron acceptor of formate dehydrogenase of *D. vulgaris*, a small cytochrome with a mass of 6.5 kDa was isolated and (in accord with its absorption maximum) termed "cytochrome c_{553}" (Yagi, 1969; Sebban et al., 1995). The purified protein could be reduced by formate dehydrogenase but not by hydrogenase (Yagi, 1979). Cytochrome c_{553} also can function as primary electron acceptor of lactate dehydrogenase (Ogata et al., 1981). Recognition of a leader sequence in the structural gene furnished evidence for a periplasmic location of cytochrome c_{553} (van Rooijen et al., 1989). Cytochrome c_{553} is a monoheme cytochrome with methionine and histidine as axial ligands (Fauque et al., 1991). The complete amino acid sequences of cytochromes c_{553} from *D. vulgaris* strains Hildenborough and Miyazaki revealed that the two proteins were not closely related (Nakano and Kikumoto, 1983). Apart from its small size, cytochrome c_{553} shows two further peculiar characteristics: 1) it has a low redox potential (ca. 0.01 V; Bertrand et al., 1982) and 2) it undergoes a conformational change during the transition from the oxidized to the reduced state (Senn et al., 1983). Structural analysis by means of NMR spectroscopy revealed that cytochrome c_{553} contains three conserved helices around the heme group, which resides in a cleft, and an additional fourth helix (Marion and Guerlesquin, 1992; Blackledge et al., 1995). In addition, the existence of two conformations of cytochrome c_{553} was recognized with NMR studies of the purified recombinant protein (Blanchard et al., 1993). The tyrosine residue Tyr64, which is positioned at the interface between the heme group and the central cleft of the protein, is thought to play a key role in structural stability (possibly affecting electron exchange with formate dehydrogenase;

Blanchard et al., 1994; Sebban-Kreuzer et al., 1998a; Sebban-Kreuzer et al., 1998b). Further studies attempting to elucidate this electron transfer involve ^{15}N labeling of cytochrome c_{553} and analysis with NMR techniques (Morelli et al., 1999).

In addition to sulfate-reduction, *Desulfomonile tiedjei* DCB-1 can also employ reductive dehalogenation as a mode of energy conservation. A new type of cytochrome was found to be co-induced with the dehalogenating activity. This cytochrome is probably located toward the periplasmic aspect of the membrane because the protein was extracted from the membrane fraction and carries an N-terminal signal sequence. The coding gene of the new cytochromes was cloned by means of primers developed from the N-terminal sequence of the purified protein. Two *c*-type heme-binding motifs were identified in the C-terminus. However, the protein sequence was found to have no substantial similarities with sequences deposited in databases. Thus this protein is considered as a new *c*-type cytochrome (Louie et al., 1997).

Ferredoxins Ferredoxins are very common in sulfate-reducing and sulfur-reducing bacteria (Probst et al., 1978; Bache et al., 1983; Gebhardt et al., 1983; LeGall and Fauque, 1988; Fauque et al., 1991). Several types have been described, but possible physiological roles are known only in a few cases. In *Desulfovibrio gigas*, ferredoxin I ($E_0' = -0.440$ V), a protein with one [4Fe-4S] cluster, is active in the cleavage of pyruvate (viz. pyruvate:ferredoxin oxidoreductase reaction). In case of the assumed tetrathionate pathway, the midpoint potential of ferredoxin I would make it an appropiate electron donor for the thiosulfate reductase reaction. Ferredoxin II ($E_0' = -0.130$ V) from *D. gigas* has one [3Fe-4S] cluster and has been suggested to function as electron donor in the reduction of bisulfite to sulfide (Fauque et al., 1991; LeGall and Fauque, 1988). There is evidence for an interconversion of the different clusters in these ferredoxins. In *Desulfobacter* as well as in the sulfur reducers (*Desulfuromonas* and *Desulfurella*), a ferredoxin is the acceptor in the 2-oxoglutarate dehydrogenase reaction (Gebhardt et al., 1983; Paulsen et al., 1986; Schmitz et al., 1990; Thauer, 1988; Thauer et al., 1989b).

Flavodoxins In some but not all *Desulfovibrio* and *Desulfomicrobium* species, flavodoxins have been found. The two oxidation states, F/FH((E_0' = –0.140 V) and FH(/FH_2 (E_0' = –0.440 V) have midpoint potentials comparable to those of ferredoxin I and II, and the corresponding proteins could replace each other in their function as electron carriers (Fauque et al., 1991). Flavodoxin was not active as electron donor for the purified thiosulfate reductase of *Desulfovibrio vulgaris* strain Miyazaki F (Aketagawa et al., 1985). The three-dimensional structure and the gene sequence of flavodoxin from *Desulfovibrio vulgaris* is known (Curley and Voordouw, 1988).

Rubredoxins Rubredoxins are low molecular mass single-iron proteins (M_r ca. 6,000) which carry only electrons, like cytochromes and ferredoxins. They are present in all *Desulfovibrio* strains studied and also in *Thermodesulfobacterium commune* (LeGall and Fauque, 1988; Shimizu et al., 1989); the amino acid sequences of some of them have been determined, and the sequence of the gene in *Desulfovibrio vulgaris* (Hildenborough) coding for rubredoxin is known (Voordouw, 1988a; Voordouw, 1988b; Shimizu et al., 1989). Because of their rather positive midpoint potentials (–0.050 to +0.005 V), questions have been raised as to the physiological role of this protein in dissimilatory sulfate reduction (LeGall and Fauque, 1988; Brumlik and Voordouw, 1989). Kremer et al. (1988b) speculated about a role of rubredoxin as electron donor in the reduction of APS to bisulfite. However, such a role would be likely only if the actual potential of APS/HSO_3^- + AMP at the in vivo concentrations is more positive than the midpoint potential (–0.060 V), which may not be the case (see Reduction of APS in this Chapter). Experimental evidence for such a role has not been found so far. Rubredoxin may play a major role in channeling electrons to O_2 consumption or O_2 detoxification (See Oxygen in this Chapter).

Rubrerythrin A high-potential redox protein, rubrerythrin (midpoint potential +0.23 V), has been purified from *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* (Fauque et al., 1991); if this redox carrier is involved in sulfate reduction, a possible function that can be envisaged is in the reduction of trithionate to thiosulfate and bisulfite ($E_0' = +0.225$ V). Recent studies indicate that the major role of rubrerythrin and nigerythrin may lie in protection against deleterious effects of O_2. The proteins from *Desulfovibrio vulgaris* were shown to have NADH peroxidase activity (Coulter et al., 1999).

Menaquinone All sulfate-reducing prokaryotes examined contain menaquinones (Collins and Widdel, 1986; Schmitz et al., 1990; Tindall et al., 1989). The number of isoprenoid units per side chain varies between 5 and 9. Terminal saturation of the side chain may occur in other bacteria

(Collins and Widdel, 1986). Judging from their general occurrence in sulfate-reducing microorganisms, menaquinones seem to be obligate components of electron transport chains. Involvement in the electron transport during oxidation of acetate (Kröger et al., 1988; Möller-Zinkhan and Thauer, 1988; Möller-Zinkhan and Thauer, 1989; Thauer et al., 1989b; Figs. 6 and 8) or lactate (Fauque et al., 1991) has been discussed.

METABOLISM OF ELECTRON DONORS AND ENERGY CONSERVATION A great variety of low molecular mass compounds serve a electron donors for dissimilatory sulfate reduction (and often simultaneously as carbon sources for cell synthesis). Many of these are products from the fermentative breakdown of biomass, which reflects the importance of sulfate-reducing bacteria as terminal degraders in anoxic, sulfate-rich habitats such as marine sediments.

The study of several electron donors of sulfate-reducing bacteria is closely connected to investigations into structure and function of special enzymes such as hydrogenase. Furthermore, the study of electron donors has led to the discovery of previously unknown anaerobic pathways or capacities (e.g., a modified, anaerobic citric acid cycle, the oxidative C_1/CO-dehydrogenase pathway, reactions at aromatic molecules or the capacity for alkane oxidation without O_2).

The bioenergetic processes of sulfate-reducing bacteria are determined by the electron donors. The catabolism of organic electron donors connected to the reduction of an external electron acceptor generally offers two advantages over a purely fermentative catabolism. First, with an external electron acceptor, substrate level phosphorylation can be performed to a larger extent than in purely fermentative metabolism in which a part of the organic substrate has to be sacrified for the disposal of surplus reducing equivalents (e.g., regeneration of $NADP^+$). With external electron acceptors, substrate-level phosphorylation and growth is even possible with non-fermentable compounds, as for instance butyrate or higher fatty acids. In the vast number of sulfate-reducing bacteria that excrete acetate, substrate-level phosphorylation via phosphate acetyltransferase and acetate kinase can be expected. Substrate-level phosphorylation in *Desulfobacter* species occurs via ATP-citrate lyase, and in sulfate-reducing bacteria employing the C_1/CO-dehydrogenase pathway most likely during formation of free formate from formyltetrahydropterin. Further substrate-level reactions may occur in the few species of sulfate-reducing microorganisms that grow with carbohydrates. Second and most importantly, the reduction of the external electron acceptor can be associated with an electron transport chain that allows generation of a transmembrane proton gradient and chemiosmotic ATP synthesis. In microorganisms utilizing organic compounds without an external electron acceptor, chemiosmotic energy conservation occurs only in special metabolic types, as for instance propionate-forming bacteria (Schink, 1988a) or methanogens. In sulfate-reducing bacteria, numerous enzymes catalyzing redox-reactions as well as potentially electron-carrying proteins and menaquinones have been studied in detail, and electron transport chains have been proposed. However, there is no unifying theory of electron transport in sulfate-reducing bacteria. In view of the various electron donors, metabolically diverse species and differences in the redox protein outfit, the development of a unifying model of electron transport is unlikely, except for steps in the pathway of sulfate reduction.

In the following, physiological, enzymatic and energetic aspects of the utilization of various electron donors for sulfate reduction are presented.

Molecular Hydrogen Molecular hydrogen (H_2) is (besides acetate) a key intermediate in the natural mineralization of organic substances in sediments, sludge digestors and other anoxic ecosystems. Also the fact that many species of various genera of sulfate-reducing bacteria utilize H_2 as sole electron donor (Table 1) reflects the ecological importance of the lightest of all molecules. Hydrogen at standard pressure is an energetically favorable electron donor ($2\ H^+/H_2$, $E_0' = -0.414$ V); the free energy change at various partial pressures is depicted in Fig. 11. Cell material during growth on H_2 and sulfate may be synthesized from acetate and CO_2 (chemolithoheterotrophic species) or alone from CO_2 (see autotrophic species; Carbon Assimiliaton).

Growth on H_2 has been observed in many of the known genera of sulfate-reducing bacteria (Table 1). Hydrogenase activities have been demonstrated in strains of the genera *Desulfovibrio* (Fauque et al., 1991), *Desulfobulbus* (Samain et al., 1986b; Kremer and Hansen, 1988a), *Desulfobacter*, *Desulfobacterium*, *Desulfosarcina* (Schauder et al., 1986), *Desulfotomaculum* (Cypionka and Dilling 1986) and *Thermodesulfobacterium* (Fauque et al., 1992). Hydrogenase activity has even been found in *Desulfobacter* species that cannot grow on H_2 (Lien and Torsvik, 1990); the role of the enzyme in such bacteria is unknown. Hydrogenases may

act not only in the uptake of H_2 at various partial pressures (see last paragraph of this section), but also in the production of H_2 during growth of certain species by fermentation or in syntrophic co-cultures (see Fermentative and Syntrophic Growth in the Absence of Sulfate in this Chapter).

Hydrogenases catalyze the reversible heterolytic cleavage of H_2 and oxidation of the resulting hydride ion, according to:

$$H_2 \rightleftharpoons H^+ + H^- \rightleftharpoons 2H^+ + 2e^- \quad (12)$$

Detailed information on the biochemistry, coding genes and mechanism of function of hydrogenases (in sulfate-reducing bacteria) is so far only available from *Desulfovibrio* species. Hydrogenases are probably the most intensely studied enzymes in sulfate-reducing bacteria. Their investigation has significantly contributed to our understanding of hydrogenases in general. The first resolution of the three-dimensional structure of a hydrogenase was achieved with the enzyme from *Desulfovibrio gigas* (Volbeda et al., 1995). Based on their metal composition, three types of hydrogenases are distinguished in *Desulfovibrio* species, the [Fe] hydrogenases (Huynh et al., 1984a), the [NiFe] hydrogenases (Teixeira et al., 1986) and the [NiFeSe] hydrogenases (Rieder et al., 1984; Teixeira et al., 1987). All three types of hydrogenases have heterodimeric αβ-structures and are mostly located in the periplasm (Odom and Peck, 1981a; Fauque et al., 1988). There are marked differences between the three types of enzymes (Table 4) with respect to their H_2-uptake and H_2-evolving activities, their sensitivity to CO, NO, NO_2^- and acetylene (e.g., He et al., 1989), and their molecular structures (Prickril et al., 1987; Fauque et al., 1988). The three types of hydrogenase are not uniformly distributed among *Desulfovibrio* species. Voordouw et al. (1990) analyzed the distribution of the hydrogenase encoding genes in 22 different *Desulfovibrio* species. The genes for the [NiFe] hydrogenase could be identified in all tested strains, whereas the distribution of [Fe] and [NiFeSe] hydrogenases were limited. Individual strains may contain only one type of hydrogenase (e.g., [NiFe] hydrogenase in *D. vulgaris* strain Groningen), two types of hydrogenases (e.g., [NiFe] and [NiFeSe] hydrogenase in *D. vulgaris* strain Miyazaki; [NiFe] and [Fe] hydrogenases in *D. desulfuricans* strain El Agheila) or all three types of hydrogenases (in *D. vulgaris* strain Hildenborough).

Genes coding for hydrogenases have been cloned and sequenced from various *Desulfovibrio* spp. and from *Desulfomicrobium baculatum* (Table 8). An extensive sequence comparison of hydrogenase genes including those from sulfate-reducing bacteria has been carried out by Wu and Mandrand (1993). The [NiFe] and [NiFeSe] hydrogenases from sulfate-reducing bacteria were related to each other and also to [NiFe] hydrogenases from species from other subclasses of the Proteobacteria such as *Rhodobacter*, *Rhizobium*, *Azotobacter*, *Escherichia* or *Wolinella*. These hydrogenases were not related to [Fe] hydrogenases from *Desulfovibrio* species, which have their own line of enzymatic evolution.

The [Fe] hydrogenases were purified from *Desulfovibrio vulgaris* strain Hildenborough (Huynh et al., 1984a), *D. desulfuricans* (Hatchikian et al., 1992) and *D. fructosovorans* (Casalot et al., 1998). In the case of [Fe] hydrogenases from *D. vulgaris* and *D. desulfuricans*, an atypical Fe-cluster and two ferredoxin-type [4Fe-4S] clusters were identified. The atypical Fe-cluster, also known as the H-cluster, is assigned to the H_2 activation site. The [4Fe-4S] clusters, which are also referred to as F-clusters, transfer electrons between the H-cluster and the external electron carrier (Adams, 1990). The crystal structure of the [Fe]hydrogenase from *D. desulfuricans* was the first to be determined of this type of hydrogenase (Nicolet et al., 1999). The three-dimensional structure revealed that this hydrogenase displays a novel protein fold, and that the H-cluster is composed of a typical [4Fe-4S] clus-

Table 4. Brief overview of characteristics of different types of hydrogenases found in *Desulfovibrio* species[a]

	[Fe]hydrogenase	[NiFe]hydrogenase	[NiFeSe]hydrogenase
Catalytic activity			
H_2 uptake	very high	moderate	low
H_2 evolution	high	moderate	moderate
Sensitivity to			
CO	very high	high	moderate
NO	very high	high	very high
Nitrite	moderate	no	moderate
Acetylene	no	high	moderate
Molecular mass (kDa)	~57	~90	~81

[a]Adapted from Fauque et al., 1991.

ter bridged to a binuclear Fe center as the active site. The two Fe ions at the active site probably possess CO and CN^- as binuclear ligands, as found in [NiFe] hydrogenases. The structural analysis of the [Fe] hydrogenase corroborates the earlier finding that one of the two active-site irons could be ligated by intrinsic CN^- and CO (Pierik et al., 1998). In contrast to the [NiFe] hydrogenases, the binuclear active site as well as the [4Fe-4S] clusters in [Fe] hydrogenase reside on one subunit. Channel-like paths have been identified that allow the transport of protons and H_2 to or from the active site buried in the center of the protein. The second subunit of the [Fe] hydrogenase from D. desulfuricans forms a belt around the other subunit. The [Fe] hydrogenase from the anaerobic Gram-positive bacterium *C. pasteurianum* has an active center similar to the one in the *D. desulfuricans* enzyme, even though the former consists of only a single polypeptide (Adams, 1990; Peters et al., 1998; Peters, 1999; Cammack, 1999). The *hydA* and hydB genes coding for the large and small subunits, respectively, of [Fe] hydrogenase in *D. vulgaris* (Hildenborough) and *D. vulgaris* subsp. oxamicus are highly homologous (Voordouw et al., 1989b); however, there is no significant homology between the [Fe] hydrogenases and the [NiFe] hydrogenases (see next paragraph). A gene probe for the [Fe] hydrogenase did not hybridize with the DNA of sulfate-reducing bacteria without a [Fe] hydrogenase (Voordouw et al., 1987). Deckers et al. (1990) demonstrated that *D. vulgaris* strain Miyazaki F lacks the [Fe] hydrogenase genes.

In *D. fructosovorans* a new type of [Fe] hydrogenase, which reacts with $NADP^+$, was identified; it may be regarded as a fourth type of hydrogenase present in *Desulfovibrio*. The $NADP^+$-reducing hydrogenase is assumed to be a heterotetrameric enzyme complex that is encoded by the hndABCD genes (Malki et al., 1995). Mutants with deleted hndABCD genes showed reduced hydrogenase activity (Malki et al., 1997). Homology studies implicated that HndA and HndC form the NADP-reducing moiety, and that HndD harbors the H_2-activating site of a [Fe] hydrogenase; the function of HndB is presently unknown. The purified HndA subunit contains a [2Fe-2S] cluster which belongs to the family of [2Fe-2S] ferredoxins (DeLuca et al., 1998a). Studies with antisera raised against the four putative subunits overexpressed in (and purified from) *Escherichia coli* demonstrated that the active $NADP^+$-reducing hydrogenase in the sulfate reducer is indeed a complex, even though the complex itself has not been purified so far (DeLuca et al., 1998b). Thus the $NADP^+$-reducing hydrogenase appears to differ structurally from the three other types of hydrogenases.

The [NiFe] hydrogenases have been purified from *D. desulfuricans* (Krüger et al., 1982), *D. gigas* (Moura et al., 1982), *D. multispirans* (Czechowski et al., 1984) and *D. africanus* (Niviere et al., 1986). A [NiFe] hydrogenase was also isolated from the thermophilic sulfate reducer *Thermodesulfobacterium mobile* (Fauque et al., 1992). The [NiFe] hydrogenase from *D. gigas* has been studied most intensively. Analysis of the coding genes, *hynA* and *hynB*, suggested that the large subunit (62 kDa) carries the Ni ion and that the small subunit (26 kDa) could ligate at least two [FeS] clusters due to the presence of 12 cysteines (Voordouw et al., 1989a). Spectroscopic analysis of [NiFe] hydrogenase from *D. gigas* indicated the presence of two [4Fe-4S] clusters, one [3Fe-xS] cluster, one Ni ion and one unknown redox component, which was hypothesized to be a special Fe ion (Huynh et al., 1987; Albracht, 1994). The structure of the [NiFe] hydrogenase from *D. gigas* was determined at 2.85 and 2.54 Å resolution (Volbeda et al., 1995; Volbeda et al., 1996). The two subunits interact extensively, and the large subunit has a unique topology. The presence of Fe as the second metal ion, besides Ni, in the active site of the large subunit was demonstrated. The distance between the two metal ions was suggested to be around 3 Å. A coordination of intermediate species of H_2 between the two metal ions (Ni and Fe) in the active site is suggested to function in catalysis (Volbeda et al., 1995). The Ni ion is anchored to the protein via sulfur bridges from two cysteine residues (Cys65 and Cys530) and coordinately bound to the Fe ion again by two sulfur bridges provided by Cys68 and Cys533. The Fe ion possesses three intrinsic dinuclear ligands, which were demonstrated to be two CN^- groups and one CO molecule (Pierik et al., 1999). High resolution X-ray structural analysis (1.8 Å) of the [NiFe] hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) indicated that this protein is similar to the [NiFe] hydrogenase from *Desulfovibrio gigas* with respect to the folding pattern, the arrangement of the metal center and the probable presence of SO, CO and CN^- as dinuclear ligands of the Ni-Fe center (Higuchi et al., 1997). These dinuclear ligands generate unusual infrared bands, which have been observed in several [NiFe] and [Fe] hydrogenases from *Desulfovibrio* species and other microorganisms like *Chromatium vinosum* (Bagley et al., 1995; van der Spek et al., 1996). Thus [NiFe] hydrogenases appear to contain $NiFeCO(CN)_2$ as prosthetic group, the finding of which would be unprecedented in the study of biological systems (Fig. 9; Happe et al., 1997); the function of the dinuclear ligands remains unclear. The three

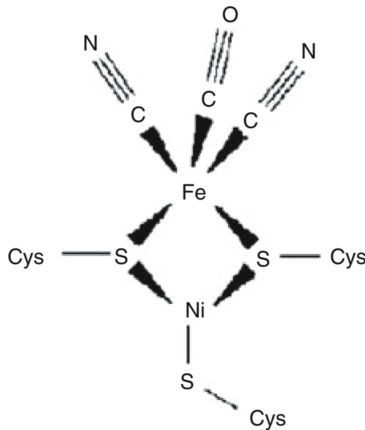


Fig. 9. Suggested prosthetic group of [NiFe] hydrogenases. Three dinuclear, non-protein ligands (2 CN^-, 1 CO) coordinate the Fe atom in the active center (Happe, 1997; Higuchi et al., 1997; Pierik et al., 1999).

[FeS] clusters of the small subunit of [NiFe] hydrogenase from *D. gigas* are arranged in one line with the two low potential [4Fe-4S] clusters at the proximal and distal sides and the high potential [3Fe-4S] cluster in the middle. It was suggested that an electron channel is formed from the center of the protein, where H_2 is oxidized at the active site, to the surface of the [NiFe] hydrogenase, where the electrons would be accepted by cytochrome c_3. However, the role of the median [3Fe-4S] cluster is uncertain, considering the high potential of this cluster for the electron transfer is unfavorable. To study the role of the [3Fe-4S] cluster in the electron transfer, the [3Fe-4S] cluster was converted to a [4Fe-4S] cluster by site-directed mutagenesis in [NiFe] hydrogenase from *D. fructosovorans.* Because the catalytic activities of this mutant were similar to those of the wild-type, it was speculated that the [3Fe-4S] cluster may serve a structural function rather than participate in electron transfer (Rousset et al., 1998b). Studies by Higuchi et al. (1994) demonstrated the presence of three [FeS] clusters and one Ni ion in the [NiFe] hydrogenase of *D. vulgaris* strain Miyazaki F, suggesting a similar structure as the one for *D. gigas* [NiFe] hydrogenase.

The first [NiFeSe] hydrogenase in sulfate-reducing bacteria was recognized by Rieder et al. (1984) in *Desulfomicrobium norvegicum*, formerly *Desulfovibrio desulfuricans* strain Norway 4 (Sharak Genthner et al., 1997). The coding genes for the small and large subunit of the [NiFeSe] hydrogenase exhibited much sequence similarity with the corresponding genes of [NiFe] hydrogenase from *Desulfovibrio gigas* (Voordouw et al., 1989a). The large subunit of [NiFeSe] hydrogenase contains equimolar amounts of selenium and nickel. Another [NiFeSe] hydrogenase was purified from *Desulfovibrio salexigens* (Teixeira et al., 1986). Spectroscopic studies suggested that selenocysteine takes part in the coordination of the active-site nickel ion in the [NiFeSe] hydrogenase of *Desulfomicrobium baculatum* (Eidsness et al., 1989), formerly *Desulfovibrio baculatus* (Rozanova et al., 1988a). Comparative studies suggest that the [NiFeSe] hydrogenases are distinct from [NiFe] hydrogenases in terms of catalytic properties (Teixeira et al., 1987). The mechanism of selenium incorporation into proteins has been well studied with formate dehydrogenase from *Escherichia coli*. Selenium is present in proteins as selenocysteine, the 21st amino acid, which is cotranslationally incorporated into the nascent polypeptide from selenocysteyl-tRNASec. This selenocysteyl-tRNASec is synthesized from seryl-tRNASec and selenophosphate by selenocysteine synthase (Böck et al., 1991; Heider and Böck, 1993). Selenocysteyl-tRNASec recognizes an in-frame UGA codon that otherwise terminates translation (Leinfelder et al., 1988). Efficient read-through of the UGA codon is dependent on a specific secondary structure of the mRNA downstream of the UGA codon (Zinoni et al., 1990). The selenocysteine-loaded tRNASec is directed to the UGA codon by a specialized elongation factor, SelB (Baron et al., 1993). The corresponding triplet was identified in the sequence of the coding gene for [NiFeSe] hydrogenase from *Desulfomicrobium baculatum* (Menon et al., 1987; 1993). The gene coding for the selenocysteine-inserting tRNASec (*selC*) was cloned and sequenced from *Desulfomicrobium baculatum* (Tormay et al., 1994). A lacZ-fusion of the gene coding for the large subunit of the [NiFeSe] hydrogenase from *Desulfomicrobium baculatum* was constructed to study its heterologous expression in *E. coli*. Interestingly, in *E. coli*, selenocysteine was not incorporated into the *D. baculatum* hydrogenase subunit, demonstrating that the UGA codon was suppressed. Gel-shift experiments showed that purified SelB from *E. coli* in comparison to that from *D. baculatum* had a lower affinity for the hydrogenase mRNA from *D. baculatum*. Thus it appears that the specific interaction of SelB and target mRNA is a prerequisite for proper synthesis of the selenoprotein (Tormay and Böck, 1997).

First evidence for a periplasmic location of a [NiFe] hydrogenases came from purification procedures that only required cells to be washed in slightly alkaline solution (Bell et al., 1974). Further studies on the localization revealed that hydrogenase in various sulfate-reducing bacteria are often localized in the periplasm. Investigated

species are *Desulfovibrio vulgaris* strain Marburg (Badziong and Thauer, 1980), *Desulfovibrio vulgaris* strain Hildenborough (van der Westen et al., 1978), *Desulfovibrio desulfuricans* (Steenkamp and Peck, 1981), *Desulfovibrio gigas* (Bell et al., 1974), *Desulfomicrobium norvegicum* (Rieder et al., 1984). Association of hydrogenases with the cytoplasmic membrane was demonstrated in *Desulfovibrio vulgaris* by means of immunocytochemical labeling and electron microscopy. In this species, the [NiFe] hydrogenase was located on the periplasmic side and the [NiFeSe] hydrogenase on the cytoplasmic side of the membrane (Rohde et al., 1990). In the case of *Desulfovibrio desulfuricans* (Essex 6) and the Gram-positive *Desulfotosporosinus orientis*, a cytoplasmic location of hydrogenase has been demonstrated by the use of inhibiting agents (Cypionka and Dilling, 1986a; Fitz and Cypionka, 1989). In the case of periplasmic hydrogenases, an export mechanism for these enzymes must exist. Indeed, the gene for the small subunit of the [Fe] hydrogenase of *D. vulgaris* was shown to encode a protein with a signal peptide of 34 amino acids (Prickril et al., 1986). There is, however, no evidence for a leader sequence in the gene for the large subunit. The situation is similar in the case of the periplasmic [NiFe] hydrogenase of *Desulfovibrio gigas* and of the [NiFeSe] hydrogenase of *Desulfomicrobium baculatum*. The mature small-subunit sequences are preceded by N-terminal signal sequences of 32 and 50 amino acids, respectively, whereas no leader sequences were found for the large subunits (Voordouw et al., 1989a; Menon et al., 1987). Also the small subunit of [NiFe] hydrogenase from *Desulfovibrio desulfuricans* contains a signal peptide with 50 amino acids (Rousset et al., 1990). The presence of an internal signal sequence in the large subunit of the *D. vulgaris* hydrogenase that might be involved in the translocation of the protein to the periplasm has been the subject of speculation. Alternatively, the immature small subunit might function as a carrier for the large subunit in the translocation process (Prickril et al., 1986). Some evidence for the latter model was presented by van Dongen et al. (1988). Homology studies revealed a consensus box containing two consecutive arginine residues in the N-terminal leader sequence of the small subunit of hydrogenases (Voordouw, 1992; Berks, 1996). A similar export mechanism was suggested for the periplasm-orientated HydB subunit of the membrane integral [NiFe] hydrogenase from the sulfur reducer *Wolinella succinogenes* (Gross et al., 1999). Fusion of the signal peptide from [NiFe] hydrogenase of *Desulfovibrio vulgaris* (Hildenborough) to the β-lactamase from *Escherichia coli* lacking its own leader sequence allowed export of the enzyme. Exchange of one of the two arginines in the leader sequence to glutamate by site-directed mutagenesis inhibited export of β-lactamase completely (Nivière et al., 1992). These results demonstrated an essential role of the two subsequent arginines of the consensus box in the export of hydrogenase (Berks, 1996). The large subunit of the [NiFe] hydrogenase from *Desulfovibrio gigas* was shown to be processed by cleavage of 15 amino acids from the carboxy terminus (Menon et al., 1993). Hatchikian et al. (1999) demonstrated that also the large subunit of [Fe] hydrogenase from *Desulfovibrio desulfuricans* is subjected to a C-terminal processing in which 24 amino acids are cleaved. This finding is in agreement with the structural analysis of the same enzyme (Nicolet et al., 1999). Hatchikian et al. (1999) speculated that the C-terminal processing may play a role in the export of the protein to the periplasm. Export of the [NiFe] hydrogenase from *Desulfovibrio fructosovorans* may employ yet another mechanism involving an additional protein. Downstream of the structural *hynA* and *hynB* genes a third open reading frame (hydC) was identified. All three genes were found to constitute a single operon with a strong (^{70}-like promoter. The HydC protein possesses an amphipathic segment and is speculated to mediate the integration of hydrogenase into the membrane or the export of the enzyme to the periplasm (Rousset et al., 1993).

Primary acceptors for the electrons produced by hydrogenase is the periplasmic cytochrome c_3 which contains multiple heme groups. Initial indication for the electron transfer between hydrogenase and cytochrome c_3 arose from the co-localization of the two proteins in the periplasm (Bell et al., 1974). The interaction between hydrogenases and cytochrome c_3 has been demonstrated with [Fe] hydrogenase from *Desulfovibrio vulgaris* strain Hildenborough (Brugna et al., 1998), [NiFe] hydrogenase from *Desulfovibrio gigas* (Moreno et al., 1993) and [NiFeSe] hydrogenase from *Desulfovibrio desulfuricans* strain Norway (Haladjian et al., 1991). The structural analysis of the cytochrome c_3 molecules from *Desulfovibrio vulgaris* strain Hildenborough (Matias et al., 1993), *Desulfovibrio desulfuricans* strain Norway (Czjzek et al., 1994) and *Desulfovibrio gigas* (Fritz et al., 1999) revealed an overall similar molecular structure and arrangement of the four heme groups. Two types of interactions were identified, one between hemes I and II and another between hemes III and IV. A point mutation of the tyrosine 73 residue in cytochrome c_3 from *Desulfovibrio desulfuricans* (Norway) resulted in a change of the heme IV environment and an

alteration of the hydrogenase-cytochrome interaction (Aubert et al., 1997; Aubert et al., 1998a). The positive charges surrounding the surface-exposed heme IV of cytochrome c_3 are supposed to mediate the contact to hydrogenase. In *Desulfovibrio vulgaris* (Hildenborough), further transfer of electrons is assumed to proceed from cytochrome c_3 to the 16 heme containing high-molecular-mass cytochrome c, termed Hmc (Pollock et al., 1991; Bruschi et al., 1992; Voordouw, 1995; Pereira et al., 1998). The Hmc, which is localized to the periplasmic aspect of the membrane, is part of a multisubunit protein complex that contains membrane integral components (Rossi et al., 1993). Electrons from reduced Hmc are proposed to be transferred via the membrane integral subunits of the Hmc complex to the [Fe-S] cluster-containing gene product of Orf6 that is also part of the Hmc complex and is located at the inner aspect of the membrane. Further transfer of electrons may proceed directly to APS reductase or sulfite reductase or may involve cytoplasmic electron carriers such as flavodoxin (Voordouw, 1995). Mutants of *Desulfovibrio vulgaris* (Hildenborough) that had an elevated expression of the *hmc* operon grew more rapidly than the wildtype on H_2, supporting the involvement of the Hmc complex in the electron transfer from H_2 to sulfate (Keon et al., 1997). In *Desulfovibrio gigas,* Hmc was shown to accept electrons directly from hydrogenase (Chen et al., 1994a). Similarily, the [NiFe] hydrogenase from *Desulfovibrio desulfuricans* can reduce the Hmc-analogous nonaheme cytochrome *c* in addition to tetraheme cytochrome c_3 (Fritz, 1999). These findings imply that cytochrome c_3 may not always be required as a connecting link for the electron transfer from periplasmic hydrogenase to membrane-localized Hmc complex.

The finding of periplasmic hydrogenase in sulfate-reducing bacteria led to the hypothesis of energy conservation by so-called vectorial electron transport, the simplest transmembrane process that can generate a proton gradient for chemiosmotic ATP synthesis. The protons from H_2 oxidation are released by hydrogenase into the periplasm, while abstracted electrons are transported via redox-active centers of transmembrane proteins to the cytoplasm (or cytoplasmic aspect of the membrane) and used for sulfate reduction. This charge separation, which is driven by the exergonic process of sulfate reduction, is compensated by a simultaneous (somewhat “retarded”) proton flow via ATPase into the cytoplasm; ATPase finally conserves the energy from the redox process in a phosphoric anhydride bond. The generation of a proton gradient by simple charge separation (vectorial electron transport) by a periplasmic hydrogenase would leave eight extracellular protons per mol sulfate to be reduced. Because (at least) one of these electrogenically produced protons enters the cell during sulfate-transport (Cypionka, 1989), no more than seven protons would be left for chemiosmotic energy conservation yielding $1^3/_4$ to $2^1/_3$ mol ATP, if one assumes a ratio of 3–4 H^+/ATP (Schink, 1988a; Thauer and Morris, 1984; Stock et al., 1999) per mol sulfate reduced with H_2 (Fig. 10). Because sulfate activation is associated with a net consumption of 2

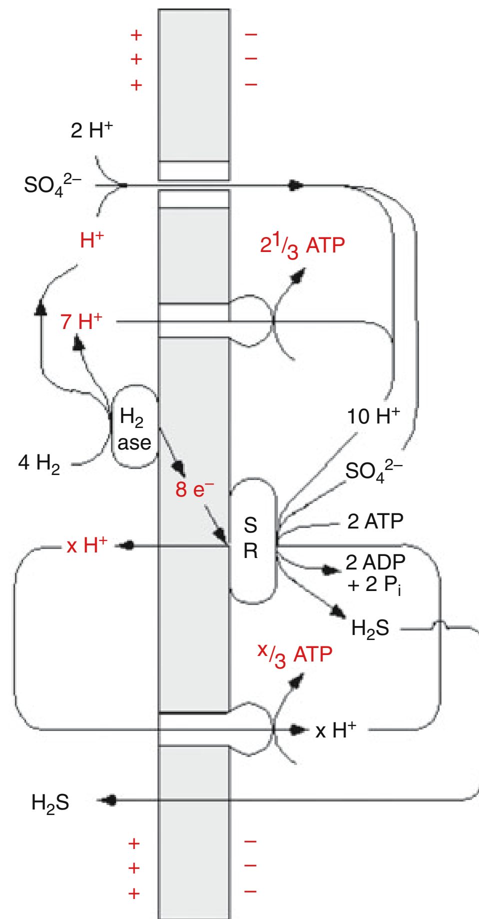


Fig. 10. Possible generation of a proton-motive force (pmf) during growth of *Desulfovibrio* on H_2 and sulfate (low concentration). Electrogenic transport of sulfate with three protons is assumed (Cypionka, 1989). In addition to a proton-translocating mechanism during sulfate reduction, vectorial electron transport from a periplasmic hydrogenase (H_2ase) via the membrane may contribute to the generation of a pmf. Periplasmic cytochrome c_3 and membrane–spanning, high-molecular-mass cytochrome (Hmc) mediate the electron flow between H_2 oxidation and sulfate reduction. Activation of sulfate consumes 2 ATP because AMP liberated by the adenosine-5′-phosphosulfate (APS) reductase from APS has to be converted to ADP by adenylate kinase (myokinase). Abbreviation: SR, enzymes and other components involved in sulfate reduction to H_2S.

ATP/SO_4^{2-}, a maximum of $^1/_3$ mol ATP would remain for cell synthesis. This is much less than the estimates from growth yields, which suggest a net synthesis of 1.3 mol ATP per mol sulfate (Nethe-Jaenchen and Thauer, 1984). Hence, a proton gradient seems to be generated in addition by proton pumping, provided the $3H^+/ATP$ ratio used in the calculations is a correct estimate. Indeed, proton translocation with H_2 and sulfate has been measured in *Desulfovibrio desulfuricans* strain Essex 6 in which the hydrogenase present under the applied growth conditions was reported to be cytoplasmic or at least on the cytoplasmic aspect of the membrane (Fitz and Cypionka, 1989). Strains of other *Desulfovibrio* species translocated protons with H_2 and nitrite, even though hydrogenase and nitrite reductase were both periplasmic enzymes (Barton et al., 1983; Steenkamp and Peck, 1981); this location excludes generation of a proton gradient by simple vectorial electron flow via the membrane. Finally, growth of the Gram-positive *Desulfotosporosinus orientis* on H_2 with high cell yields demonstrated that chemiosmotic ATP synthesis does not require a periplasmic hydrogenase (Cypionka and Pfennig, 1986). Hence, vectorial electron transport due to periplasmic hydrogenase appears to be only an additional mechanism for energy conservation in a number of *Desulfovibrio* species. The main mechanism is obviously vectorial proton transport (e.g., by proton-pumping redox proteins or "Mitchell-type" loops, involving the menaquinones that are commonly present in sulfate-reducing bacteria). Nothing is known about the possibility of a Q-cycle (Peck and Lissolo, 1988); considering this translocates two protons for one electron, the process would require significant differences in the redox potential between two couples, which is not very likely in the anaerobic respiratory chain in sulfate-reducing bacteria.

An energetically intriguing, not sufficiently understood, aspect is the growth of sulfate-reducing bacteria with H_2 over a wide range of partial pressures. At standard pressure, H_2 is one of the energetically most favorable electron donors ($\Delta G°' = -152.2$ kJ/mol sulfate). However, *Desulfovibrio* was shown to scavenge H_2 below 10 Pa (10^{-4} atm; Cord-Ruwisch et al., 1988). In natural anoxic habitats where sulfate-reducing bacteria thrive, even H_2 partial pressures as low as >5 Pa ($>5 \cdot 10^{-5}$ atm; Sørensen et al., 1981) $2.5 \cdot 10^{-2}$ Pa ($2.5 \cdot 10^{-7}$ atm, would be at the thermodynamic equilibrium; Scranton et al., 1984) and 1.1 Pa ($1.1 \cdot 10^{-5}$ atm; Lovley et al., 1982) have been measured. The free energy of sulfate reduction with H_2 at varying partial pressure is depicted in Fig. 11. Assuming that net ATP synthesis coupled to any catabolic overall

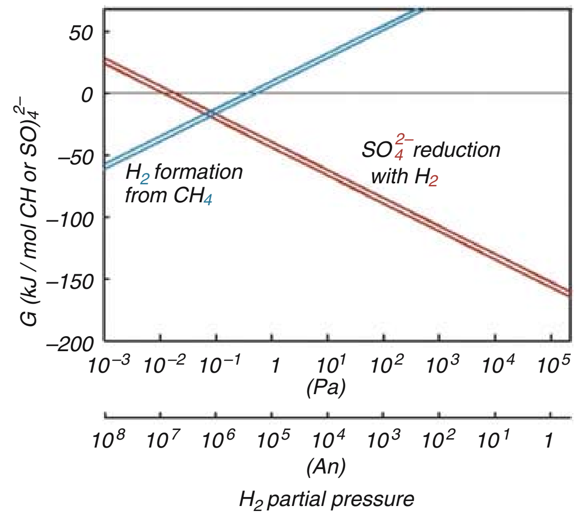
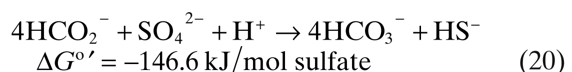


Fig. 11. Free energy change of sulfate reduction and "reverse" methanogenesis related to H_2 partial pressure. Reduction of SO_4^{2-} with H_2 is shown in red line; H_2 formation from CH_4 is shown in blue lines. Full lines represent values calculated for pH 7 and dashed lines those for pH 8.

reaction has irreversible character and requires around 70 kJ/mol ATP, the ATP gain at the natural, low H_2 pressures has to be much less than the gain measured with optimal supply of the electron donor. Hence, electrons from H_2 at low partial pressure cannot be transported via a chain with the same number of energy-conserving steps ("coupling sites") as electrons from H_2 at standard pressure. One may speculate that electrons from H_2 at various partial pressures enter the electron transport chain at different levels, and that different types of hydrogenases are involved.

Formate Formate is a fermentation product in several anaerobic bacteria, as for instance in enterobacteria. In addition, formate has been discussed as a means for an interspecies transfer of reducing equivalents and as an alternative to H_2 in natural anaerobic bacterial communities (Thiele et al., 1988a; Thiele and Zeikus, 1988b); formate transfer was most likely to occur in a sulfate-reducing coculture (Zindel et al., 1988). However, syntrophisms based on interspecies H_2 transfer are more important (Schink, 1997). Also energetically, formate may be regarded as an electron donor that is equivalent to H_2. The redox potential of the couples 2 H^+/H_2 and $HCO_3^-/HCOO^-$ are very similar ($E^{0\prime}$ around –0.41 V). Hence, formate is a favorable electron donor:



$$4HCO_2^- + SO_4^{2-} + H^+ \rightarrow 4HCO_3^- + HS^- \quad \Delta G°' = -146.6 \text{ kJ/mol sulfate} \tag{20}$$

The ability to grow with formate has been observed in most genera of sulfate-reducing eubacteria. Formate dehydrogenase has been found in *Desulfovibrio* (Fauque et al., 1991; LeGall and Fauque, 1988) and in completely oxidizing sulfate reducers except for *Desulfobacter* (Schauder et al., 1986; Spormann and Thauer, 1988; Aeckersberg et al., 1991; Rabus et al., 1993; Fukui et al., 1999).

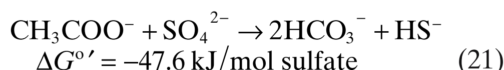
Formate dehydrogenase in *Desulfovibrio* is a periplasmic protein (Odom and Peck, 1981a). It was partially purified from *Desulfovibrio vulgaris* (Miyazaki); purified cytochrome c_{553} functioned as an electron acceptor but cytochrome c_3 did not (Yagi, 1979). The formate dehydrogenase of *Desulfovibrio gigas* is thought to use cytochrome c_3 as electron acceptor (Riederer-Henderson and Peck, 1986). The periplasmic formate dehydrogenase of *Desulfovibrio vulgaris* (Hildenborough) was purified by Sebban et al. (1995). The enzyme is composed of three subunits. The large 83.5-kDa subunit contains a molybdenum cofactor and most likely presents the active site. A 27-kDa subunit with an [Fe-S] center is similar to the [Fe-S]-containing subunit of the formate dehydrogenase from *Escherichia coli*. The 14-kDa subunit holds a *c*-type heme. Cytochrome c_{553} is thought to be the natural electron acceptor of this formate dehydrogenase (Sebban-Kreuzer et al., 1998b). Recently, a tungsten-containing formate dehydrogenase was purified from *Desulfovibrio gigas* and characterized. This protein was found to have a heterodimeric structure (subunits 92 kDa and 27 kDa) and to contain approximately seven Fe per molecule most probably in two [4Fe-4S] clusters; the tungsten is most likely bound to a molybdopterin guanine dinucleotide-type cofactor (Almendra et al., 1999). This is the second W-protein that has been isolated from *Desulfovibrio gigas* (see dissimilation of ethanol). The formate dehydrogenase of *Desulfovibrio desulfuricans* was also found to contain molybdenum, iron-sulfur centers and heme (Costa et al., 1997).

Completely oxidizing sulfate-reducing bacteria employ the C_1/CO dehydrogenase pathway for acetyl-CoA oxidation, always contain formate dehydrogenase (see next section) and often grow on formate. Growth of the completely oxidizing *Desulfotomaculum acetoxidans* on formate is poor and difficult to achieve (Widdel and Pfennig, 1981b), despite the high formate dehydrogenase activity (Spormann and Thauer, 1988). This can be explained by the lack of a proper transport system. Formic acid is less lipophilic and has a lower pK_a value (3.75) than acetic acid (4.75) and probably cannot enter the cell by diffusion via the membrane. Formate dehydrogenases that are part of the C_1/CO-pathway were found to be membrane-associated, probably with the reactive site toward the cytoplasm. Their natural electron acceptor is not known. The reduction of NAD^+ with formate probably occurred via a transhydrogenase.

Terminal Oxidation and Utilization of Acetate

The oxidation of organic substrates in sulfate-reducing bacteria may be complete, leading entirely to CO_2, or incomplete with acetate being the end product; in the latter case a mechanism for acetyl-CoA oxidation is lacking. A complete oxidation of organic compounds by sulfate reducers without the capacity for acetate oxidation is possible only with C_1-compounds such as formate or methanol (Klemps et al., 1985; Nanninga and Gottschal, 1987; Ollivier et al., 1988), or with C_2-compounds that are more oxidized than acetate (e.g., glycine; Stams et al., 1985), glycolate (Friedrich and Schink, 1995) or oxalate (Postgate, 1963).

The capacity for complete oxidation of various organic substrates, viz. the presence of a pathway for acetyl-CoA oxidation, usually includes also the ability to use free acetate as a growth substrate:



$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad \Delta G^{o\prime} = -47.6 \text{ kJ/mol sulfate} \qquad (21)$$

Desulfobacter species use acetate preferentially or even exclusively. In certain complete oxidizers, however, growth on acetate may be very poor, even though other compounds are readily oxidized to CO_2. Complete oxidizers may excrete acetate if growing, e.g., on ethanol or butyrate (Imhoff-Stuckle and Pfennig, 1983; Laanbroek et al., 1984; Schauder et al., 1986; Widdel and Pfennig, 1981b). With limiting amounts of substrates, the excreted acetate may be oxidized further. Species using acetate very poorly may leave the acetate once formed almost untouched (Imhoff-Stuckle and Pfennig, 1983). An explanation for the acetate excretion by complete oxidizers is that formation of acetyl-CoA proceeds faster than its terminal oxidation. The formation of 1 mol acetate per mol butyrate oxidized has been explained by the use of 1 mol acetyl-CoA (from 2 mol formed per mol of butyrate) for the activation of butyrate by a CoA transferase (Schauder et al., 1986); see also section "Butyrate and other fatty acids"). The marginal capacity or inability of some complete oxidizers to use free acetate is not clearly understood.

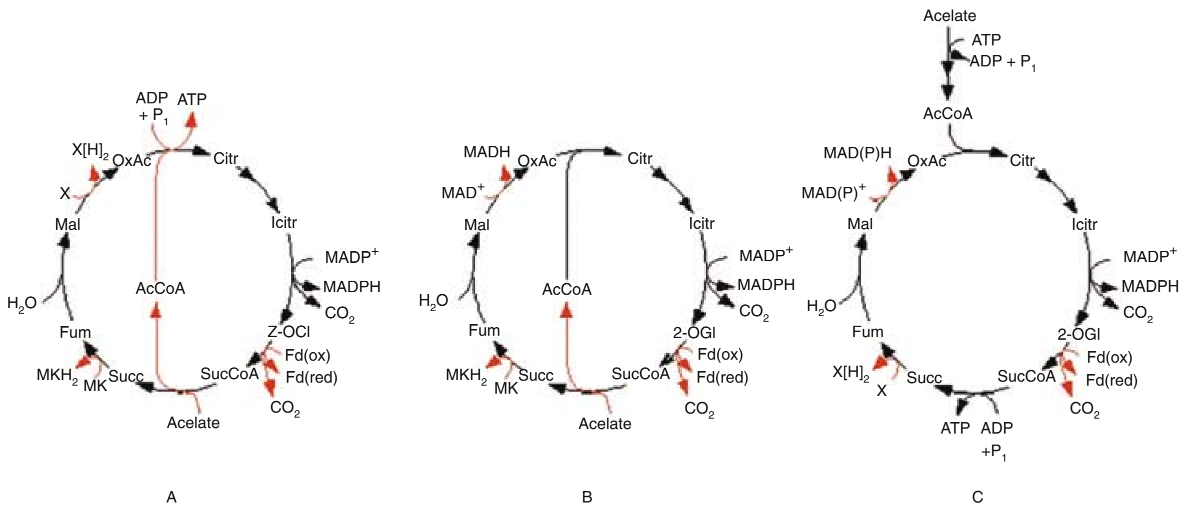


Fig. 12. Modifications of the citric acid cycle for the anaerobic oxidation of acetate in three species of sulfate- and sulfur-reducing bacteria: (A) *Desulfobacter postgatei*; (B) *Desulfuromonas acetoxidans*; and (C) *Desulforella acetivorans*. The reactions leading from citrate to succinyl-CoA are the same in all three cycles. The H^+ ions, water, and some other reactants are not indicated. Abbreviations: AcCoA, acetyl-CoA; Citr, citrate; Fd(ox), oxidized ferredoxin; Fd(red), reduced ferredoxin; Fum, fumarate; Icitr, isocitrate; Mal, malate; MK, menaquinone; 2-OGl, 2-oxoglutarate (α-ketoglutarate); OxAc, oxaloacetate; Succ, succinate; SucCoA, succinyl-CoA; X, unknown physiological electron or hydrogen carrier. Adapted from Thauer (1988) and Thauer et al. (1989b).

Organic end products other than acetate are formed in incomplete or complete oxidizers if substrate oxidation leads to products that cannot be degraded further by the enzymatic outfit. Examples are the oxidation of *n*-propanol, *n*-butanol or isobutanol to propionate, butyrate or isobutyrate, respectively (Mechalas and Rittenberg, 1960), the formation of propionate from C-odd fatty acids (Pfennig and Widdel, 1981b; Widdel, 1980; Widdel and Pfennig, 1981b) or of benzoate from phenylpropionate (Brysch et al., 1987).

The pathways for acetyl-CoA oxidation have been elucidated by enzymatic measurements and growth experiments with ^{14}C-labeled substrates.

In *Desulfobacter postgatei*, all enzymes of a citric acid cycle were found (Brandis-Heep et al., 1983; for an overview see Kröger et al., 1988; Thauer, 1988; Thauer, 1989a; Thauer et al., 1989b). Also with position-labeled [^{14}C]-acetate as growth substrate, the labeling pattern in aspartate and glutamate that are derived from oxaloacetate and 2-oxoglutarate were in agreement with an operating citric acid cycle (Gebhardt et al., 1983). The cycle differs in some aspects from the cycles in mitochondria and aerobic bacteria. Acetate in *Desulfobacter* is not activated via acetate thiokinase (acetyl-CoA synthetase) or acetate kinase and phosphotransacetylase (phosphate acetyltransferase), but via succinyl-CoA:acetate CoA transferase (Fig. 12). Dehydrogenation of isocitrate occurs with $NADP^+$, as in most eubacteria. However, the conversion of 2-oxoglutarate to succinyl-CoA does not couple to NAD^+, but rather to a ferredoxin, as electron acceptor. The hydrogen acceptor for succinate oxidation to fumarate, so far known, is menaquinone and not ubiquinone, as in mitochondria and most Gram-negative bacteria. A remarkable finding was that condensation of acetyl-CoA and oxaloacetate to citrate in *Desulfobacter* is associated with ATP synthesis (Möller et al., 1987). The enzyme, ATP-citrate lyase, enables the conservation of the energy of the thioester; the citrate synthase reaction in the common citric acid cycle wastes this energy by hydrolysis of the intermediary citryl-CoA. The ATP-citrate lyase reaction is reversible ($\Delta G^{\circ\prime}$ (0 kJ). Indeed, before being found in *Desulfobacter*, the reaction was only known to proceed in vivo in its opposite direction. In the cytosol of eukaryotic cells, ATP-citrate lyase cleaves citrate that functions as the acetyl carrier across the two mitochondrial membranes. Green sulfur bacteria fix CO_2 via a reverse citric acid cycle which was found to include the ATP-citrate lyase reaction (Ivanovsky et al., 1980). Citrate formation in *Desulfobacter* species occurs with Si-face stereospecificity. The acceptor for malate-dehydrogenase is neither NAD^+ nor $NADP^+$. The reduction of the artificial acceptor 2,6-

dichlorophenol indophenol (DCPIP) was inhibited by the menaquinone antagonist 2(n-heptyl)-4-hydroxyquinoline-N-oxide (HQNO). From this and the occurrence of the activity in the membrane, one may speculate that menaquinone serves as hydrogen acceptor in malate oxidation. However, in vitro tests with substitutes for menaquinone (naphthoquinone, dimethylnaphthoquinone) yielded no or marginal activity (Möller-Zinkhan and Thauer, 1988). Still, it is most likely that *Desulfobacter* employs a more positive acceptor for malate oxidation than other bacteria. The reversible, energy-conserving ATP-citrate lyase reaction in *Desulfobacter* necessitates the use of a more positive acceptor for malate oxidation to favor the concentration of the product. The citric acid cycle in *Desulfobacter hydrogenophilus* has the same reactions as in *D. postgatei* (Schauder et al., 1987). A comparison of the modifications of the citric acid cycle found in sulfate- and sulfur-reducing bacteria is presented in Fig. 12.

In completely oxidizing sulfate reducers other than *Desulfobacter*, 2-oxoglutarate dehydrogenase could not be detected (Schauder et al., 1986). Inasmuch as most completely oxidizing sulfate reducers grow very poorly on acetate, [3-^{14}C]-pyruvate was used for them as growth substrate in labeling studies (Schauder et al., 1986). For *Desulfotomaculum acetoxidans*, [^{14}C]-acetate could be used. The labeling in aspartate and glutamate showed that a citric acid cycle was not operating. Citrate synthase of the incomplete cycle seemed to have re-specificity. All complete oxidizers without 2-oxoglutarate dehydrogenase contained high activities of CO dehydrogenase, which was absent in *Desulfobacter* species. In labeling experiments, cell extracts of species without 2-oxoglutarate dehydrogenase catalyzed an equilibrium exchange of the C_1-position in acetyl-CoA with free CO_2. Furthermore, these species formed traces of methane indicating a reactive methyl group as an intermediate; such a mini-methane formation was not observed in *Desulfobacter*. All these findings led to the conclusion that completely oxidizing genera other than *Desulfobacter*, viz. the majority of sulfate reducers, cleave acetyl-CoA into bound CO and a bound methyl group; both C_1 units are then oxidized to CO_2 (Schauder et al., 1986; Spormann and Thauer, 1988; Fig. 13). The carrier of the methyl group in *Desulfotomaculum acetoxidans* was tetrahydrofolate (Spormann and Thauer, 1988). For two steps, namely the dehydrogenation of methylenetetrahydrofolate and formate, NAD^+ wasthe natural electron acceptor. The conversion of formyl-tetrahydrofolate to free formate is associated with ATP synthesis. In *Desulfobacterium autotrophicum*, the C_1-carrier is a homologue of tetrahydrofolate, tetrahydropteroyltetraglutamate, which has four glutamate residues instead of one (Länge et al., 1989). Dehydrogenation of the methylene group in this species occurs with $NADP^+$ (Schauder et al., 1989). The results demonstrated for the first time that the pathway of acetyl-CoA synthesis known from homoacetogenic bacteria (Fuchs, 1986; Wood et al., 1986) can operate in the reverse direction for terminal oxidation of organic substrates. Thereafter, the pathway was also found in a syntrophic thermophile that oxidized acetate to CO_2 and H_2 of low partial pressure (Lee and Zinder, 1988), and in the archaeal sulfate reducer, *Archaeoglobus* (Möller-Zinkhan et al., 1989).

The bioenergetic implications of the pathways for acetate oxidation and terminal oxidation of other organic compounds in sulfate- and sulfur-reducing bacteria have been discussed (Kröger et al., 1988; Thauer, 1988; Thauer et al., 1989b). The free energy gain from reduction with acetate (equation 21) is much lower than from sulfate reduction with H_2 at standard pressure (Fig. 11). However, due to the stoichiometric factors in the equations, the free energy per mol of sulfate reduced is less concentration-dependent in the case of acetate oxidation than in the case of hydrogen oxidation.

Net ATP yields available for cell synthesis may be estimated from growth yields. The highest growth yield measured with an acetate-oxidizing sulfate reducer, *Desulfobacter postgatei*, in batch cultures was 4.8 g dry mass/mol acetate (or sulfate). Theoretical maximum growth yields (Y_{max}) from extrapolation to infinite growth rates ($\mu = \infty$) have not been determined. Nevertheless, the growth yield may be compared to that of other bacteria at similar growth rates. The doubling time of *D. postgatei* was around 20 hours. At this doubling time, *Desulfovibrio vulgaris* growing on H_2 and sulfate with acetate as carbon source for cell synthesis would have a growth yield of 7.7 g dry weight/mol sulfate (calculated from Nethe-Jaenchen and Thauer, 1984). A yield of 1.3 mol ATP/mol sulfate was estimated for *D. vulgaris* (Nethe-Jaenchen and Thauer, 1984). *Desulfobacter postgatei* should thus gain around 0.8 mol ATP/mol acetate.

Comparison of the free energy available from reactions (21) with the generally observed requirement of >70 kJ/mol ATP (Schink, 1988a; Thauer et al., 1977) again indicates that acetate-oxidizing sulfate-reducing bacteria obtain less than 1 mol ATP/mol sulfate.

In *Desulfobacter*, ATP-citrate lyase enables net gain of 1 ATP by substrate-level phosphorylation during acetate oxidation; however, 2 ATP are needed for sulfate activation (assuming that pyrophosphate is irreversibly hydrolyzed). The energy requirement for sulfate transport in

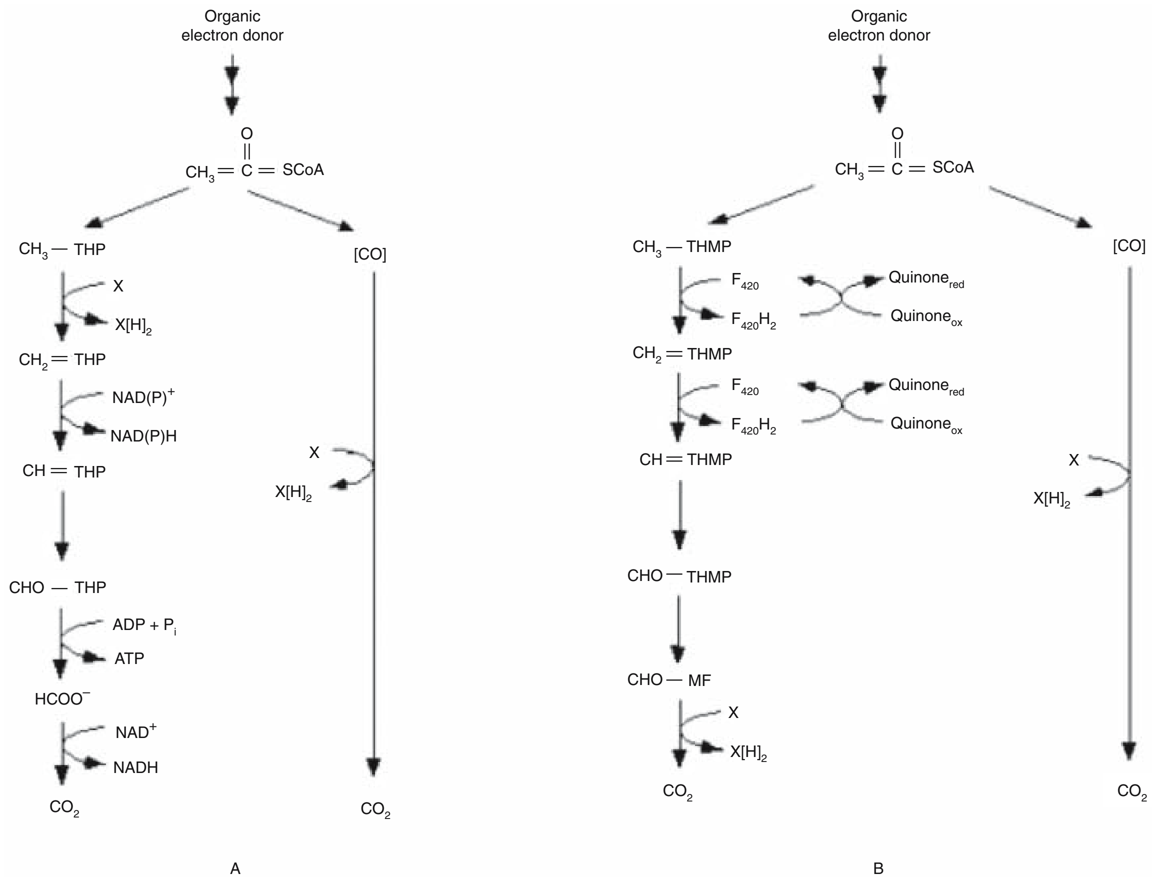


Fig. 13. Terminal oxidation of acetyl-CoA via the C_1/carbon monoxide dehydrogenase pathway in sulfate-reducing bacteria. The H^+ ions, water, and some other reactants are not indicated. (A) Reactions in *Desulfotomaculum acetoxidans*, *Desulfobacterium autotrophicum*, and presumably other completely oxidizing sulfate-reducing bacteria (except for *Desulfobacter* species). The former species uses NAD^+, and the latter $NADP^+$ for dehydrogenation of the methylene group; in *Desulfotomaculum*, NAD^+ is probably not the direct electron acceptor for formate dehydrogenase but reduced via an unknown, primary acceptor. THP is tetrahydrofolate in *D. acetoxidans*, and tetrahydropteroyltetraglutamate in *D. autotrophicum*. (B) Reactions in the archaeon *Archaeoglobus fulgidus*. Abbreviations: [H], unknown physiological electron or hydrogen carrier; MF, methanofuran; THMP, tetrahydromethanopterin; THP, tetrahydropterin; F_{420}, formate dehydrogenase. Adapted from Thauer (1988) and Thauer et al. (1989b).

Desulfobacter is unknown. *Desulfobacter* occurs mainly in brackish or marine environments with high sulfate concentrations. It is therefore likely that an electrogenic transport of sulfate (Warthmann and Cypionka, 1990) is not required under such conditions. Thus, for a net ATP gain, more than 1 ATP has to be synthesized by chemiosmosis. In *Desulfuromonas*, there is no substrate-level phosphorylation. In *Desulfobacter postgatei*, the transport of reducing equivalents from NADPH to MK-7 ($\Delta E_0' =$ 0.25 V) could be associated with a translocation of 2 H^+/2 [H], which are 4 H^+/acetate (Kröger et al., 1988; Thauer, 1988). The preceding reduction of $NADP^+$ with ferredoxin ($\Delta E_0' = 0.1$ V) has been discussed as another energy-conserving step allowing the translocation of 1 H^+ (Fig. 14A). With the assumed requirement of 3–4 H^+/ATP, chemiosmosis in *Desulfobacter postgatei* should produce $^5/_4$ to $^5/_3$ ATP and thus allow a net ATP gain of $^1/_4$ to $^2/_3$ per sulfate for cell synthesis. The latter value is more likely in view of the aforementioned calculations based on growth yields. In *Desulfobacter*, succinate oxidation to fumarate ($E_0' = +0.033$ V) with menaquinone ($E_0' = -0.074$ V) is endergonic from the viewpoint of standard potentials. Still, the reaction appears possible with shifted concentration ratios of involved redox couples, or by specific coupling to a favorable redox reaction of the sulfate reduction pathway. It is true that from a mere thermodynamic viewpoint any unfavorable partial reaction is rendered possible if embedded in an exergonic overall reaction. In biological systems,

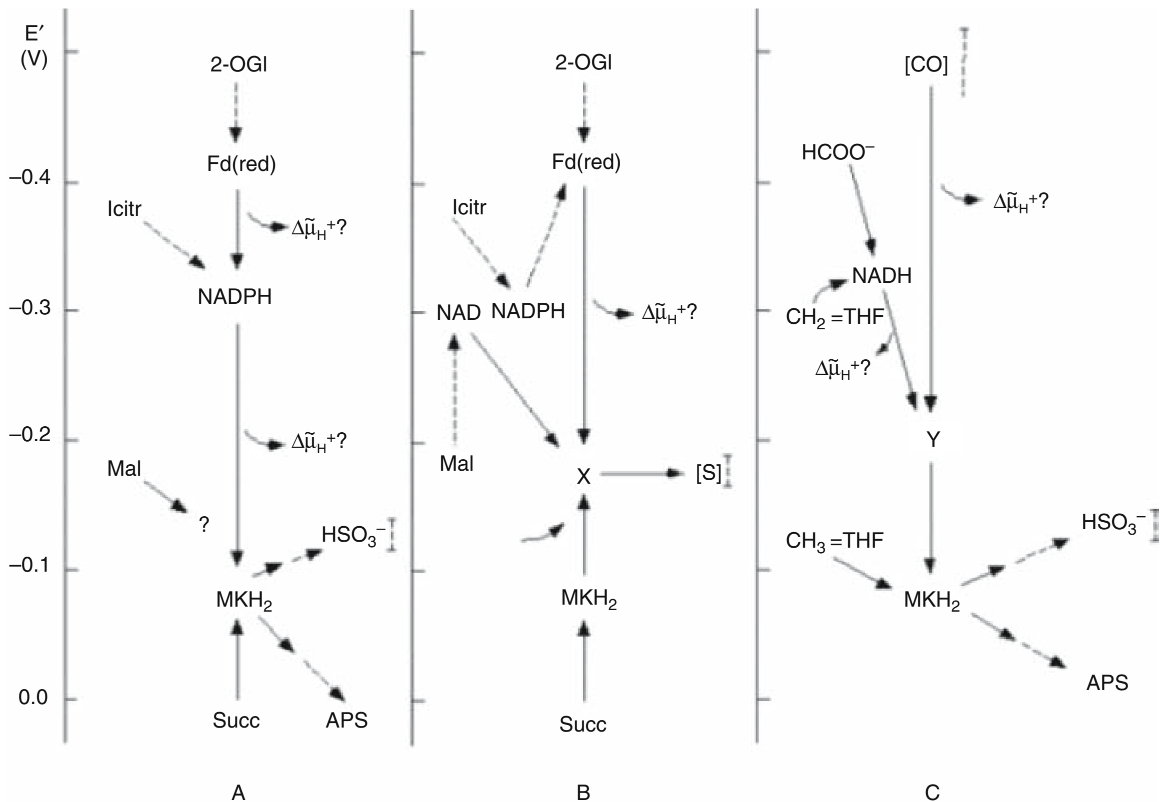


Fig. 14. Flow of reducing equivalents during terminal oxidation of *acetyl-CoA* in sulfate- and sulfur-reducing bacteria. Electron-donating and electron-accepting redox couples are presented only as the reduced or oxidized forms, respectively. In most cases, the midpoint potential is indicated (often concentration-independent, with E' being identical to E_0'); the exact redox potential in the cell may differ, according to concentrations of reaction partners. The redox potential of APS reduction refers to concentrations of 1 mM. The redox potential of bisulfite reduction given for a six-electron step refers to 1 mM HSO_3^- and a range of 1 to 10 mM H_2S (dotted redox span). The concentration range of H_2S for sulfur reduction is also 1 to 10 mM. Bound CO probably has a less negative midpoint potential than free CO. The value of the former is not exactly known. (A) *Desulfobacter postgatei*, growing on acetate and sulfate. (B) *Desulfuromonas acetoxidans*, growing on acetate and sulfur. X is a carrier, presumably a cytochrome *c* that donates electrons to sulfur reduction. (C) *Desulfotomaculum acetoxidans*, growing on acetate and sulfate. Y is an unknown electron carrier. Symbols and abbreviations: arrows (in full lines), reactions catalyzed by membrane-associated enzymes; arrows (in dashed lines), reactions catalyzed by soluble enzymes; APS, adenosine-5′-phosphosulfate. Fd(red), reduced ferredoxin; Icitr, isocitrate; Mal, malate; Succ, succinate; THF, tetrahydrofolate; 2-OGl, 2-oxoglutarate. Adapted from Möller-Zinkhan and Thauer (1988) and Thauer (1988).

however, also the rates are important. In a thermodynamically very unfavorable partial reaction, the very low product concentration may not be sufficient to allow appropriate rates with the enzyme of the subsequent reaction. The equilibrium of unfavorable reactions can in principle be shifted by an input of energy, which in case of redox reactions is known as reversed electron transport. Indeed, a membrane preparation catalyzed a strictly ATP-dependent oxidation of succinate with sulfur or NAD^+ (Paulsen et al., 1986). The reaction was sensitive to the ATPase inhibitor DCCD6 or to the protonophore TTFP7, indicating that ATP acted indirectly via formation of a proton gradient as the driving force for succinate oxidation. The primary hydrogen acceptor of succinate oxidation was apparently menaquinone (MK-8); its analogue dimethylnaphthoquinone was reduced with succinate without addition of ATP, as in *Desulfobacter*. Hence, the proton gradient-driven reaction is probably the oxidation of menaquinone with the subsequent electron carrier that feeds into sulfur reductase. This electron carrier might be one of the membrane-bound *c*-type cytochromes with a midpoint potential more negative than –200 mV. It has been estimated that two to three protons have to reenter the cell to promote the oxidation of one molecule of succinate (Kröger et al., 1988; Thauer, 1988). With the remaining one to two protons, the net energy conservation in the sulfur

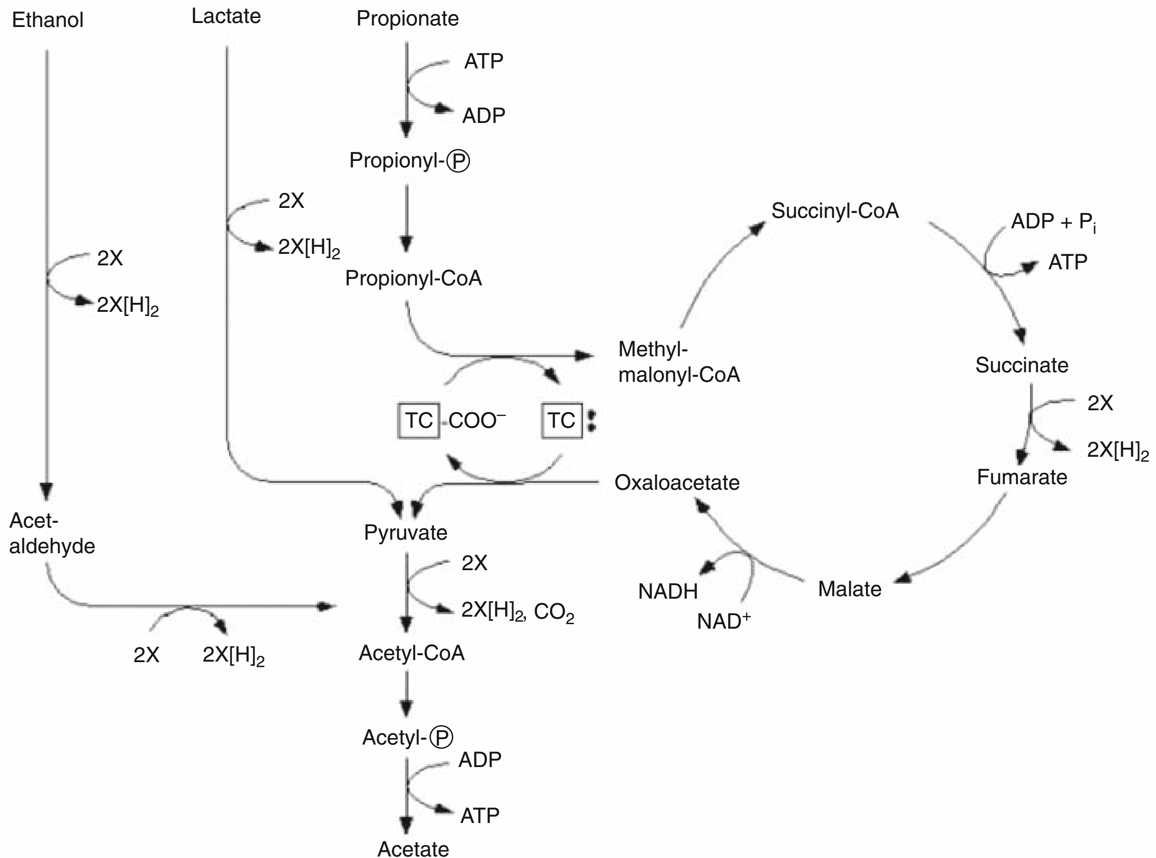


Fig. 15. Oxidation of ethanol, lactate, and propionate to acetate in *Desulfobulbus propionicus*. Methylmalonyl-CoA is formed by carboxylation of propionyl-CoA with CO_2 bound to transcarboxylase (TC). The "X" is the unknown physiological electron or hydrogen carrier.

reducer would be $^1/_3$ to $^2/_3$ ATP per molecule of acetate.

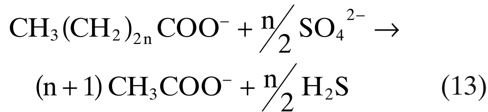
Suggested proton-translocating reactions in the C_1/CO-dehydrogenase-pathway are indicated in Fig. 14C.

Propionate Propionate serves as electron donor and carbon source for the incompletely oxidizing *Desulfobulbus* species and several completely oxidizing sulfate reducers (Table 2). Propionate in *Desulfobulbus* is oxidized to acetate via a randomizing pathway with succinate, a symmetric molecule, as free intermediate (Kremer and Hansen, 1988a; Fig. 15). The principle of this pathway in *Desulfobulbus* was first elucidated in its reverse direction, the formation of propionate from fermentable substrates in the absence of sulfate (see Fermentative and Syntrophic Growth in the Absence of Sulfate). A succinate dehydrogenase/fumarate reductase was purified from *Desulfobulbus elongatus*; it consists of three subunits and contains one cytochrome *b*, flavin and eight non-heme iron atoms (Samain et al., 1987). The oxidation of propionate to CO_2 by *Desulfococcus multivorans* was also shown to proceed via the succinate pathway (Stieb and Schink, 1989). Unlike *Desulfobulbus*, *Desulfococcus* can oxidize the pyruvate formed via the C_4-dicarboxylic acid sequence further than the acetate level; acetyl-CoA is oxidized to CO_2 via the C_1/CO-dehydrogenase-pathway (see preceding section). Not unexpectedly therefore, the molar growth yield of *Desulfococcus*, if related to propionate, was more than twice as high as that of *Desulfobulbus* (approximately 10 and 4 g dry mass per mol propionate, respectively; Stieb and Schink, 1989; Stams et al., 1984); if related to sulfate, the yields are rather similar (approximately 5.7 and 5.3 g dry mass per mol sulfate, respectively).

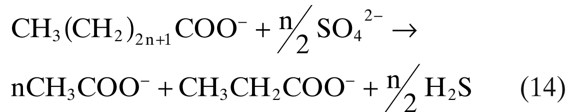
Butyrate and Other Fatty Acids Butyrat and higher fatty acids are oxidized by many incompletely and completely oxidizing sulfate-

reducing bacteria (Widdel, 1980; Pfennig and Widdel, 1981b; Widdel, 1988; Table 2).

The incomplete oxidation of C-even fatty acids yields only acetate. The C-odd fatty acids are oxidized to acetate and propionate. Measured degradation balances were in agreement with the following general equations:



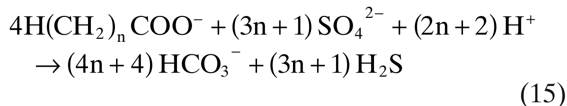
$$CH_3(CH_2)_{2n}COO^- + \frac{n}{2}SO_4^{2-} \rightarrow (n+1)\,CH_3COO^- + \frac{n}{2}H_2S \quad (13)$$



$$CH_3(CH_2)_{2n+1}COO^- + \frac{n}{2}SO_4^{2-} \rightarrow nCH_3COO^- + CH_3CH_2COO^- + \frac{n}{2}H_2S \quad (14)$$

The ratio is explained by a β-oxidation. In the case of C-odd fatty acids, propionyl-CoA is left, which obviously cannot be metabolized by most incomplete fatty acid oxidizers and therefore has to be excreted. If 2-methylbutyrate is used by incomplete oxidizers, propionate is formed too. β-Oxidation of acetyl-CoA is in principle not hampered by a 2-methyl group; this leads to formation of propionyl-CoA rather than acetyl-CoA from the first part of the fatty acid chain.

Most complete oxidizers can degrade the propionyl residue; therefore, also C-odd fatty acids and, if metabolized, 2-methylbutyrate are oxidized like C-even fatty acids:



$$4H(CH_2)_nCOO^- + (3n+1)\,SO_4^{2-} + (2n+2)\,H^+ \rightarrow (4n+4)\,HCO_3^- + (3n+1)\,H_2S \quad (15)$$

Nevertheless, complete oxidizers may excrete acetate, probably as a result of an "imbalance" between β-oxidation and acetyl-CoA oxidation. *Desulfobacterium autotrophicum* formed one mol acetate per mol butyrate (Schauder et al., 1986). It is concluded from this ratio that acetate was formed by CoA transfer from acetyl-CoA to activate butyrate. Free acetate is used very poorly by this sulfate reducer. *Desulfotomaculum acetoxidans*, which is a complete oxidizer but cannot utilize propionate, oxidizes valerate to propionate (Widdel and Pfennig, 1982).

Sulfate reducers using isobutyrate and 3-methylbutyrate (isovalerate) are always complete oxidizers. The pathway of isobutyrate degradation has been elucidated in a *Desulfococcus multivorans* strain (Stieb and Schink, 1989). The reactions are in principle the same as found in aerobic organisms' metabolism of valine. The initial degradation steps in *Desulfococcus* were mediated by two enzymes that are involved in the catabolism of *n*-butyrate. Isobutyryl-CoA is first converted via butyryl-CoA dehydrogenase and enoyl-CoA hydratase to 3-hydroxyisobutyryl-CoA, which is then hydrolyzed to the free acid and oxidized to methylmalonate semialdehyde. CoA-dependent dehydrogenation of the semialdehyde and decarboxylation leads to propionyl-CoA. This is oxidized to acetyl-CoA as in *Desulfobulbus propionicus* (see foregoing section). Acetyl-CoA is then oxidized via the C_1-pathway (Schauder et al., 1986). By means of a succinyl-CoA:acid CoA transferase, the conversion of succinyl-CoA to succinate is coupled to the activation of the free isobutyrate to isobutyryl-CoA. In contrast to the sulfate-reducing culture, a methanogenic coculture isomerized isobutyrate to butyrate that was oxidized to two acetate residues (Stieb and Schink, 1989). The pathway for isovalerate degradation has not been examined in sulfate-reducing bacteria.

Lactate and Pyruvate Lactate, the "classical" substrate for cultivation of sulfate-reducing bacteria, is utilized by most species of almost each genus and may be oxidized completely or incompletely (equations 1 and 2, respectively). *Desulfoarculus baarsii* (formerly *Desulfovibrio*; Widdel, 1980), several *Desulfobacter* species (Widdel and Pfennig, 1981a; Widdel, 1987) and some species of the genera *Desulfobacterium*, *Desulfonema* (Widdel et al., 1983) and *Desulfotomaculum* (Widdel and Pfennig, 1981b) cannot use lactate.

The oxidation of L- and D-lactate to pyruvate is mediated by $NAD(P)^+$-independent lactate dehydrogenases that occur mainly membrane-bound. None of the enzymes from sulfate-reducing bacteria have been purified to homogeneity. D-Lactate dehydrogenase of *Desulfovibrio desulfuricans* was present in the particulate fraction, and detergents were required for its solubilization (Czechowski and Rossmoore, 1980), but in *Desulfovibrio vulgaris* strain Miyazaki, part of the enzyme activity was soluble (Ogata et al., 1981). L-lactate dehydrogenase activities were demonstrated in a *Desulfomicrobium baculatum*-like strain (formerly a *Desulfovibrio desulfuricans* strain; Stams and Hansen, 1982), *Desulfovibrio desulfuricans*, *Desulfovibrio gigas* (Peck and LeGall, 1982b) and *Desulfovibrio vulgaris* (Pankhania et al., 1988).

In *Desulfovibrio vulgaris* strains, pyruvate has been shown to be oxidatively decarboxylated to acetyl-CoA with ferredoxin or flavodoxin as electron acceptor (Suh and Akagi, 1966; Ogata and Yagi, 1986). The low potential ferredoxin I was found to be particularly active in the pyruvate:acceptor oxidoreductase reaction of *Desulfovibrio gigas* (LeGall and Fauque, 1988; Fauque et al., 1991). Pyruvate:ferredoxin oxi-

doreductase (POR) has been purified from *Desulfovibrio africanus*. The enzyme is a homodimer of 256 kDa and contains thiamine pyrophosphate (TPP) and three iron-sulfur clusters. Spectroscopic analysis of the activated enzyme indicated the presence of a free radical (Pieulle et al., 1995). A catalytic mechanism involving a free radical had been demonstrated before for the POR from the extremely halophilic bacterium *Halobacterium halobium* (Cammack et al., 1980). The gene for POR of *D. africanus* was cloned and the enzyme overexpressed in *E. coli* (Pieulle et al., 1997) so that enzyme quantities sufficient for crystallization could be obtained. The enzyme from *D. africanus* is the first POR to have its crystal structure determined (Chabriere et al., 1999a; Chabriere et al., 1999b; Pieulle et al., 1999a; for review see Charon et al., 1999). The substrate pyruvate is bound at the active site in the proximity of TPP. The three [4Fe-4S] clusters are located between TPP and the protein surface, indicating that this arrangement serves as the path for electron transfer within the protein. Further transfer of electrons from POR to the external ferredoxin probably requires electrostatic interactions (Pieulle et al., 1999b).

In the incomplete oxidation of organic substrates, acetyl-CoA produced from pyruvate is converted to acetate by means of phosphotransacetylase and acetate kinase (Brown and Akagi, 1966; Ogata and Yagi, 1986), which allows phosphorylation of ADP to ATP.

The incomplete oxidation of lactate to acetate in *Desulfovibrio* species gave the first hint that oxidation of organic substrates in sulfate-reducing bacteria is associated with chemiosmotic energy conservation (formerly, electron transport phosphorylation) in addition to substrate-level phosphorylation. A simple yet basic calculation (Peck, 1966) revealed, that the net ATP gain by substrate-level phosphorylation during growth of *Desulfovibrio* on lactate and sulfate is zero. The two molecules of lactate oxidized per molecule of reduced sulfate (equation 1) yield two molecules of ATP during liberation of acetate via acetate kinase; these two ATP molecules are consumed for the activation of sulfate, namely one for the ATP sulfurylase reaction and one for regeneration of ADP from AMP (adenylate kinase reaction) formed during APS reduction (see Activation of Sulfate in this Chapter). Hence, there has to be an additional mechanism for ATP formation.

A unique mechanism for generation of a proton gradient with lactate as electron donor was suggested by Odom and Peck (1981b). Their so-called hydrogen-cycling model for growth on lactate of a *Desulfovibrio* involved the cytoplasmic production of H_2 as a result of the oxidation of lactate to pyruvate and pyruvate to acetyl-CoA; after diffusion through the cytoplasmic membrane, the H_2 would be oxidized in the periplasm as described for H_2 as electron donor. An involvement of periplasmic [Fe] hydrogenase in growth of *Desulfovibrio vulgaris* (Hildenborough) on lactate was also suggested by van den Berg et al. (1991). Reduction of the amount of this hydrogenase by means of antisense RNA resulted in a pronounced reduction of growth yields on lactate. The [NiFeSe] hydrogenase, which is located at the cytoplasmic aspect of the cytoplasmic membrane, might function as the H_2-evolving hydrogenase, and the [Fe] and the [NiFe] hydrogenases are thought to function as the H_2-oxidizing enzymes (Rohde et al., 1990). On the other hand, there are also arguments against free H_2 as an obligatory intermediate in the catabolism of lactate. Important in this respect is the lack of a strong inhibition of lactate oxidation by an H_2 atmosphere, unlike what should be expected for thermodynamic reasons in the H_2-cycling model (e.g., Pankhania et al., 1986); furthermore, a *Desulfovibrio* mutant was isolated that does not grow on H_2 plus sulfate but does grow on lactate plus sulfate (Odom and Wall, 1987). Also, there are other sulfate reducers growing well on lactate and other substrates without possessing hydrogenase, e.g., *Desulfobotulus sapovorans* or *Desulfococcus multivorans*. Hydrogen production linked to the oxidation of lactate to pyruvate has been even shown to be an energy-dependent process (Pankhania et al., 1988). The investment of energy makes H_2 cycling as a mode of energy conservation on lactate unlikely. It is true that, with pyruvate as growth substrate for *Desulfovibrio vulgaris*, H_2 cycling was directly demonstrated by employing membrane-inlet mass spectrometry (Peck et al., 1987). However, under natural conditions, cycling by *Desulfovibrio* of H_2 from pyruvate oxidation is probably not a significant reaction. In natural habitats, pyruvate is probably not a major free product of fermentative bacteria and a less important substrate, if at all, for sulfate reducers than lactate. With pyruvate added to artificial media, a rapid pyruvate:ferrodoxin oxidoreductase (PFOR) reaction may cause a burst of H_2 which is then scavenged mainly by periplasmic hydrogenase (Tsuji and Yagi, 1980). To our understanding, the production of some H_2 during growth on lactate and sulfate (Lupton et al., 1984) is neither a proof for H_2 cycling nor a proof for a specific mechanism that controls the redox state of electron carriers involved in lactate oxidation. One possible explanation is that part of the reducing power during growth on lactate and sulfate simply diffuses off via a constitutive hydrogenase; the H_2 partial pressure may reflect the degree of

imbalance between electron-producing and electron-consuming reactions. Another explanation can be given in view of the capacity of *Desulfovibrio* species to grow by interspecies H_2-transfer in sulfate-free cocultures with methanogens. Lactate conversion to acetate, H_2 and CO_2 in the absence of sulfate seems to be one of the ecological roles of *Desulfovibrio* species (Bryant et al., 1977; Zellner et al., 1987; Zellner and Winter, 1987; section Fermentative growth and syntrophy). In the presence of sulfate, the H_2-evolving system (Pankhania et al., 1988) may not be completely suppressed and lead to a minor loss of reducing power as H_2.

Ethanol and Acetaldehyde Ethanol is a very common electron donor and carbon source for incompletely and completely oxidizing sulfate reducers (Table 1). Ethanol is oxidized via acetaldehyde to acetate, which may be further oxidized. Some *Desulfovibrio* species can oxidize choline to acetate and trimethylamine. There is some evidence that acetaldehyde formed as the first intermediate from choline degradation is oxidized to acetate via acetyl-CoA (Hayward, 1960).

With primary alcohols as electron donors, some sulfate-reducing bacteria form strong smelling byproducts which might be chemical adducts of sulfide and aldehydes that are formed as free intermediates (F. Widdel, unpublished observation).

During growth on ethanol plus sulfate, *Desulfovibrio gigas* and three other examined *Desulfovibrio* strains contained high NAD^+-dependent alcohol dehydrogenase activities. In lactate-grown cells, these activities were lower or absent. NAD^+-dependent alcohol dehydrogenases have been purified from *Desulfovibrio gigas* and from *Desulfovibrio strain* HDv; the latter organism is now known as *Desulfovibrio burkinensis* (Ouattara et al., 1999). Both enzymes were oxygen-labile; the proteins were decameric, with subunits of 43 and 48 kDa, respectively, and contained zinc (Hensgens et al., 1993; Hensgens et al., 1995a). The first 21 N-terminal amino acids of the enzyme from *Desulfovibrio* strain HDv were identical to those of the alcohol dehydrogenase from *Desulfovibrio gigas*; on the basis of the N-terminal amino acid sequences, the enzymes are members of the so-called family III of alcohol dehydrogenases which is not related to the family that includes the major yeast and mammalian alcohol dehydrogenases (Reid and Fewson, 1994). The alcohol dehydrogenases from *Desulfovibrio* are only highly active toward short primary alcohols; unlike the decameric family III enzyme from *Bacillus methanolicus*, however, they show no activity with methanol.

A molybdenum iron-sulfur protein from *D. gigas* was shown to have some aldehyde dehydrogenase activity (Turner et al., 1987). Somewhat later, aldehyde oxidation was studied in more detail (Kremer et al., 1988b). Coenzyme A or phosphate dependency was not found, indicating that acetyl-CoA and acetyl phosphate are not intermediates in the conversion of acetaldehyde to acetate (Kremer et al., 1988b). Furthermore, it was shown that acetaldehyde can be oxidized in *Desulfovibrio gigas* by two completely different enzymes, a molybdenum-containing enzyme which can be assayed with DCPIP as artificial electron acceptor, and a tungsten-containing enzyme reacting with benzylviologen as an acceptor; the latter is strongly stimulated by K^+ ions (Kremer et al., 1988b). The synthesis of the enzymes appeared to be strongly affected by the presence of molybdate and tungstate in the growth media (Hensgens et al., 1994). Extracts of cells grown in the presence of both tungstate and molybdate have only very low levels of the DCPIP-dependent enzyme. The benzylviologen-linked tungsten-containing aldehyde dehydrogenase allows much faster growth with ethanol than the molybdenum enzyme. During growth on ethanol of *Desulfovibrio gigas*, in media without tungstate, transient excretion of acetaldehyde was observed. The molydenum-containing aldehyde oxidoreductase is a homodimer of subunits with 907 amino acid residues and contains a molybdopterin cofactor and two different [2Fe-2S] centers. It is a member of the xanthine oxidase family, and it is the first molybdenum enzyme with a molybdopterin cofactor (the crystal structure of which was determined; Romão et al., 1995). The tungsten-containing aldehyde oxidoreductase of *Desulfovibrio gigas* was active towards several aldehydes. This enzyme consists of two subunits of 62 kDa and was found to contain approximately 0.7 W, 4.8 Fe and 3.2 labile S per subunit; EPR studies indicated the presence of a [4Fe-4S] center (Hensgens et al., 1995b). Most likely, the tungsten-containing aldehyde oxidoreductase is related to similar enzymes from hyperthermophilic archaea and from Gram-positive anaerobic bacteria (Kletzin and Adams, 1996; Romão et al., 1997; Hu et al., 1999). The presence of a tungsten-containing aldehyde dehydrogenase, supposedly using flavins as natural cofactors, in *Desulfovibrio* simplex was demonstrated in experiments with cell-free extracts (Zellner and Jargon, 1997).

Other Monovalent Alcohols and Polyols Methanol is a less common electron donor for sulfate-

reducing bacteria and growth on methanol is usually slower. Enrichment cultures with methanol usually select for methanogenic bacteria, despite the presence of sulfate. Methanol can be used by some *Desulfotomaculum* species such as the mesophilic *Desulfotosporosinus orientis* (Klemps et al., 1985), the thermophilic *Desulfotomaculum kuznetsovii* (Nazina et al., 1988), a few *Desulfovibrio* species (e.g., *Desulfovibrio carbinolicus*; Nanninga and Gottschal, 1987), *Desulfobacterium anilini* (Schnell et al., 1989) and *Desulfobacterium catecholicum* (Szewzyk and Pfennig, 1987). The mechanism of methanol oxidation is unknown.

Primary alcohols higher than ethanol, for instance 1-propanol and 1-butanol, can also act as H_2 donors for sulfate-reducing bacteria. Oxidation by *Desulfovibrio* species is incomplete and leads to the formation of the corresponding acids (propionate, butyrate, respectively; Mechalas and Rittenberg, 1960). *Desulfobulbus* strains oxidize 1-propanol incompletely to acetate (Widdel and Pfennig, 1982). Species of other genera may oxidize these alcohols completely.

Certain *Desulfovibrio* strains were shown to dehydrogenate a secondary alcohol such as 2-propanol to acetone (Widdel, 1986; Zellner et al., 1989a; Tanaka, 1992) or 2-butanol to 2-butanone (Tanaka, 1992). *Desulfococcus biacutus* (Platen et al., 1990) and *Desulfococcus multivorans* strains except for the type strain (Widdel, 1988) oxidize 2-propanol completely to CO_2.

Metabolism of diols by *Desulfovibrio* strains involves either an initial oxidation of the primary alcohol group yielding an hydroxyaldehyde, or the dehydration of the diol to an aldehyde. Thus, 1,2-propanediol can be metabolized to acetate with lactaldehyde as a presumed intermediate, or to propionate via propanal (see Hansen, 1994). Oxidation of 1,3-propanediol leads to 3-hydroxypropionate or to acetate production as the major product (Nanninga and Gottschal, 1987; Qatibi et al., 1991; Tanaka, 1990; Tanaka, 1992); oxidation of 1,4-butanediol and 1,5-pentanediol yielded the corresponding hydroxyacids (Tanaka, 1992). Oppenberg and Schink (1990) suggested a pathway involving malonylsemialdehyde for the conversion of 1,3-propanediol to acetate by *Desulfovibrio* strain OttPd1.

Some *Desulfovibrio* species were shown to grow on glycerol (e.g., Stams et al., 1985; Kremer and Hansen, 1987; Nanninga and Gottschal, 1987; Ollivier et al., 1988). In two marine *Desulfovibrio strains*, glycerol is degraded to acetate and CO_2 via glycerol-3-phosphate, dihydroxyacetone phosphate and subsequent reactions known from the glycolytic pathway (Kremer and Hansen, 1987). *Desulfovibrio carbinolicus* oxidizes glycerol to 3-hydroxypropionate (Nanninga and Gottschal, 1987). In the case of *Desulfovibrio fructosovorans*, acetate is the normal product during sulfate reduction, but during syntrophic growth with a methanogenic archaeon as H_2 scavenger, glycerol is oxidized to 3-hydroxypropionate (Qatibi et al., 1998).

Sugars Batch enrichment cultures with sugars commonly select for fermentative bacteria rather than for sulfate reducers, due to faster growth of the former. Nevertheless, some species of sulfate reducers isolated on other substrates were shown to use fructose in the absence or presence of sulfate (Klemps et al., 1985; Ollivier et al., 1988; Zellner et al., 1989a; Trinkerl et al., 1990). *Desulfotomaculum nigrificans* was originally reported to utilize glucose (Campbell et al., 1957; Akagi and Jackson, 1985). However, later growth tests with filter-sterilized sugars revealed that fructose rather than glucose is utilized by this species. When glucose had been autoclaved rather than filter sterilized, growth was observed, indicating partial conversion to a utilizable sugar, probably fructose (Klemps et al., 1985).

Acetone Desulfococcus biacutus (Platen et al., 1990) and *Desulfococcus multivorans* strains other than the type strain (Widdel, 1988) used acetone that was completely oxidized to CO_2. *Desulfobacterium cetonicum* is the other known sulfate-reducing bacterium that can grow with acetone as sole source of carbon and energy (Galushko and Rozanova, 1991). Acetone degradation was shown to depend on CO_2 in cell suspensions of *Desulfococcus biactus*. Enzyme studies with the same microorganism indicated that it metabolized acetone via initial carboxylation to acetoacetyl-CoA. The latter is then thiolytically cleaved to two acetyl-CoA, which are further oxidized to CO_2. The energy gain with acetone as substrate is low because degradation requires carboxylation and activation to acetoacetyl-CoA (Platen et al., 1990; Janssen and Schink, 1995a). Similar results were obtained for acetone metabolism of *Desulfobacterium cetonicum* (Janssen and Schink, 1995b). An ATP-dependent carboxylation of acetone under anaerobic conditions was also demonstrated in cell-free extracts of the photosynthetic bacterium *Rhodobacter capsulatus* (Birks and Kelly, 1997) and other bacteria (Ensign et al., 1998).

Recently, an enrichment culture of sulfate-reducing bacteria was described that could utilize the long-chain ketones hexadecan-2-one and 6,10,14-trimethylpentadecan-2-one. The oxida-

tion of these ketones also involved a carboxylation reaction (Hirschler et al., 1998).

Glycolate Glycolate is a widespread byproduct of autotrophic organisms (e.g. cyanobacteria and algae) in oxic environments with limiting CO_2 concentrations. Ribulose-1,5-bisphosphate carboxylase of the Calvin cycle may incorporate O_2 instead of CO_2 in the substrate, such that one moiety is released as glycolate. It can be oxidized completely to CO_2 by *Desulfofustis glycolicus*, an organism which was isolated from marine sediment (Friedrich et al., 1996). With methylene blue as an electron acceptor, a rather high activity of a membrane-bound glycolate dehydrogenase was detected (Friedrich and Schink, 1995).

Malate, Fumarate, Succinate and Other Dicarboxylic Acids Dicarboxylic acids known from the citric acid cycle are relatively common substrates of incompletely and completely oxidizing sulfate reducers (Postgate, 1984a; Postgate, 1984b; Widdel, 1988). Growth on fumarate and malate is usually faster than on succinate. Some species may utilize only one or two of these compounds because certain transport systems might be limited or lacking. Growth yields of *Desulfovibrio* strains on succinate are far lower than on malate (Kremer et al., 1989). This may be explained by a partial investment of the conserved energy for reverse electron transport from the oxidation of succinate (fumarate/succinate, $E_0' = +0.033$ V).

In various *Desulfovibrio* strains, the C_4-dicarboxylic acids are oxidized via a reaction sequence with an $NADP^+$-dependent malic enzyme (a decarboxylating malate dehydrogenase); the activity was dependent on divalent cations (Mn^{2+} or Mg^{2+}) and stimulated by K^+ (Kremer et al., 1989). The $NADP^+$-dependent malic enzyme of *Desulfovibrio gigas* was found to be a monomeric 45-kDa protein (Chen et al., 1995).

The C_5 and C_7 dicarboxylic acids, glutarate and pimelate, respectively, can serve as substrates for some complete oxidizers (Bak and Widdel, 1986b; Imhoff-Stuckle and Pfennig, 1983; Schnell et al., 1989; Szewzyk and Pfennig, 1987).

Amino Acids Utilization of amino acids as electron donors and carbon sources mainly has been reported for marine species. They include several *Desulfovibrio* strains; alanine utilization seems to be widespread and has been reported for *Desulfotomaculum ruminis* (Coleman, 1960), *Desulfovibrio salexigens* (Zellner et al., 1989a; van Niel et al., 1996), *Desulfovibrio strains* 20020 and 20028 (Stams et al., 1985), *Desulfovibrio acrylicus* (van der Maarel et al., 1996c), and *Desulfovibrio zosterae* (Nielsen et al., 1999). *Desulfocella halophila*, which was isolated from sediment of the Great Salt Lake, is also able to use L-alanine as an electron donor (Brandt et al., 1999). *Desulfovibrio acrylicus* and *Desulfovibrio strains* 20020 and 20028 also have been shown to utilize serine, glycine and cysteine; even other amino acids are used by *Desulfovibrio* strains 20020 and 20028. Several *Desulfobacterium* strains use glutamate (Imhoff-Stuckle and Pfennig, 1983; Bak and Widdel, 1986b; Brysch et al., 1987; Szewzyk and Pfennig, 1987; Heijthuijsen and Hansen, 1989; Schnell et al., 1989; van der Maarel et al., 1996a; Rees et al., 1998). Some other amino acids were also utilized by *Desulfobacterium* strain PM4, the *Desulfobacterium*-like strain WN, and *Desulfobacetrium vacuolatum* (Heijthuijsen and Hansen, 1989; van der Maarel et al., 1996a; Rees et al., 1998). *Desulfovibrio aminophilus*, which was isolated from an anaerobic lagoon of a dairy wastewater plant, degraded six amino acids including alanine in the presence of sulfate (Baena et al., 1998).

L-Alanine was found to be oxidized to pyruvate by an NAD^+-dependent alanine dehydrogenase in *Desulfovibrio* strains 20020 and 20028 and in *Desulfotomaculum ruminis* (Stams and Hansen, 1986).

Furfural Desulfovibrio furfuralis has been isolated with furfural; several other, previously known species of this genus also turned out to use this compound (Folkerts et al., 1989).

On the basis of feeding experiments with ^{13}C-labeled furfural, a reaction sequence was postulated for the breakdown of the substrate in *D. furfuralis* with succinic semialdehyde as a key intermediate (Folkerts et al., 1989); furfuryl alcohol and 2-furoic acid transiently accumulated in the culture supernatants as important intermediates.

Methylated N- and S-compounds (Glycine, Betaine, Dimethylsulfoniopropionate and Dimethylsulfide) Glycine betaine (trimethylglycine) is widely used as an osmolyte in many bacteria (for summary, see Galinski, 1995). Dimethylsulfoniopropionate is an osmolyte in many marine algae.

Growth with glycine betaine as organic substrate has been demonstrated for a number of isolates belonging to the *Desulfobacterium*/*Desulfobacter* cluster of the δ-Proteobacteria; they include *Desulfobacterium autotrophicum*, *Desulfobacterium niacini*, *Desulfobacterium vacuolatum*, a strain named WN which clusters in

between *Desulfobacterium* and *Desulfobacter* (Heijthuijsen and Hansen, 1989; van der Maarel et al., 1996a), and *Desulfospira joergensenii* (Finster et al., 1997a). Glycine betaine (trimethylglycine) was demethylated to dimethylglycine as end product. It was speculated that the oxidation of the methyl group in these strains, which contain CO dehydrogenase, is mediated via the methyl branch of the oxidative C_1-pathway normally used for the oxidation of the methyl moiety of the acetyl group in acetyl-CoA (Heijthuijsen and Hansen, 1989). Most organisms that can oxidize glycine betaine also grow by demethylation of dimethylsulfoniopropionate (DMSP) with 3-methylthiopropionate as the product; *Desulfobacterium autotrophicum* did not grow on DMSP (van der Maarel et al., 1996a). In cell extracts of DMSP-grown strain WN and other strains, a high DMSP:tetrahydrofolate methyltransferase activity was detected (Jansen and Hansen, 1998). Certain sulfate-reducing bacteria have been shown to metabolize DMSP in a different way, namely by cleaving the DMSP to acrylic acid and dimethylsulfide and by reducing the acrylate to propionate (van der Maarel et al., 1996c; see below for a discussion of acrylate reduction).

Dimethylsulfide is a widespread degradation product of dimethylsulfoniopropionate in marine environments. As a trace gas in the atmosphere, dimethylsulfide leads to sulfuric acid that forms condensation nuclei for water and thus influences cloud formation. Dimethylsulfide oxidation by mesophilic sulfate-reducing bacteria from marine sediments was inferred from experiments with labeled substrates and inhibitor studies (Kiene et al., 1986). Oxidation of dimethylsulfide by a thermophilic *Desulfotomaculum* strain has been reported (Tanimoto and Bak, 1994) but utilization of dimethylsulfide by pure cultures of mesophilic sulfate reducers remains to be demonstrated.

Polar Aromatic Compounds (Non-Hydrocarbons) The utilization of various nonfermentable aromatic compounds in the absence of O_2 or nitrate seems to be one of the domains of sulfate-reducing bacteria. In contrast, aromatic compounds with more than two hydroxyl groups (e.g. gallic acid, pyrogallol or phloroglucinol) are readily degraded by fermentative bacteria (Schink and Pfennig, 1982; Schink, 1988a; Schink, 1988b). Several new types of sulfate-reducing bacteria have been directly isolated with aromatic compounds (Widdel, 1980; Imhoff-Stuckle and Pfennig, 1983; Widdel et al., 1983; Bak and Widdel, 1987; Szewzyk and Pfennig, 1987; Schnell et al., 1989; Kuever et al., 1993; Gorny and Schink, 1994). Most of these isolates are very versatile sulfate reducers that also use many aliphatic compounds. Benzoate is the most commonly and most readily utilized aromatic substrate. Representatives of other classes of aromatic compounds oxidized by sulfate reducers are phenol, *p*-cresol (Bak and Widdel, 1986b), aniline (Schnell et al., 1989), and the N-heterocyclic compounds nicotinate (Imhoff-Stuckle and Pfennig, 1983), indole and quinoline (Bak and Widdel, 1986a). An overview of non-hydrocarbon aromatic substrates utilized by pure cultures of sulfate-reducing bacteria is presented in Table 5.

So far, most sulfate-reducing bacteria that degrade aromatic compounds are complete oxidizers. The only known exception is *Desulfovibrio inopinatus*, which degrades the relatively oxidized compound hydroxyhydroquinone (1,2,4-trihydroxybenzene) incompletely to acetate (Reichenbecher and Schink, 1997). Little is known about reactions at the aromatic ring in sulfate-reducing bacteria. Aerobic bacteria employ oxygenases which require O_2 (as co-substrate) to activate (hydroxylate) and cleave the aromatic ring. The pathways in the anaerobic sulfate-reducing bacteria are therefore expected to be completely different from those of aerobic bacteria and to involve novel biochemical reactions. Most insights into the degradative pathways of aromatic compounds under anoxic conditions were obtained from studies with denitrifying and phototrophic bacteria (for overview see e.g., Berry et al., 1987; Evans and Fuchs, 1988; Tschech, 1989a; Heider and Fuchs, 1997a; Heider and Fuchs, 1997b; Harwood et al., 1999). Important principles of aromatic compound degradation recognized in the nonsulfate-reducing bacteria are that the degradative pathways can be classified into three categories. First, many aromatic compounds are converted via so-called peripheral reactions to a central intermediate, benzoyl-CoA. Second, a central sequence of reactions abolishes aromaticity of benzoyl-CoA and leads to ring cleavage. Third, certain polyhydroxybenzoates or polyhydroxybenzenes undergo reactions that lead to aliphatic intermediates without the involvement of benzoyl-CoA.

Examples of peripheral reactions are phosphorylation/carboxylation to convert phenol to *p*-hydroxybenzoate (Knoll and Winter, 1989; Tschech and Fuchs, 1989b; Lack and Fuchs, 1992; Lack and Fuchs, 1994), the involvement of an α-oxidation reaction in the conversion of phenylacetate to benzoyl-CoA (Mohamed et al., 1993), the reductive removal of hydroxyl groups (Tschech and Schink, 1986; Gibson et al., 1997). Free benzoate is simply activated to benzoyl-CoA in an ATP-consuming reaction (Geissler et al., 1988; Altenschmidt et al., 1991). Some

Table 5. Sulfate-reducing bacteria with the capacitiy to use aromatic compounds as growth substrates.

Organism	Aromatic substrate[a]	Reference
Non-hydrocarbon aromatic compounds		
Desulfonema magnum	Benzoate, 4-hydroxybenzoate, phenylacetate, 3-phenylpropionate, hippurate	Widdel et al., 1983
Desulfococcus niacim[b]	Nicotinic acid, 3-phenylpropionate	Imhoff-Stuckle and Pfennig, 1983
Desulfobacterium indolicum[c]	Indole, 2-aminobenzoate, quinoline	Bak and Widdel, 1986a
Desulfobacterium phenolicum[d]	Phenol, *p*-cresol, benzoate, phenylacetate, indole, 4-hydroxyphenylacetate, 2-hydroxybenzoate, 4-hydroxybenzoate, phenylalanine, 2-aminobenzoate	Bak and Widdel, 1986b
Desulfobacterium catecholicum[e]	Catechol, resorcinol, 4-hydroxybenzoate, hydroquinone, benzoate 2-aminobenzoate, protocatechuate, phloroglucinol, pyrogallol	Szewzyk and Pfennig, 1987
Desulfobacterium anilini[c]	Aniline, 2-aminobenzoate, 4-aminobenzoate, indolylacetate, quinoline	Schnell et al., 1989
strain Cat2[c]	Phenol, catechol, *m*-cresol, *p*-cresol, benzoate, phenylacetate, phenylpropionate 4-hydroxybenzoate, 3,4-dihydroxybenzoate, phenylalanine	Schnell et al., 1989
strain SAX[c]	Benzoate, *p*-hydroxybenzoate, phenol, phenylacetate, phenylalanine	Drzyzga et al., 1993
Desulfotomaculum strain Groll[f]	Catechol, phenol, *m*-cresol,, *p*-cresol, benzoate, 3-hydroxybenzoate, benzaldehyde benzyl alcohol, phenylacetate, phenylpropionate	Kuever et al., 1993
Desulfovibrio inopinatus	Hydroxyhydroquinone (1,2,4-trihydroxybenzene)	Reichenbecher and Schink, 1997
Aromatic hydrocarbons		
"Desulfobacula toluolica"	Toluene, *p*-cresol, benzaldehyde, benzoate, phenylacetate, *p*-hydroxybenzoate *p*-hydroxybenzaldehyde	Rabus et al., 1993
strain PRTOL1[g]	Toluene, *p*-cresol, benzaldehyde, benzoate, phenylacetate, phenylpropionate *p*-hydroxybenzoate	Beller et al., 1996
strain mXyS1[c]	Toluene, *m*-xylene, *m*-ethyltoluene, *m*-isopropyltoluene, benzoate, *m*-methylbenzoate	Harms et al., 1999
strain oXyS1[h]	Toluene, *o*-xylene, *o*-ethyltoluene, *o*-methylbenzyl alcohol benzoate, *o*-methylbenzoate, benzylsuccinate	Harms et al., 1999
strain NaphS2[h]	Naphthaline, 2-naphthoate, benzoate	Galushko et al., 1999

[a]Information about additional aromatic substrates is provided in the respective references.
[b]Has to be reclassified as "*Desulfobacterium niacim*" (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[c]Has to be classified/reclassified as new genus (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[d]Has to be reclassified as "*Desulfobacula phenolicum*" (J. Kuever, F. A. Rainey and F. Wuddel, personal communication).
[e]Has to reclassified (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[f]Has been classified as *Desulfotomaculum gibsoniae* (Kuever et al., 1999).
[g]Has to be classified as a new species of the genus *Desulforhabdus* (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[h]Has to be classfied as a new species of the genus *Desulfosarcina* (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[i]Strain NaphS2 affiliates closely with strain mXyS1 (Galushko et al., 1999) and will therefore be classified into the same new genus (J. Kuever, F. A. Rainey and F. Widdel, personal communication).

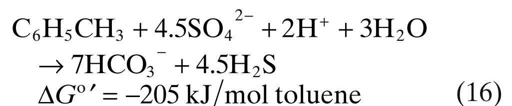
peripheral reactions for the degradation of aromatic compounds also have been suggested thus far in sulfate-reducing bacteria. Degradation of aniline by *Desulfobacterium anilini* is initiated by a carboxylation probably yielding 4-aminobenzoate, which via ligation with acetyl-CoA and reductive deamination is supposed to yield benzoyl-CoA (Schnell and Schink, 1991). *p*-Cresol was first suggested to be converted to *p*-hydroxybenzyl alcohol by an anaerobic *p*-cresol methylhydroxylase (McIntire et al., 1985; Suflita et al., 1989); further oxidation to the corresponding aldehyde and acid, ligation with coenzyme A and reductive dehydroxylation (or vice versa) could yield benzoyl-CoA. Such a pathway would be in agreement with the ability of *p*-cresol-utilizing sulfate reducers to grow with benzoate. Furthermore, an anaerobic degradation of *m*-cresol by *Desulfotomaculum* strain (Groll) is proposed to proceed via a methyl-group oxidation to 3-hydroxybenzoate because the latter compound was detected in *m*-cresol-

degrading cultures (Londry et al., 1997). However, in the light of recent findings about the anaerobic activation of toluene by methyl condensation with fumarate (see Aromatic Hydrocarbons) methyl hydroxylation reactions may be questioned and reactions analogous to toluene activation may be assumed. Indeed, an activation of *m*-cresol by a fumarate-dependent reaction to 3-hydroxybenzylsuccinate was demonstrated in cell-free extracts of *Desulfobacterium cetonicum* (Müller et al., 1999). Catechol degradation by *Desulfobacterium* strain Cat2 was proposed to be initiated by a carboxylation to protocatechuate, because high activities of a protocatechuate decarboxylase and low activities of an ATP/HCO_3^--dependent protocatechuyl-CoA-forming enzyme synthetase could be measured in extracts of catechol grown cells. Further degradation to benzoyl-CoA would involve reductive dehydroxylation reactions (Gorny and Schink, 1994).

The further metabolism of benzoyl-CoA has been studied most intensely in denitrifying *Thauera aromatica* strain K172. The stable aromatic state is abolished by benzoyl-CoA reductase. This novel enzyme contains FAD as prosthetic group and uses ferredoxin as the natural electron donor. It requires two ATP to generate and transfer two electrons into the ring of benzoyl-CoA to yield cyclohexa-1,5-diene-1-carboxyl-CoA (Boll and Fuchs, 1995; Boll and Fuchs, 1998); the first electron has to have an extremely negative redox potential. Different enzymes have been measured and isolated from *Thauera aromatica* (Laempe et al., 1998) and *Rhodopseudomonas palustris* (Perrotta and Harwood, 1994; Pelletier and Harwood, 1998) that are involved in further reduction and cleavage of the ring structure to yield the open chain pimelyl-CoA, which can be further degraded to acetyl-CoA via reactions such as β-oxidation. Thus, somewhat different pathways for benzoate degradation are employed by these two organisms (Harwood and Gibson, 1997; Harwood et al., 1999), suggesting that variations of pathways exist for the anaerobic degradation of benzoate. Considering the high energy requirement of anaerobic benzoate degradation in the aforementioned microorganisms, it appears rather unlikely that sulfate-reducing bacteria with their relatively low ATP yield employ the same reactions for ring reduction. *Desulfococcus multivorans* required selenite in addition to molybdate for the degradation of benzoate, but not for growth on aliphatic substrates (Widdel, 1980). However, neither the role of these trace elements in *Desulfococcus* nor the pathway of benzoate degradation is known.

Heterocyclic aromatic compounds utilized by sulfate-reducing bacteria are nicotinate (Imhoff-Stuckle and Pfennig, 1983), indole and quinoline (Bak and Widdel, 1986a). *Desulfobacterium niacini* requires traces of selenite for the oxidation of nicotinate (Imhoff-Stuckle and Pfennig, 1983), as *Clostridium barkeri* does for fermentation of this compound. In the latter, the degradation of nicotinic acid is initiated by a conversion to 6-hydroxynicotinate via nicotinate dehydrogenase, which is probably a selenoenzyme (Imhoff and Andreesen, 1979). Nicotinate dehydrogenase, also detected in *Desulfobacterium niacini* (W. Buckel, personal communication), may explain the selenium requirement.

Aromatic Hydrocarbons Aromatic hydrocarbons as apolar molecules are biochemically less reactive than their aromatic counterparts carrying functional groups. Degradation of aromatic hydrocarbons under anaerobic conditions was long considered to be impossible. However, studies with anaerobic sediment and enrichment cultures of mixed methanogenic cultures (Gribc-Galic and Vogel, 1987), denitrifying (Kuhn et al., 1988) and sulfate-reducing bacteria (Edwards et al., 1992) demonstrated that aromatic hydrocarbons such as toluene were indeed degradable under anoxic conditions. The first pure cultures that could anaerobically degrade toluene were obtained under denitrifying (Dolfing et al., 1990; Altenschmidt and Fuchs, 1991; Evans et al., 1991; Schocher et al., 1991) and ferric-iron reducing (Lovley et al., 1990) conditions. The first pure culture of a toluene-degrading sulfate-reducing bacterium, "*Desulfobacula toluolica*," was isolated from marine sediment (Rabus et al., 1993). This new isolate oxidized toluene completely to CO_2 according to equation (16), as demonstrated by measurement of the degradation balance. Another toluene-degrading sulfate reducer, strain PRTOL1, was isolated from fuel-contaminated subsurface soil (Beller et al., 1996).



$$C_6H_5CH_3 + 4.5SO_4^{2-} + 2H^+ + 3H_2O \rightarrow 7HCO_3^- + 4.5H_2S \quad \Delta G^{o\prime} = -205\ \text{kJ/mol toluene} \quad (16)$$

A marine enrichment culture that grew anaerobically on crude oil with concomitant sulfate reduction to sulfide (Rueter et al., 1994) was the source for the isolation of the *o*-xylene-degrading strain oXyS1 and the *m*-xylene-degrading strain mXyS1 (Harms et al., 1999). Both strains also used toluene for growth by sulfate reduction. Furthermore, strain oXyS1 oxidized *o*-ethyltoluene, and strain mXyS1 oxidized *m*-ethyltoluene and *m*-isopropyltoluene anaerobically. Sulfate-reducing strain NaphS2 was isolated as the first pure culture which can

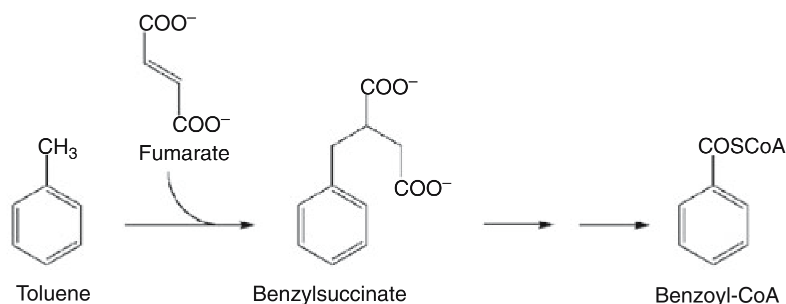


Fig. 16. Anaerobic, fumarate-dependent activation of toluene to benzylsuccinate in *Desulfobacula toluolica* and strain PRTOL1. Further degradation of benzylsuccinate to the central intermediate benzoyl-CoA is not completely understood.

utilize the bicyclic aromatic hydrocarbon naphthalene (Galushko et al., 1999). Anaerobic degradation of other aromatic hydrocarbons with sulfate as electron acceptor has been demonstrated in enriched sediment communities, but not so far in pure cultures. These hydrocarbons are benzene (Lovley and Phillips, 1995b; Phelps et al., 1996) and the polyaromatic hydrocarbons phenanthrene and fluorene (Coates et al., 1997). Oxidation was shown by the formation of $^{14}CO_2$ from the ^{14}C-labeled hydrocarbon substrates.

The best known anaerobic pathway of an aromatic hydrocarbon is that of toluene. Understanding of anaerobic toluene metabolism has greatly benefited from studies with denitrifying bacteria. Benzylsuccinate, first identified as a metabolite in toluene-grown cultures of a denitrifyer (Evans et al., 1992), a sulfate-reducing enrichment culture (Beller et al., 1992) and *Desulfobacula toluolica* (Rabus and Widdel, 1995), was shown to be the initial activation product in denitrifying bacteria (Biegert et al., 1996; Beller and Spormann, 1997a). It was formed from toluene and fumarate. Fumarate-dependent formation of benzylsuccinate from toluene was subsequently reported with permeabilized cells of sulfate-reducing strain PRTOL1 (Beller and Spormann, 1997b) and in cell-free extracts of *Desulfobacula toluolica* (Rabus and Heider, 1998). The further demonstration of benzylsuccinate formation in a toluene-utilizing phototroph that is unrelated to denitrifying or sulfate-reducing bacteria (Zengler et al., 1999b) suggests that this is a general anaerobic activation mechanism for toluene, a naturally widespread trace hydrocarbon (Heider et al., 1999). Genetic analysis of genes underlying the benzylsuccinate-forming enzyme (benzylsuccinate synthase) indicates that this is a glycyl radical enzyme (Coschigano et al., 1998; Leuthner et al., 1998); the radical is supposed to attack the methyl group of toluene yielding a benzyl radical which then combines with fumarate (Fig. 16). Further degradation of benzylsuccinate is proposed to proceed via reactions analogous to β-oxidation of α-methyl-branched fatty acids and to yield benzoyl-CoA as a central intermediate. In agreement with this, toluene-utilizing sulfate-reducing bacteria can also grow on benzoate.

Hints as to the initial reaction in anaerobic degradation of naphthalene were obtained from enriched sediment communities under sulfate-reducing conditions. The finding of 2-naphthoate (naphthalene-2-carboxylate) suggested a carboxylation as the initial activation of the bicyclic aromatic hydrocarbon (Zhang and Young, 1997). In agreement with this, naphthalene-degrading strain NaphS2 is able to grow on 2-naphthoate, but not on 1-naphthoate (Galushko et al., 1999). A different initial mechanism of anaerobic napthalene degradation was suggested in a study of freshwater microcosms under conditions of sulfate reduction; in these communities, a naphthol (isomer unknown) was detected as a possible intermediate (Bedessem et al., 1997).

Saturated Hydrocarbons Saturated hydrocarbons (n-alkanes, branched-chain alkanes and cycloalkanes) are the chemically least reactive organic compounds. The chemical recalcitrance is explained by the exclusive presence of apolar s bonds. Because of these structural properties and the fact that aerobic bacteria initiate alkane activation always with O_2 as co-substrate (monoxygenase reaction), the possibility of an anaerobic alkane oxidation has often been doubted. Nevertheless, evidence for the anaerobic oxidation of alkanes in enriched microbial communities and pure cultures has been repeatedly provided.

In the 1940s, enrichment cultures and pure cultures of *Desulfovibrio* strains were reported to grow or to reduce sulfate with long-chain alkanes (Novelli and ZoBell, 1944; Rosenfeld, 1947). The techniques available at that time to guarantee strictly anoxic conditions in the experiments were not described in detail. The cultures have not been preserved. In experiments with suspensions of other *Desulfovibrio* species, sulfate reduction was stimulated by octadecane and a

Table 6. Sulfate-reducing bacteria with the capacitiy to use aliphatic hydrocarbons as growth substrates.

		Aliphatic hydrocarbon utilized[a]		
Organism	Optimum temperature (°C)	*n*-Alkanes	1-Alkenes	Reference
Hxd3[b]	28–30	C_{12}–C_{20}	C_{14}, C_{16}, C_{18}	Aeckersberg et al., 1991
Pnd3[c]	30	C_{14}–C_{17}	C_{14}, C_{16}, C_{18}	Aeckersberg et al., 1994, 1998
TD3c[d]	55–65	C_6–C_{16}, 3-methyloctane		Rueter et al., 1994 Ehrenreich 1996
AK01[e]	26–28	C_{13}–C_{18}	C_{15}, C_{16}	So and Young, 1999a

[a]So far tested; more detailed information on growth substrates can be obtained from the respective references.
[b]Has tentatively been classified as "*Desulfobacterium oleovorans*" (Aeckersberg et al., 1991). The genus name has to be reclassified (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[c]Has to be classified as a new genus (J. Kuever, F. A. Rainey and F. Widdel, personal communication).
[d]Has been tentatively classified as "*Desulfothermus naphthae*" (Ehrenreich, 1996).
[e]Has to be classified into the same new genus as strain Hxd3 (J. Kuever, F. A. Rainey and F. Widdel, personal communication).

small part (around 0.4%) of ^{14}C-labeled alkane was recovered as CO_2 (Davis and Yarbrough, 1966). The possibility of anaerobic alkane oxidation with sulfate was again examined in connection with a study of sulfate reducers in oil fields. A sulfate-reducing bacterium that nutritionally and morphologically differed from *Desulfovibrio* was isolated with hexadecane (Aeckersberg et al., 1991). Quantitative degradation experiments in anoxic, fused glass (air-excluded) ampullas showed that up to ca. 90% of the added hexadecane was oxidized with sulfate. A control experiment with a *Desulfovibrio* strain did not reveal alkane utilization. Three other pure cultures of alkane-degrading sulfate-reducing bacteria, two mesophilic strains (Aeckersberg et al., 1998; So and Young, 1999a) and a thermophilic strain (Rueter et al., 1994) were subsequently described. The range of alkanes utilized by these isolates and other characteristics are summarized in Table 6. Anaerobic utilization of various long-chain *n*-alkanes was also observed with enriched communities in marine sediment under conditions of sulfate reduction (Caldwell et al., 1998).

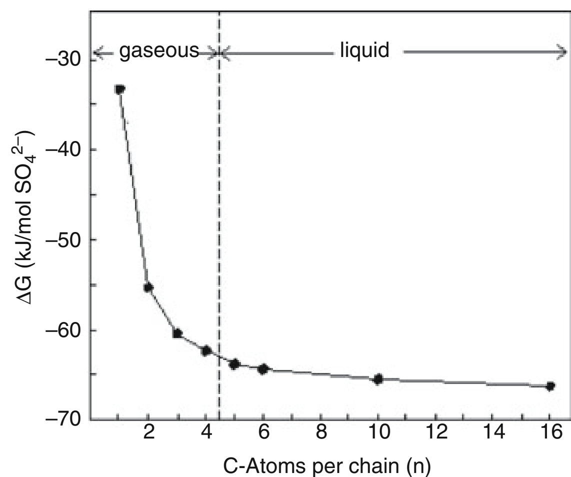


Fig. 17. Free energy change of sulfate reduction with *n*-alkanes of various chain lengths (methane through hexadecane) at 25°C, pH = 7, SO_4^{2-} and HCO_3^- concentrations = 10^{-2} M, and HS^- concentration = 10^{-3} M. Individual stoichiometric equations are derived from $CnHn+_2 + (3n+1)/4\ SO_4^{2-} \rightarrow n\ HCO_3^- + (3n+1)/4\ HS^- + (n-1)/4\ H^+ + H_2O$, with n being the number of carbon atoms per chain. Free energy values were calculated from data given by Dean (1992); Thauer et al. (1977) and Zengler et al. (1999a).

The biochemical problem in anaerobic alkane degradation is the first step, the activation of an apolar molecule, rather than in the free energy change of the overall reaction. With the exception of methane oxidation (see next section), the amount of free energy per mol of sulfate reduced with alkanes (see Fig. 17) is comparable to that available from acetate or propionate oxidation. The activation has to start with a cleavage of a C-H bond that is not activated. A resulting alkyl radical would not have the possibility for stabilization by delocalization, as in the case of an aryl radical (e.g., benzyl radical; see preceding section). Studies on changes in the cellular fatty acid composition in response to the growth substrate provided first hints at possible activation reactions of alkanes in a sulfate-reducing bacterium, strain Hxd3 (Aeckersberg et al., 1998). If cells were grown on hexadecane ($C_{16}H_{34}$), the chains of cellular fatty acids were mainly C-odd. Conversely, cells grown on heptadecane ($C_{17}H_{36}$) contained mainly C-even fatty acids in the lipid fraction. It was concluded from these results that the alkane chain was altered by a C_1-unit during activation, possibly by the terminal addition of a C_1-compound. However, with a second, phylogenetically related alkane-degrading sulfate reducer, strain Pnd3, a C-even alkane yielded C-even fatty acids, and a C-odd alkane yielded C-odd fatty acids. Assuming a mechanism principally as in strain Hxd3, activation at the second carbon atom was proposed as one possible explanation for the findings with strain Pnd3.

Also, addition to fumarate was discussed as hypothetical activation mechanism, which could differ from toluene activation by a lack of radical stabilization in the substrate molecule (Aeckersberg et al., 1998). Chemical analysis with a third alkane-degrading sulfate reducer, strain AK-01, yielded methyl-branched cellular fatty acids resulting from the *n*-alkane provided as substrate (So and Young, 1999b). Labeling studies suggested that a carbon compound, which is not derived from bicarbonate, is subterminally added to the alkane such that the terminal methyl group of the *n*-alkane becomes a methyl branch in the fatty acid formed via subsequent reactions.

Methane Methane, the only existent stable C_1-hydrocarbon, can be regarded as the first member of the homologous series of alkanes. It is chemically even somewhat more stable than higher alkanes. Methane is formed as an end product of anaerobic degradation processes involving methanogenic archaea in sediments that are depleted of electron acceptors other than CO_2. Because of the important role of methane in the carbon cycle in aquatic habitats and on a global scale, the possibility of an anaerobic oxidation of this hydrocarbon has been frequently investigated. In anoxic marine habitats, sulfate would be the most important terminal electron acceptor for anaerobic methane oxidation.

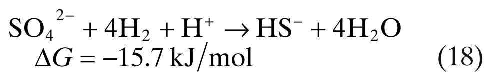
Hints on an anaerobic methane oxidation came mostly from biogeochemical investigations in marine sediments. Geochemical evidence is based on three different observations. First, methane in marine habitats often disappears far below the oxic zone, and the depth profile of the methane concentration exhibits a concave-up curvature, which indicates a methane sink (Devohl and Ahmed, 1981; Reeburgh, 1976; Barnes and Goldberg, 1976; Martens and Berner, 1977; Alperin and Reeburgh, 1984); an increase ("second maximum") of the sulfate reduction rate in the depth profile was observed to coincide with the zone of anaerobic methane depletion (Alperin and Reeburgh, 1985; Iversen and Jørgensen, 1985; Reeburgh and Alperin, 1988; Hansen et al., 1998). Second, $^{13}C/^{12}C$ analyses are in favor of an anaerobic methane oxidation. Residual methane in the zone of its anaerobic depletion is ^{13}C-enriched (and ^{2}H-enriched), indicating a biochemical consumption reaction (Alperin et al., 1988). In addition, inorganic carbon (CO_2, HCO_3^-, CO_3^{2-}) in the zone of methane depletion was shown to be relatively poor in ^{13}C (Reeburgh, 1980; Reeburgh and Alperin, 1988; Blair and Aller, 1995); this finding suggested that oxidation of isotopically light methane added to the signature of the isotopically heavier background of inorganic carbon. Third, after addition of ^{14}C-labeled methane to anoxic marine sediment cores or slurries, formation of radioactive CO_2 could be measured (Reeburgh, 1980; Iversen and Blackburn, 1981; Alperin and Reeburgh, 1984; Alperin and Reeburgh, 1985; Iversen and Jørgensen, 1985; Hansen et al., 1998). The rates of anaerobic methane oxidation calculated from data of the biogeochemical investigations were always rather low; they ranged between 1 and 67 μmol $\cdot$liter^{-1} $\cdot$day^{-1}, or were even lower. However, at a gas seep, volumetric sulfate reduction rates as high as 2.5 mmol $\cdot$liter^{-1} $\cdot$day^{-1} were attributed to methane as the electron donor (Aharon and Baoshun, 2000); this implies that methane oxidation at this site has the same rate.

An organism that can consume methane anaerobically has not been enriched and isolated thus far. A partial conversion of $^{14}CH_4$ to $^{14}CO_2$ during methanogenesis but no net oxidation of methane has been measured in cultures of methanogenic archaea (Zehnder and Brock, 1979; Zehnder and Brock, 1980), which provided the first hint of a "reverse methanogenesis". Because biologically produced methane, which is usually used for labeling experiments, may contain traces of CO as a by-product, ^{14}C-methane was purified from this by-product and applied to active methanogenic bacteria (Harder, 1997). Again, a partial oxidation of methane without net consumption was demonstrated. The reaction was not detectable in cultures of sulfate-reducing and homoacetogenic bacteria.

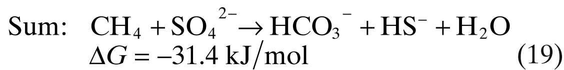
The assumption that anaerobic oxidation of methane is a reversed methanogenesis and catalyzed by methanogenic archaea (or at least by a phylogenetically closely related group) is supported by microbiological in situ analysis of bacterial populations on the basis of biomarkers and 16S rRNA gene sequences. Special isoprene lipids and hydrocarbons such as crocetane (2,6,11,15-tetramethylhexadecane) that occurred in the zone of methane depletion and exhibited an unusually low $^{13}C/^{12}C$-ratio were assumed to belong to the methane-utilizing anaerobes (Elvert and Suess, 1999; Hinrichs et al., 1999); also retrieved 16S rRNA gene sequences forming a distinctive cluster within the *Methanosarcinales* were tentatively assigned to these microorganisms (Hinrichs et al., 1999). From these and earlier studies (Hoehler et al., 1994; Hansen et al., 1998), it was concluded that methane is not directly utilized by sulfate-reducing bacteria, but rather by a group of archaea (eventually identified as methanogens)

that convert methane in a "reversed methanogenesis" to CO_2 and an intermediate, possibly H_2; the latter is then scavenged and kept at low concentration by the activity of sulfate-reducing bacteria. The free energy yield from anaerobic methane oxidation with sulfate near natural concentrations is relatively low ($\Delta G = -33$ kJ/mol sulfate). This amount would have to be shared between two partners (Fig. 11). Assuming an equal share of the free energy with H_2 as the intermediate (conditions see below following equations), the partial pressure of the latter would have to be around 0.12 Pa (corresponding to: $0.9 \cdot 10^{-9}$ M dissolved H_2; $E' = -0.269$ V at pH 7.5) to render methane oxidation thermodynamically feasible.

$$CH_4 + 3H_2O \rightarrow HCO_3^- + 4H_2 + H^+ \quad \Delta G = -15.7\ \text{kJ/mol} \qquad (17)$$



$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O \quad \Delta G = -15.7\ \text{kJ/mol} \qquad (18)$$



$$\text{Sum:}\ CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \Delta G = -31.4\ \text{kJ/mol} \qquad (19)$$

(calculated for 25°C; pH = 7.5; CH_4 partial pressure = 10^5 Pa; H_2 partial pressure = 0.12 Pa; SO_4^{2-} concentration = $2 \cdot 10^{-2}$ M; HCO_3^- concentration = 10^{-2} M; HS^- concentration = $2 \cdot 10^{-3}$ M; activity coefficients of SO_4^{2-}, HCO_3- and HS^- in seawater of 0.1, 0.5 and 0.5, respectively; data for calculation from Stumm and Morgan, 1981, and Thauer et al., 1989b).

Measurement of H_2 at partial pressures in the indicated range is technically possible. However, because the partial pressure is the result of a dynamic equilibrium between production and consumption, sampling procedures that affect substrate availability are expected to have a significant influence on the H_2 partial pressure. Hydrogen partial pressures reported for conditions of sulfate reduction were 5 Pa in marine sediment (Sørensen et al., 1981), 0.17 Pa in sulfate-amended lake sediment (Lovley et al., 1982), and between 0.05 and 0.4 Pa in the anoxic seawater of Cariaco Trench (Scranton et al., 1984). Hence, the partial pressures determined in the latter samples would be roughly in the range required if anaerobic methane oxidation occurred via free H_2.

Sulfate reduction at the calculated very low H_2 concentrations is expected to be very slow, even if sulfate-reducing bacteria are closely associated with the H_2-producing partners. With the most favorable kinetic parameters reported for cells of sulfate-reducing bacteria, viz. a maximum rate (V_{max}) of 90 mol H_2 $g^{-1} \cdot h^{-1}$ (see Overview of Principal Properties, Sulfate-Reducing Bacteria and Archaea in this Chapter) and a half-saturation constant (K_M) of $0.7 \cdot 10^{-6}$ mol $\times$ H_2 l^{-1} (Widdel, 1988), the specific rate (related to cell dry mass) of H_2 oxidation would be 0.12 mol $\times$ $g^{-1} \cdot h^{-1}$; hence the rate of sulfate reduction or methane oxidation would be 0.03 mol $g^{-1} \cdot h^{-1}$. (The rate at substrate concentrations K_M is calculated by multiplication of the first-order rate constant, V_{max}/K_M, with the substrate concentration.) Since members of the *Methanosarcinales* are metabolically versatile, also a transfer of metabolites other than H_2 may be assumed. However, organic compounds known as methanogenic substrates would require concentrations even lower than that of hydrogen to allow reverse methanogenesis and an approximately equal energy share of both partners (acetate, $3 \cdot 10^{-11}$ M; concentrations of methanol and methylsulfide even lower). Hydrogen or electron carriers with midpoint potentials close to the redox potential calculated above (–0.269 V) would allow kinetically more favorable concentrations for a transfer of their oxidized and reduced forms between the partners.

Special Inorganic Electron Donors (Other than H_2) An economically important inorganic electron donor for sulfate-reducing bacteria is metallic iron. Oxidation of metallic iron with sulfate as electron acceptor is regarded as the principal reaction in anaerobic corrosion (Hamilton, 1985; Postgate, 1984c; Sequeira and Tiller, 1988; Von Wolzogen Kuhr and van der Vlught, 1934; Widdel, 1992). Anaerobic corrosion, a process with significant economic impact, has been frequently observed to cause pitting and destruction of pipelines and other iron and steel constructions exposed to sulfate-containing, oxygen-depleted waters. Because of the negative redox potential (Fe^{2+}/Fe, $E_0 = -0.44$ V; even more negative in carbonate-rich or sulfidic medium), iron can liberate H_2 ($2H^+/H_2$, $E_0' = -0.41$ V) in aqueous surroundings (according to $2\ Fe + 2\ H^+ \rightarrow 2\ Fe^{2+} + H_2$) and may in this way indirectly act as an electron donor for sulfate-reducing bacteria that possess hydrogenase (Cord-Ruwisch and Widdel, 1986). However, a direct utilization of electrons (liberated according to $Fe \rightarrow Fe^{2+} + 2\ e^-$) by cells associated with the iron surface and involving redox proteins at the cell surface (outer membrane) has been discussed as another mechanism in anaerobic corrosion (Van Ommen Kloeke et al., 1995; Widdel, 1992). Such a direct withdrawal of electrons may be kinetically more favorable than consumption of the electrochemically formed H_2.

Another inorganic, unique electron donor for dissimilatory sulfate reduction is phosphite

($H_2PO_3^-$), that has been used for the enrichment and isolation of a novel type of sulfate-reducing bacterium (Schink and Friedrich, 2000). The isolate is phylogenetically related to *Desulfobacula* and *Desulfospira*. Phosphite was oxidized to phosphate ($H_2PO_4^-$). The natural role of this capacity is unknown. The occurrence of a "dissimilatory phosphate reduction" in natural habitats as a source of reduced phosphorous compounds is very unlikely, because the redox potentials of the reduction steps of phosphorus (ranging from +V to –III) are extremely low (E_0' lower than –0.48 V; Schink and Friedrich, 2000; Widdel, 1992). Reduction of H+ to H_2 would be easier to achieve (see redox potential above).

Fermentative and Syntrophic Growth in the Absence of Sulfate In the absence of sulfate or other inorganic electron acceptors, several types of sulfate reducers can grow by fermentation of several organic substrates. Some *Desulfovibrio* species (for overview see Widdel, 1988), *Desulfobacterium* species (Brysch et al., 1987) and *Desulfosarcina variabilis* (Widdel, 1980) ferment fumarate and, with the exception of the latter species, malate; the fermentation products are succinate, acetate, CO_2, and sometimes propionate.

Pyruvate is easily fermented by many sulfate-reducing bacteria that can use lactate. Pyruvate fermentation by *Desulfovibrio desulfuricans* produces acetate, CO_2 and H_2 (e.g., Postgate, 1984a; Stams et al., 1985). In *Desulfovibrio sapovorans*, which also ferments pyruvate but does not possess a hydrogenase (Widdel, 1980), lactate, acetate and CO_2 are the expected fermentation products.

Lactate or ethanol plus CO_2 allow fermentative growth of some *Desulfobulbus* strains that form propionate and acetate (Laanbroek et al., 1982; Widdel and Pfennig, 1982). Propionate is formed in *Desulfobulbus* via a randomizing pathway involving a methylmalonyl-CoA:pyruvate transcarboxylase and free succinate as a symmetric molecule (Stams et al., 1984). This pathway is very similar to the one used by Propionibacterium except that the activation of succinate to succinyl-CoA is not directly linked to the formation of propionate from propionyl-CoA. The succinate pathway in the inverse direction also is used for the oxidation of propionate to acetate and CO_2 in the presence of sulfate (see Propionate in this Chapter; Kremer and Hansen, 1988a; Fig. 15).

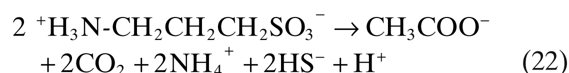
Desulfovibrio desulfuricans can ferment choline to trimethylamine, ethanol, and acetate (e.g., Fiebig and Gottschalk, 1983). *Desulfovibrio fructosovorans* and *Desulfotomaculum nigrificans* fermented fructose in the absence of sulfate (Klemps et al., 1985; Ollivier et al., 1988). The former was shown to form succinate, acetate and ethanol.

Furthermore, a fermentation of cysteine with liberation of sulfide and ammonia has been reported for a sulfate reducer, probably a *Desulfovibrio* strain (Senez and Leroux-Gilleron, 1954b).

Desulfotosporosinus orientis grew slowly by converting formate, methanol, or the methyl groups of 3,4,5-trimethoxybenzoate via a homoacetogenic metabolism to acetate (Klemps et al., 1985). Lactate was fermented by this species to acetate as the only organic product, which is in agreement with the observed de novo acetate formation as it occurs in homoacetogenic bacteria. Also in *Desulfobacterium* species, fermentation of lactate and malate yielded an acetate to substrate ratio that can only be explained by an additional de novo synthesis of acetate from reducing equivalents and CO_2 (Brysch et al, 1987; F. Widdel, unpublished observation).

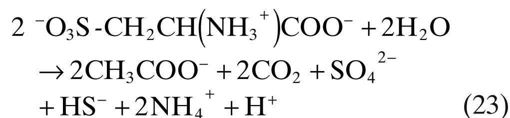
Desulfovibrio carbinolicus and *Desulfovibrio fructosovorans* ferment glycerol to 1,3-propanediol and 3-hydroxypropionate (Nanninga and Gottschal, 1987; Ollivier et al., 1988).

The marine sulfate-reducing bacterium *Desulforhopalus singaporensis* was isolated from an anaerobic enrichment culture with taurine (2-aminoethanesulfonate) as the only source of carbon, energy, and nitrogen (Lie et al., 1999). The degradation of taurine, that includes a reduction of the oxidized sulfur, could be described by the following equation (22):



$$2\ {}^{+}H_3N\text{-}CH_2CH_2CH_2SO_3^{-} \rightarrow CH_3COO^{-} + 2CO_2 + 2NH_4^{+} + 2HS^{-} + H^{+} \quad (22)$$

Another sulfonate that was reported to be fermented by a sulfate reducer is cysteate (Laue et al., 1997a). The fermentation of cysteate by *Desulfovibrio* strain GRZCYSA could be approximated by the following equation (23):



$$2\ {}^{-}O_3S\text{-}CH_2CH(NH_3^{+})COO^{-} + 2H_2O \rightarrow 2CH_3COO^{-} + 2CO_2 + SO_4^{2-} + HS^{-} + 2NH_4^{+} + H^{+} \quad (23)$$

Desulfovibrio species may grow with ethanol or lactate in the absence of sulfate if co-cultured with H_2-scavenging methanogenic bacteria (Bryant et al., 1977). In these syntrophic associations, the sulfate reducers serve as syntrophic, H_2-producing acetogenic bacteria. Without a H_2-scavenging partner in the absence of sulfate,

Desulfovibrio forms H_2-partial pressures of up to 1.5 kPa, without growth (Pankhania et al., 1988). Hydrogen formation in *Desulfovibrio* from lactate in the absence of sulfate was inhibited by protonophores and inhibitors of proton-translocating ATPase, whereas H_2 formation from pyruvate was not inhibited under such conditions (Pankhania et al., 1988). This observation indicated that the reducing equivalents from lactate dehydrogenation were converted to H_2 in an energy-driven process, as also suggested by the concerning redox couples (pyruvate/lactate, $E_0' = -0.190$ V; 2 H^+/H_2, $E_0' = -0.414$ V). This process may be a reversed electron transport driven by the proton gradient, as in substrate oxidation of other syntrophic bacteria (Schink, 1997). The assumption of a chemiosmotically driven dehydrogenation of lactate is further supported by the fact that lactate dehydrogenase is associated with the cytoplasmic membrane (see section on lactate). The energy for lactate dehydrogenation in *Desulfovibrio* is presumably derived from the subsequent exergonic conversion of pyruvate via acetyl-CoA (Acetyl-CoA + CO_2/pyruvate, $E_0' = -0.5$ V) and acetyl phosphate to free acetate; the latter step allows substrate-level phosphorylation and generation of a proton gradient through ATP hydrolysis at the ATPase.

In a coculture with a methanogenic bacterium, a *Desulfovibrio* species converted choline to trimethylamine, acetate, and H_2; the latter was used by the methanogenic partner (Fiebig and Gottschalk, 1983).

Desulfonema limicola, *Desulfosarcina variabilis*, and species of the genera *Desulfobulbus*, *Desulfobacterium*, and *Desulfotomaculum* did not grow in cocultures with methanogens in sulfate-free medium on lactate, ethanol, fatty acids or benzoate, even though the tested sulfate reducers possessed hydrogenase (C. Schneider and F. Widdel, unpublished observation). Apparently, there is no special mechanism in these sulfate reducers for the transfer of reducing equivalents to the redox-level of H_2, as it probably occurs in *Desulfovibrio* (Pankhania et al., 1988).

However, further sulfate-reducing bacteria that grow syntrophically with H_2 scavengers were detected during investigations on methanogenesis from propionate. The propionate-utilizing, syntrophic partners, that were named as Syntrophobacter species, turned out to be sulfate-reducing bacteria and members of the δ-subclass (Dörner, 1992; Harmsen et al., 1993; Wallrabenstein et al., 1995; for review see Schink, 1997). Growth with propionate and sulfate was extremely slow. If simultaneously an H_2-scavenging *Desulfovibrio* strain was present in sulfate-containing medium, the propionate-oxidizing strains grew syntrophically by interspecies H_2 transfer rather than by utilizing sulfate themselves. Apparently, the pathway of sulfate reduction is poorly developed in the propionate oxidizers.

Carbon Assimilation

Heterotrophic Growth The organic compounds utilized as electron acceptors by sulfate-reducing bacteria serve simultaneously as carbon sources for cell synthesis. Carbon dioxide is an important additional carbon source for various carboxylation reactions during biosynthesis. With several H_2-utilizing species, the capacity for autotrophic growth with CO_2 as the only carbon source was demonstrated (see next section). Hydrogen-utilizing sulfate-reducing bacteria of the genus *Desulfovibrio*, which are complete oxidizers, require acetate in addition to CO_2 for cell synthesis (Mechalas and Rittenberg, 1960; Postgate, 1960; Sorokin, 1966a; Sorokin, 1966b; Sorokin, 1966c; Badziong et al., 1979; Brandis and Thauer, 1981; Brysch et al., 1987). Also species of the genera *Desulfobulbus* and *Thermodesulfobacterium*, and *Desulfomicrobium norvegicum* (formerly *Desulfovibrio* desulfuricans strain Norway 4) required acetate as organic carbon source (Brysch et al., 1987; F. Widdel, unpublished observation). The observation that approximately one third of cell carbon is derived from CO_2 and two thirds derived from acetate (or molar CO_2 : acetate = 1 : 1; Sorokin, 1966a; Sorokin, 1966b; Sorokin, 1966c) is explained by the pyruvate synthase reaction. Pyruvate synthase or pyruvate:ferredoxin oxidoreductase (PFOR), that carboxylates acetyl CoA reductively (acetyl-CoA + CO_2 + $2e^-$ + $2H^+ \rightarrow$ 2 pyruvate + CoA) is a central metabolic enzyme (Badziong et al., 1979; Brandis-Heep et al., 1983; Schauder et al., 1987). The biosynthetic reaction is always required if the carbon source is acetate or a substrate that yields exclusively acetyl-CoA, e.g., ethanol or C-even fatty acids (or CO_2 in autotrophs; see below). Hence, sulfate-reducing bacteria growing on ethanol or C-even fatty acids without the capacity for complete oxidation are expected to strictly require external CO_2 for growth. It is unknown whether sulfate-reducing bacteria employ the same PFOR for acetyl-CoA assimilation and for pyruvate oxidation, e.g., during growth on lactate, or whether there are specifically regulated isoenzymes.

Several further assimilatory enzymes have been studied in *Desulfobacter* species that employ a citric acid cycle for acetyl-CoA oxidation (Brandis-Heep et al., 1983; Schauder et al., 1987). In addition to pyruvate synthase, anaplerotic reactions include acetate activation via acetyl-CoA synthetase (acetate + ATP $\rightarrow$ acetyl-

CoA + PP_i; in addition to succinyl-CoA: acetate transferase), phosphoenolpyruvate (PEP) synthetase (pyruvate + ATP → PEP + AMP + P_i), and PEP carboxylase (PEP + HCO_3^- → oxaloacetate + P_i) to compensate for the withdrawal of α-ketoacids for biosynthesis. Phosphoenolpyruvate is also expected to serve for synthesis of triose and higher sugar phosphates. In incompletely oxidizing sulfate-reducing bacteria, similar reactions may provide PEP and oxaloacetate; further biosynthetic precursors can then be synthesized via sequences of an incomplete citric acid cycle. A citrate synthase with (R)-specificity has been studied in incompletely oxidizing *Desulfovibrio* species (Gottschalk, 1968).

Autotrophic Growth When sulfate-reducing bacteria were shown to use H_2 as an inorganic electron donor, also the possibility that cell synthesis might occur autotrophically from CO_2 became of interest (Stephenson and Strickland, 1931). Carbon autotrophy was reported for sulfate-reducing enrichment cultures (Wight and Starkey, 1945) and *Desulfovibrio* strains (Butlin and Adams, 1947; Sisler and ZoBell, 1951). Later however, growth experiments and labeling studies with *Desulfovibrio* species revealed repeatedly that these sulfate reducers were lithoheterotrophs that required acetate in addition to CO_2 for cell synthesis (Mechalas and Rittenberg, 1960; Postgate, 1960; Sorokin, 1966a; Sorokin, 1966b; Sorokin, 1966c; Badziong et al., 1979; Brandis and Thauer, 1981; Brysch et al., 1987). However, several newly isolated, completely oxidizing sulfate-reducing bacteria grew with H_2 plus CO_2 (or formate) in the absence of other carbon compounds (Widdel, 1980; Widdel and Pfennig, 1981; Pfennig et al., 1981c). Labeling studies with formate-utilizing *Desulfoarculus* (formerly *Desulfovibrio*) *barsii* (Jansen et al., 1984; 1985) and H_2-utilizing *Desulfobacterium* species, *Desulfobacter hydrogenophilus*, *Desulfosarcina variabilis*, *Desulfonema limicola* and *Desulfotosporosinus orientis* (Brysch et al., 1987) clearly demonstrated the capacity for autotrophic growth. Several autotrophic strains excreted traces of acetate if incubated with H_2 plus CO_2 (or formate), with limiting sulfate concentrations. Enrichment cultures with H_2 plus CO_2 in sulfate-containing media were shown to yield mixed cultures of non-autotrophic *Desulfovibrio* species and autotrophic homoacetogenic bacteria, the latter providing acetate to the former (Brysch et al., 1987). Such mixed cultures grew faster than truly autotrophic sulfate-reducing bacteria; hence direct enrichment of the latter (autotrophic bacteria) from natural samples under autotrophic conditions is unlikely.

With the exception of *Desulfotosporosinus orientis*, the facultative lithoautotrophic sulfate reducers are complete oxidizers (Brysch et al., 1987). Indeed, the mechanisms of CO_2 fixation were found to be reverse reactions from the pathways which during organotrophic growth serve for acetyl-CoA oxidation. Whereas *Desulfobacter hydrogenophilus* assimilated CO_2 via a reductive citric acid cycle (Schauder et al., 1987), *Desulfobacterium autotrophicum* used the inverse C_1-pathway or Wood pathway (Schauder et al., 1989), viz. a sequence of reactions observed in homoacetogenic bacteria. The former has to reactivate acetate liberated in the succinyl-CoA:acetate CoA-transferase reaction. Formed acetyl-CoA from both pathways is then converted to pyruvate (see preceding section). Lithoautotrophically and organotrophically grown cells of *D. autotrophicum* exhibited different patterns of CO dehydrogenase aggregates during gel electrophoresis (Schauder et al., 1989). Obviously, the reductive and oxidative pathway, respectively, employed somewhat different enzymes. This indicates that formation of enzymes for the reductive and the oxidative pathways is regulated depending on whether H_2 or organic electron donors are present. *Desulfotosporosinus orientis* can grow autotrophically but cannot oxidize organic substrates completely to CO_2 (Klemps et al., 1985). The CO-dehydrogenase activity (R. Klemps and F. Widdel, unpublished observation) and a weak capacity for homoacetogenic growth (Klemps et al., 1985) suggests that this sulfate reducer also uses the C_1-pathway for CO_2 fixation. It is not understood why the assimilatory pathway in this species cannot be reversed for acetyl-CoA oxidation. Another incomplete oxidizer, *Desulfomicrobium apsheronum* also has been reported to grow autotrophically (Rozanova et al., 1988a).

Assimilation of Nitrogen Compounds Ammonium represents the most readily used nitrogen source for sulfate-reducing bacteria and for other bacteria. Ammonium ions are common in anoxic habitats as a result of biomass degradation. In cultivation media for sulfate-reducing bacteria, ammonium salts are usually included. In sulfate reducers that can use nitrate as electron acceptor, its dissimilatory reduction to ammonium provides simultaneously a nitrogen source.

Diazotrophic growth has been demonstrated in species of the genera *Desulfovibrio* (Riederer-Henderson and Wilson, 1970; Lespinat et al., 1987; Postgate and Kent, 1985; Moura et al., 1987), *Desulfobacter* (Widdel, 1987), *Desulfobulbus* (Bomar M. and F. Widdel, unpublished

observation) and *Desulfotomaculum* (Postgate, 1970). The DNA carrying *nifH*/*nifD* hybridized with DNA from 13 diazotrophic strains of *Desulfovibrio* belonging to 5 different species; from *D. gigas*, the *nifH* gene coding for the Fe protein of the nitrogenase system was sequenced (Postgate et al., 1988; Kent et al., 1989).

SULFATE-REDUCING ARCHAEA *Archaeoglobus fulgidus* was isolated from a submarine hydrothermal area and was identified as the first representative of the archaeal domain of life that could conserve energy via dissimilatory sulfate reduction (Stetter et al., 1987; Stetter, 1988; Zellner et al., 1989b). Two other *Archaeoglobus* species, *A. profundus* (Burggraf et al., 1990) and *A. lithotrophicus* (Stetter et al., 1993), are further archaeal sulfate reducers. A fourth *Archaeoglobus* species, *A. veneficus*, uses sulfite but not sulfate as electron acceptor (Huber et al., 1997). *Archaeoglobus* species typically grow optimally at temperatures above 80°C and require at least 10 g NaCl/liter for growth (Stetter, 1992). Phylogenetic analyses revealed that the genus Archaeglobus represents a lineage within the Euryarchaeota (Woese et al., 1991) with particular relationships to methanogenic archaea; Archaeglobus is unrelated to the sulfur-metabolizing and fermentative extreme thermophiles of the Crenarchaeota. An overview of physiological properties of sulfate-reducing *Archaeoglobus* species is given in Table 7. Another member of this lineage is the hyperthermophilic archaeum Ferroglobus placidus, which can use thiosulfate as electron acceptor for the oxidation of H_2 (Hafenbradl et al., 1996).

Reduction of Sulfate to Sulfide Transport of sulfate has not been studied so far in *Archaeoglobus*. The general pathway of sulfate reduction to sulfide in *Archaeoglobus* is analogous to the one established for sulfate-reducing bacteria (Dahl and Trüper, 1999b). The presence of the enzymatic activities essential for dissimilatory reduction of sulfate (ATP sulfurylase, APS reductase and sulfite reductase) were demonstrated in *A. fulgidus* (Speich and Trüper, 1988; Dahl et al., 1994). In Table 3, the sulfite-reductase from *A. fulgidus* is compares the sulfite-reductase from other prokaryotes mentioned.

Activation of Sulfate Prior to reduction, sulfate is activated in an ATP-dependent reaction to APS, a reaction catalyzed by ATP sulfurylase. The dissimilatory ATP sulfurylase was purified from *A. fulgidus* and found to have a molecular weight of about 150 kDa (Dahl et al., 1988;

Table 7. Physiological properties of sulfate-reducing *Archaeoglobus* species.[a]

Species	Temp. Opt. [°C]	Organic substrates utilized with SO_4^{2-} and/or $S_2O_3^{2-}$: Formate	Acetate	Lactate	Pyruvate	Others	H_2 utilization: Lithoautotrophic with SO_4^{2-}	Lithoautotrophic with $S_2O_3^{2-}$	Lithoheterotrophic (+acetate) with SO_4^{2-}
A fulgidus strain VC-16[b]	83	+	nr	+	nr	Formamide, glucose, starch, peptone, methanol, ethanol	−	+	nr
A fulgidus strain Z[b]	75–80	+	−	+	+	2,3-Butandiol, fumarate	−	+	nr
A fulgidus strain 7342[b]	76	−	−	+	+	Valerate	−	−	+
A profundus strain AV18[b]	82	nr	+[c]	+[c]	+[c]		−	−	+ (obligate lithoheterotrophic)
A lithotrophicus	80	nr	nr	nr	nr		+	nr	nr

Symbols: +, utilized; −, not utilized; nr, not reduced.

[a]Species of the genus *Archaeglobus* are the only sulfate-reducing Archaea known so far. *A veneficus* strain SNP6 does not reduce sulfate, even though this species is capable of sulfite and thiosulfate reduction (Huber et al., 1997).

[b]Data obtained from: *A fulgidus* strain VC-16 (Stetter, 1988), *A fulgidus* strain Z (Zellner et al., 1989b), *A fulgidus* strain 7342 (Beeder et al., 1994), *A profundus* strain AV18 (Burggraf et al., 1990), *A lithotrophicus* (Stetter et al., 1993).

[c]Utilization strictly dependent on the presence of H_2. It is presently unknown whether these compounds are co-metabolically utilized as electron donors, or only as carbon sources.

1990). The coding gene for sulfate adenylyltransferase (*sat*) was cloned and found to exhibit homology with the coding genes of homo-oligomeric ATP sulfurylases from various bacteria and eukaryotes. The *sat* gene was cloned and overexpressed in *E. coli* and the recombinant protein was purified. It was found to be a homodimer. Activity testing proved that the recombinant protein could indeed form ATP from APS and PP_i (Sperling et al., 1998; Sperling et al., 1999).

Reduction of APS The enzyme APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP. The enzyme was purified from *A. fulgidus* and characterized. An apparent molecular mass of 160 kDa was determined and the protein was found to contain one FAD and [FeS] clusters (Speich and Trüper, 1988; Dahl et al., 1994). Spectroscopic studies of the purified enzyme demonstrated that the enzyme contained two distinct [4Fe-4S] clusters which showed similarity to the ones identified in the APS reductase from *Desulfovibrio gigas* (Lampreia et al., 1991). Analysis of the purified APS reductase on SDS-PAGE revealed two bands corresponding to molecular masses of 80 kDa and 18.5 kDa. Taking the apparent molecular mass of the holoenzyme into account, this finding suggested a $\alpha_2\beta$ structure for the enzyme. The presence of two different subunits was confirmed by the analysis of the genes coding for the α- and β-subunit, *aprA* and *aprB*, respectively. The *aprA* and *aprB* genes encoded a 73.3 kDa and a 17.1 kDa polypeptide, respectively. The *aprA* gene product showed homologies to flavoproteins from *Escherichia coli* and *Bacillus subtilis*, whereas the *aprB* gene contained sequences for cysteine clusters that could ligate the [FeS] centers identified by the spectroscopic analyses (Speich et al., 1994).

Reduction of Sulfite The six-electron reduction of sulfite to sulfide is catalyzed by the sulfite reductase. This enzyme was purified from *A. fulgidus* and exhibited characteristics similar to those of dissimilatory sulfite reductases from other bacteria. The native enzyme had an apparent molecular mass of 218 kDa and consisted of two subunits with molecular masses of 40 and 50 kDa, suggesting a $\alpha_2\beta_2$ structure. Furthermore, the holoenzyme contained two sirohemes and six [4Fe-4S] clusters. The genes encoding the α- and β-subunit, *dsrA* and *dsrB*, were cloned, and found to be arranged in an operon structure. The deduced DsrA peptide contains the cysteine residues required for the coordination of siroheme-[4Fe-4S] complexes. Furthermore, both deduced peptides, DsrA and DsrB, contain additional cysteine residues which are characteristic of binding motifs for ferredoxin-like [4Fe-4S]-clusters. Thus the findings of the sequence analyses corroborated the biochemical data directly obtained from the purified protein. The *dsrA* and *dsrB* genes showed a high degree of similarity suggesting that these genes arose by duplication from an ancestral gene. Comparative sequence analyses of sulfite reductases from various microorganisms revealed that only sulfite reductases from *A. fulgidus* and *Salmonella typhimurium* contained a ferredoxin-like domain in the proximity of the conserved putative siroheme-[4Fe-4S]-binding cysteine residues (Dahl et al., 1993; 1994). The *dsrAB* genes of *A. fulgidus* were also highly homologous to the dsvAB genes that code for desulfoviridin of *Desulfovibrio vulgaris* (Karkhoff-Schweizer et al., 1995).

A dissimilatory sulfite reductase was also isolated from the hyperthermophilic archaeon *Pyrobaculum islandicum*. This archaeon cannot reduce sulfate; however, it is capable of organotrophic growth with sulfite as electron acceptor (Huber et al., 1987). The purified sulfite reductase was shown to have a $\alpha_2\beta_2$ structure and to contain siroheme and [FeS] clusters. Two coding genes (*dsrA* and *dsrB*) could be cloned and found to be organized in an operon. Downstream of the *dsrB* gene, a third gene, *dsrC*, was identified which was homologous to the proposed γ-subunit of the sulfite reductase from *Desulfovibrio vulgaris* (Molitor et al., 1998; Dahl et al., 1999a).

Electron Acceptors Other Than Sulfate In addition to sulfate, *A. fulgidus* can utilize thiosulfate and sulfite as electron acceptor. The utilization of sulfite is understandable because it is an intermediate during sulfate reduction. The reduction of thiosulfate in *A. fulgidus* has not been studied in more detail.

Electron Carriers

Ferredoxin Ferredoxin is an electron carrier which has been frequently encountered in sulfate-reducing bacteria. It has also been identified in *A. fulgidus*. Ferredoxin is involved in catabolic reactions in *A. fulgidus*, such as pyruvate:ferredoxin oxidoreductase (Kunow et al., 1995) and the acetyl-CoA decarbonylase/synthase (CO-dehydrogenase-containing) complex (Dai et al., 1998), and possibly also in pyruvate synthesis from acetyl-CoA in lithoheterotrophic species that use acetate as organic carbon sources.

Menaquinone Tindall et al. (1989) discovered a novel menaquinone in *A. fulgidus*. This menaquinone possesses a fully saturated hepta-

prenyl side chain ($MK\text{-}7H_{14}$) and is the major lipoquinone in *A. fulgidus*.

Metabolism of Electron Donors *Archaeoglobus* species may grow chemolithoautotrophically with H_2 and CO_2, chemoorganotrophically on formamide, lactate, pyruvate, glucose and complex organic substrates (starch, peptone), or lithoheterotrophically on H_2 and acetate, lactate, pyruvate or other organic compounds (Stetter, 1992). An overview of the metabolism of electron donors by *Archaeoglobus* species is given in Table 7. The occurrence of *Archaeoglobus* species in marine and terrestrial oil-field waters has been reported several times (Stetter et al., 1993; Beeder et al., 1994; L'Haridon et al., 1995) and has suggested that *Archaeoglobus* species may utilize constituents of crude oil. However a utilization of hydrocarbons, the main constituents of crude oil, could not be demonstrated.

Lactate, Pyruvate, and Acetate *A. fulgidus* completely oxidizes lactate to CO_2 with sulfate as electron acceptor (Möller-Zinkhan et al., 1989; Zellner et al., 1989b). Lactate is oxidized to acetyl-CoA via lactate-dehydrogenase and pyruvate:ferredoxin oxidoreductase (PFOR; Möller-Zinkhan et al., 1989). Based on its predicted function as lactate dehydrogenase, a gene (*dld*) was cloned from the completely sequenced genome of *A. fulgidus* (Klenk et al., 1997) and heterologously overexpressed in *Escherichia coli*. The purified recombinant protein possessed D-lactate dehydrogenase activity, contained Zn^{2+} and the flavin cofactor FAD (Reed and Hartzell, 1999). The PFOR has been purified from *A. fulgidus* and found to have an apparent molecular mass of 120 kDa, a heterotetrameric ($\alpha\beta\gamma\delta$) structure and to contain thiamine pyrophosphate and iron-sulfur clusters (Kunow et al., 1995). Further oxidation of acetyl-CoA to CO_2 proceeds via a C_1/CO-dehydrogenase pathway that may be regarded as an archaeal analogue of the pathway in sulfate-reducing bacteria (Fig. 11B). A unique characteristic of the archaeal pathway is the involvement of the cofactors F_{420}, tetrahydromethanopterin and methanofuran that had been detected before in methanogenic archaea (Stetter et al., 1987; Möller-Zinkhan et al., 1989; Möller-Zinkhan and Thauer, 1990). The CO dehydrogenase is part of a multienzyme complex termed acetyl-CoA decarbonylase synthase (ACDS) that was isolated and characterized (Dai et al., 1998). This multienzyme complex consists of five different subunits ranging from 18.5 to 89 kDa in molecular mass and catalyzes the cleavage of acetyl-CoA into a bound methyl-group and bound CO, or the reverse reaction. The methyl carrier is tetrahydromethanopterin (H_4MPT); also, tetrahydrosarcinopterin reacts as methyl carrier with the complex. Ferredoxin is employed as electron carrier by this multienzyme complex. Prior to the study presented by Dai et al. (1998), ACDS complexes had been detected only in methanogens. Structural and functional properties of the ACDS complex from *A. fulgidus* are similar to those of the complex from methanogens. Therefore much insight into the function of the ACDS complex in *A. fulgidus* is based on the studies of this complex in the methanogens. The complex from *Methanosarcina barkeri*, which has been studied best, also consists of five subunits and has a $(\alpha\beta\gamma\delta)_6$ structure, giving rise to the remarkable total molecular mass of ca. 2.0 MDa for the entire complex (Grahame, 1991). Carbon monoxide and CO_2 can be used for carbonylation of methylated tetrahydrosarcinopterin. A hydrogenase that was resolved from the multienzyme complex was capable of reconstituting the acetyl-CoA synthesis of the complex (Grahame and DeMoll, 1995). Separation of the ACDS complex from *Methanosarcina* by limited proteolytic digestion allowed specific catalytic functions to individual subunits: the CO-dehydrogenase reaction is performed by the α component; the methyltransferase is located on the γ-subunit and parts of the δ-subunit; and the binding of CoA or acetyl-CoA occurs on the β-subunit (Grahame and DeMoll, 1996). The CH_3-group from acetyl-CoA cleavage in *Archaeoglobus fulgidus* is further oxidized to CO_2 via N^5,N^{10}-methylene-H_4MPT reductase, N^5,N^{10}-methylene-H_4MPT dehydrogenase, N^5,N^{10}-methenyl-H_4MPT cyclohydrolase, formylmethanofuran:H_4MPT formyltransferase and formylmethanofuran dehydrogenase (Möller-Zinkhan et al., 1989). Purification of the corresponding enzymes from *A. fulgidus* allowed the C_1-pathway of methyl oxidation to be unequivocally established and demonstrated that the enzymes from *A. fulgidus* had very similar molecular and catalytic properties as those of the acetate-degrading methanogens (Schmitz et al., 1991; Klein et al., 1993; Schwörer et al., 1993). Factor F_{420} serves as H_2 acceptor for N^5,N^{10}-methylene-H_4MPT reductase and N^5,N^{10}-methylene-H_4MPT dehydrogenase. The natural electron acceptor for formylmethanofuran dehydrogenase is unknown. A membrane-bound $F_{420}H_2$:quinone oxidoreductase complex was purified from *A. fulgidus*. This enzyme complex is presumed to be involved in the chemiosmotic conservation (Kunow et al., 1994).

Similarities in the enzymes and cofactors of the C_1-pathway in *Archaeoglobus* and methanogens suggest a metabolic relationship. Indeed, *Archaeoglobus* was suggested to represent a link between hyperthermophilic sulfur-reducing (nonsulfate-reducing) and methanogenic

archaea. However, *Archaeoglobus* does not possess the cofactors (mercaptoethanesulfonate, mercaptoheptanoyl threonine phosphate) and enzymes (methyltransferase, methyl-CoM reductase, heterodisulfide reductase) that are involved in the terminal step of CH_4 formation from the H_4MPT-bound methyl group. The formation of low amounts of CH_4 observed in *Archaeoglobus* (Stetter et al., 1987) (and in sulfate-reducing bacteria; Schauder et al., 1986) is a by-reaction of the methyl group transferred by CO dehydrogenase.

Malate, Isocitrate and Glutamate Even though *Archaeoglobus* performs oxidation of acetate via the C_1-pathway and not via the TCA cycle, activities of malate dehydrogenase and isocitrate dehydrogenase were measured in cell extracts of (Möller-Zinkhan et al., 1989). These enzymes presumably function in biosynthesis (Langelandsvik et al., 1997; Steen et al., 1997). Both enzymes were purified and found to possess pronounced thermostability. Malate-dehydrogenase was specific for NAD^+, whereas isocitrate dehydrogenase has a high preference for $NADP^+$. Also a thermostable $NADP^+$-specific glutamate dehydrogenase was purified from this archaeon. This enzyme accounts for 0.8% of the total cell extract protein, which is relatively large in view of the assumed function in the assimilation of ammonia (Aalén et al., 1997).

Autotrophic Growth Autotrophic growth on H_2 and sulfate as energy source and CO_2 as carbon source was studied in *Archaeoglobus lithotrophicus*. All enzymatic activities and coenzymes required for the fixation of CO_2 via the reductive CO dehydrogenase pathway were demonstrated in cell extracts of *A. lithotrophicus* (Vorholt et al., 1995). This reductive CO dehydrogenase pathway is, in principle, the reverse of the oxidative C_1/CO-dehydrogenase pathway employed by *A. fulgidus* for the oxidation of acetyl-CoA. The same study by Vorholt et al. (1995) showed that CO dehydrogenase was lacking in *A. profundus*, which explained why this archaeon requires acetate for biosynthesis during growth on H_2.

Genome *A. fulgidus* strain VC-16 is the first sulfate- and sulfur-reducing microorganism and the second archaeon after *Methanococcus jannaschii* (Bult et al., 1996) the complete genomic sequence of which has been determined (Klenk et al., 1997).

The genome consists of a single chromosome of about 2.2 Mb and has an average G+C content of 48.5 mol%. A total of 2,436 ORFs were identified with an average size of 822 bp. Putative functions could be assigned to 1,797 ORFs, whereas the remaining 639 ORFs had no database matches. Two thirds of these unidentified ORFs are shared with *M. jannaschii*. Of the ORFs with assigned function, 719 genes can be classified into 242 families. The largest of these families is the superfamily of ATP-binding subunits of ABC transporters, which comprises 40 members in *A. fulgidus*. The genome of *A. fulgidus* contains three regions of short repeats (>40 bp), which are similar to those found in *M. jannaschii*, and nine classes of long repeated sequences (<500 bp).

Synthesis of Basic Macromolecules (Examples), and Chemotaxis Putative components of the replicatory, DNA-repair and cell division machinery were identified that showed sequence homology to those known from either *M. jannaschii*, bacteria or eukaryotes. For example, the B-type DNA polymerase of *A. fulgidus* is related to the δ polymerase from eukaryotes. The genes for gyrases, topoisomerases and a type I restriction-modification system were identified in *A. fulgidus*.

Genes for 46 tRNAs were identified, however no gene with homology to the gene *selC* was detected that codes for selenocysteine-inserting tRNASec in *E. coli*. The presence of complex sensory and regulatory networks in *A. fulgidus* is suggested by presence of genes resembling those of the bacterial chemotaxis system (Che); furthermore these chemotaxis-related genes are located adjacent to an operon that was assigned to the flagellar biosynthesis.

Transporters Around 120 ORFs were identified that may code for proteins functioning in the transport of amino acids, peptides, amines, carbohydrates, alcohols and acids. The presence of transporters for various organic compounds is in agreement with the observation that *A. fulgidus* grows optimally in media with complex organic nutrients such as yeast extract. Transport of inorganic cations and anions is supposed to be mediated by 10 distinct types of transporters, among them also members of the large superfamily of ABC transporters. Genes belonging tentatively to the latter show homologies to the *cysA* and *cysT* genes, coding for the ATP-binding protein and permease, respectively, of the sulfate transporter in bacteria. However, it is unknown whether *A. fulgidus* indeed transports sulfate via an ATP-driven transporter. This would result in the consumption of as much as three molecules of ATP for the formation of one molecule of APS (assuming requirement of two ATP for activation). Subsequent APS reduction to sulfide with

a suitable electron donor would have to yield more than three ATP.

Catabolic and Biosynthetic Metabolism Several genes coding for acetyl-CoA synthetase, which converts acetate and ATP to acetyl-CoA, AMP and PP_i, were identified. However, growth with acetate as an externally added electron donor and carbon source has not been demonstrated. Many genes were recognized that were similar to those coding for β-oxidation enzymes in bacteria but have not been described in archaea. Many of the genes encoding β-oxidation enzymes in *A. fulgidus* are present in multiple copies, suggesting a considerable metabolic differentiation. For instance, there are 10 copies of 3-hydroxyacyl-CoA dehydrogenase encoded. Again, growth with fatty acids as electron donors and carbon sources, which would require the pathway of β-oxidation, has not been reported for *A. fulgidus*. Genes coding for enzymes of central pathways of the intermediary sugar metabolism as known from bacteria were also identified in *A. fulgidus*. According to such genes, glycolysis, the pentose-phosphate pathway, the Entner-Doudoroff pathway and reactions for gluconeogenesis are expected to be either completely or partially present. Interestingly, no gene coding for a glutamate dehydrogenase was identified, even though such an enzyme has been purified from a different strain (strain 7324) of *A. fulgidus* (Aálen et al., 1997).

Biosynthetic pathways for amino acids, cofactors, carriers, purines and pyrimidines were also identified on the gene level. Many of the recognized biosynthetic pathways show a high degree of similarity with those identified in *M. jannaschii*.

Energy Conservation Apart from the F_{420}: quinone oxidoreductase complex, no electron transport components involved in chemiosmotic energy conservation have been identified on the protein level in *Archaeoglobus*. However, the genomic sequence revealed genes tentatively coding for heterodisulfide reductase, and for several molybdopterin-containing oxidoreductases that react with polysulfide, NO_3^-, DMSO and $S_2O_3^{2-}$ as potential electron acceptors. This finding indicates that *A. fulgidus* may have more electron transport systems than known from physiological studies.

Comparison to the Genome of *M. jannaschii* A comparison of the genomes from *A. fulgidus* and *M. jannaschii* revealed that various degrees of conservation exist in different categories of genes. Among the genes of *A. fulgidus* that are related to either translation, replication or biosynthetic pathways, 80% are homologous to genes of the same categories in *M. jannaschii*. The conservation of genes related in the central metabolism of low molecular mass intermediates (essentially catabolism and energy metabolism) is far lower. Only 35% of those genes from *A. fulgidus* have homologues in *M. jannaschii*, which is in agreement with the distinct types of energy metabolism in these archaea. The methanogen *M. jannaschii* utilizes H_2 and CO_2 as substrates, but cannot degrade acetate. However, because it is an autotroph, *M. jannaschii* is expected to possess the enzymes for the reductive C_1/CO-dehydrogenase pathway for acetyl-CoA synthesis.

Sulfur-Reducing Bacteria In research of the biochemistry of sulfur-reducing bacteria, the reduction of the electron acceptor and the associated process of electron transport and energy conservation has been a main topic. This has been mainly studied in *Wolinella succinogenes*. In addition, oxidation pathways of acetate, viz. the capacity for terminal oxidation of organic substrates, have been investigated in *Desulfuromonas* and *Desulfurella*. The metabolism of other organic substrates in sulfur-reducing bacteria, in contrast to that in sulfate-reducing bacteria, has been of marginal research interest.

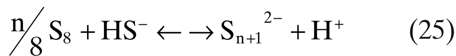
Sulfur reduction not coupled to a respiratory mechanism (for example, the incidental reduction found in various organisms) may result from its reaction with sulfhydryl groups of proteins or glutathione that are oxidized to disulfides as shown below (Roy and Trudinger, 1970; Starkey, 1937).

$$2RSH + S \longleftrightarrow RSSR + H_2S \qquad (24)$$

Because research of the biochemistry of sulfur reducers has focused on fewer organisms and fewer substrates, organization of the following section is somewhat different from those treating the sulfate-reducing bacteria.

Bioavailability of Elemental Sulfur In contrast to sulfate, elemental sulfur is chemically relatively reactive and requires no energy-dependent activation before a reduction can take place. However, a problem in the utilization of elemental sulfur is usually seen in its low solubility in water (5 µg or 0.16 µmol per liter at 25°C; Boulégue, 1978). It has therefore been questioned whether pure elemental sulfur (e.g., rhombic sulfur) can directly serve as the substrate for sulfur reductase. A form that is more

likely to be available in aqueous medium is the so-called "hydrophilic sulfur", which is elemental sulfur associated with small portions of oxocompounds such as polythionates ($^{-}O_3S\text{-}S_n\text{-}SO_3^{-}$) that may allow some pseudosolubilization. Aqueous suspensions of sulfur, especially chemically precipitated colloidal ones, and sulfur formed by sulfide-oxidizing microorganisms, contain varying portions of such hydrophilic sulfur (Steudel et al., 1988; Steudel, 1989a; Steudel et al., 1989b). Another possibility for solubilization of sulfur exists, if sulfide is present, which is usually the case in the environment of sulfur reducers. In aqueous solutions of sulfide (H_2S/HS^-, pK_{a1} = 7.02), the S_8-ring of elemental sulfur is cleaved by a nucleophilic attack of the HS^- anion which results in the formation of polysulfide (Steudel et al., 1986) according to equation (25).



$$\frac{n}{8} S_8 + HS^- \leftarrow\rightarrow S_{n+1}^{2-} + H^+ \qquad (25)$$

In media around a pH of 7, the predominant species of polysulfide are tetrasulfide (S_4^{2-}) and pentasulfide (S_5^{2-}). These two polysulfides interconvert rapidly, as shown in equation 25, and are also in equilibrium with lower concentrations of other polysulfides. Therefore it is unknown which of the two polysulfides is the preferred substrate for polysulfide reductase.

PP_i formed from sulfide and tetrathionate was shown to be reduced by formate-utilizing cells of *W. succinogenes* (Klimmek et al., 1991). The concentration of polysulfide in medium containing 1 mM sulfide (HS^- + H_2S) at pH > 6 was shown to exceed 10 µM (Schauder and Müller, 1993), which is close to the apparent K_M (about 20 µM) determined for polysulfide respiration in *W. succinogenes* (Klimmek et al., 1998). Thus there is evidence for the use of polysulfide as the actual electron acceptor in sulfur respiration.

In the study of sulfur reduction, it must not be forgotten that other bacteria grow with elemental sulfur without the possibility of solubilization in the form of polysulfide. These are anaerobes that disproportionate elemental sulfur in the presence of sulfide-scavenging ferric minerals (Thamdrup et al., 1993) or aerobic bacteria that oxidize extracellular sulfur. It is unknown how these bacteria cope with the low solubility of sulfur in water. In photoautotrophic purple bacteria and possibly in aerobic sulfide oxidizers forming intracellular sulfur globules as intermediates, the sulfur is topologically periplasmatic ("extracytoplasmatic") and associated with proteins; these complexes are assumed to control formation or further oxidation of the sulfur (Dahl, 1999b). Examples of other microorganisms growing with insoluble substrates are bacteria reducing ferric minerals, or bacteria or yeasts oxidizing long-chain alkanes. Ferric minerals are probably reduced in direct contact with the cells (Lovley, 1995a), or by an extracellular cytochrome (Seeliger et al., 1998). Long-chain alkanes are also utilized in direct contact with the cells, or via pseudosolubilization with biotensides (Bühler and Schindler, 1984).

Research on Wolinella succinogenes *Wolinella* was originally isolated as a fumarate-reducing bacterium utilizing H_2 or formate as electron acceptor. The capacity for microaerobic growth has been formerly mentioned but not the subject of more recent studies. *Wolinella* was then shown to reduce sulfur (Macy et al., 1986), like *Sulfurospirillum deleyanium*, the former *spirillum* 5175 (Wolfe and Pfennig, 1977), a facultative microaerophile. In addition to H_2 or formate, both sulfur reducers oxidize some other organic compounds such as lactate. Oxidation is incomplete and leads to acetate. *Wolinella* and *Sulfurospirillum* are not only metabolically, but also phylogenetically related; they belong to the ε Proteobacteria.

The Sud Protein In a study in which the involvement of polysulfide in sulfur respiration of *Wolinella succinogenes* was questioned, Fe^{2+} [as $Fe(OH)_2$] was added to the medium to precipitate all sulfide formed by *W. succinogenes* as FeS and thus prevent formation of polysulfide. Under these conditions, *W. succinogenes* still grew anaerobically with formate and elemental sulfur, indicating that sulfur reduction is in principle possible without the involvement of polysulfide as an intermediate (Ringel et al., 1996). From the iron(II)-containing culture of *W. succinogenes*, a soluble sulfur-containing fraction was isolated that by treatment with CN^- could be separated further into a yet unidentified sulfur species and the so-called Sud protein (Hedderich et al., 1999). The coding *sud* gene was isolated from *W. succinogenes* and its sequence indicated the presence of a signal peptide and only one cysteine in the polypeptide chain. The recombinant Sud protein was purified after heterologous expression in *Escherichia coli*. The enzyme consists of two identical subunits (14 kDa), lacks any prosthetic groups or heavy metals and is located in the periplasm. The synthesis of the Sud protein is induced during growth on elemental sulfur and polysulfide (Kreis-Kleinschmidt et al., 1995). Further studies with a His-tagged Sud protein (Sud-His_6), which was also purified from *E. coli*, demonstrated a catalysis of the formation of thiocyanate from cyanide and polysulfide with an apparent K_M of less than 20 µM polysulfide. The monomer of Sud-His_6 was found to bind up to 10 sulfur atoms from polysulfide. Addition of small amounts of Sud-His_6 to membrane fractions of

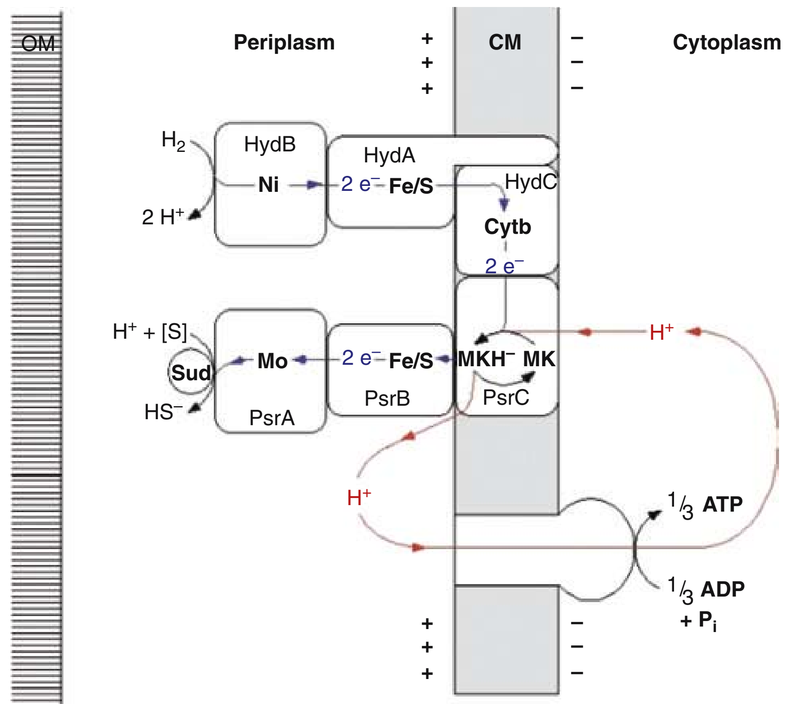


Fig. 18. Possible generation of a proton-motive force (pmf) during growth of *Wolinella succinogenes* or other spirilloid sulfur reducers on H_2 and sulfur. Diffusion and collision of HydC and PsrC is assumed to be required for electron transfer. The mechanism for generation of a proton gradient is not known. Possibly, protons are translocated via protein-bound menaquinone to the periplasm. Abbreviations: HydABC, subunits of hydrogenase; PsrABC, subunits of polysulfide reductase; Sud, protein that increases the availability of polysulfide (formerly termed "sulfide dehydrogenase"). [S] indicates a soluble form of sulfur, most probably polysulfide. The scheme was adapted from Hedderich et al. (1999).

W. succinogenes stimulated the electron transport from H_2 to polysulfide (Klimmek et al., 1998). A deletion mutant of the *sud* gene (Δ*sud*) was constructed in *W. succinogenes* by homologous recombination. However, growth of the Δ*sud* deletion mutant on formate and polysulfide as compared to that of the wild type was not affected (Kotzian et al., 1996). By site directed mutagenesis, the single cysteine residue in the Sud protein (Cys109) was replaced by a serine residue. The modified Sud protein (C109S)Sud-His_6 showed marked differences from the Sud-His_6 protein. The (C109S)Sud-His_6 protein neither catalyzed formation of thiocyanate from cyanide and polysulfide nor stimulated the electron transport to polysulfide. Moreover, the Cys109 residue was found to be required for binding polysulfide-sulfur to the Sud protein. Despite some inconsistent results from growth experiments, the Sud protein is assumed to function in transferring sulfur from aqueous polysulfide to the active site of polysulfide reductase (Klimmek et al., 1999). The Sud protein and polysulfide reductase (Psr) were present in nearly equimolar amounts when *W. succinogenes* was grown on polysulfide, and it is assumed that Sud is bound to Psr (Fig. 18; Hedderich et al., 1999).

Polysulfide Reductase The enzyme that catalyzes the reduction of polysulfide sulfur to sulfide is termed "polysulfide reductase" (Psr). This enzyme is encoded by the polysulfide reductase operon (*psrABC*; Krafft et al., 1992). The nucleotide sequence of the *psrABC* genes indicates that Psr is a heterotrimer consisting of three subunits (PsrA, B and C). The PsrA (81 kDa) and PsrB (21 kDa) subunits are hydrophilic proteins, whereas PsrC (34 kDa) is of hydrophobic nature with eight putative transmembrane-spanning segments and is assumed to function as membrane anchor of the Psr holoenzyme. The PsrA subunit was found to be homologous to known molybdoenum-containing oxidoreductases, such as formate dehydrogenase of *E. coli*. Indeed, a molybdopterin guanine dinucleotide was identified in the purified protein (Jankielewicz et al., 1994). The PsrA subunit is assumed to be the catalytic subunit of the Psr protein. The *psrA* gene also includes the coding sequence for a leader peptide indicating an orientation of the PsrA subunit toward the periplasm. Moreover, PsrA was identified in the periplasmic fraction of a Δ*psrC* mutant. Based on the predicted presence of 16 cysteine residues, PsrB is assumed to contain several [FeS] clusters involved in electron transfer. Even though the *psrB* gene does not contain a coding sequence for a leader peptide, a periplasmic orientation of PsrB is postulated (Krafft et al., 1992). The purified Psr contains 1 mol menaquinone per mol of protein. Menaquinone is assumed to serve as acceptor for electrons transferred from hydrogenase and as direct electron donor for polysulfide/sulfur reduction.

The purified Psr protein catalyzes the reduction of polysulfide to sulfide with BH_4^- as hydride donor, and the oxidation of sulfide to polysulfide by 2,3-dimethyl-1,4-naphthoquinone, a soluble analogue of menaquinone (Krafft et al., 1992). In wild type *W. succinogenes*, polysulfide reductase activity is still present and active when cells are grown with fumarate as electron acceptor (Lorenzen et al., 1993). Deletion mutants of Psr (Δ*psrC*, Δ*psrBC* and Δ*psrABC*) were grown with fumarate and cell fractions were analyzed for their capacity to oxidize sulfur and to transfer electrons from formate to polysulfide (Krafft et al., 1995). The Δ*psrC* mutant catalyzed the oxidation of sulfide with dimethylnaphthoquinone, which was not observed with the Δ*psrABC* or Δ*psrBC* mutant. This indicated that PsrA and PsrB, but not PsrC, were directly involved in the transfer of reducing equivalents to a quinone site. However, the capacity of the Δ*psrC* mutant to perform the entire electron transfer from formate to polysulfide was only 5% of the wild type activity, suggesting that PsrC is required for further electron transport reactions. If the Δ*psrABC* mutant was grown on polysulfide instead of fumarate, activity for sulfide oxidation and polysulfide reduction could still be measured. A so far unidentified protein could be extracted from the membranes of the polysulfide-grown mutant that seems to replace polysulfide reductase.

Electron Transport from Formate or H_2 to Polysulfide *Wolinella succinogenes* utilizes either H_2 or formate as electron donors (Macy et al., 1986). The same hydrogenase and formate dehydrogenase are operative if either sulfur or fumarate are used as electron acceptors (Schröder et al., 1988). A hydrogenase deletion mutant (Δ*hydABC*) did not grow with H_2 and polysulfide, or with H_2 and fumarate. Growth could be restored by complementing the mutant with the *hydABC* operon (Gross et al., 1998a; Gross et al., 1998b). Electrons from hydrogenase and formate-dehydrogenase have to be transferred to polysulfide reductase.

Electron transfer reactions were most intensely studied with formate. Even though the substrate-binding sites of formate dehydrogenase and hydrogenase are both orientated toward the periplasm (Kröger and Winkler, 1981), formate does not diffuse through membrane bilayers and thus allows more defined studies in vesicles than H_2. Electron transfer from formate was studied in vesicles as a function of the ratio between phospholipid and membrane proteins, by dilution of the membrane fraction of *W. succinogenes* with phospholipid. Based on these experiments, a model of diffusion and collision was suggested. Collision of hydrogenase or formate dehydrogenase, respectively, with the polysulfide reductase is regarded as a requirement for this electron transfer. In addition to the collision of proteins, menaquinone bound to PsrC is essential (Hedderich et al., 1999). In contrast, electron transport to fumarate reductase in the cytoplasmic membrane of *W. succinogenes* does not involve direct collision of proteins but rather occurs via freely diffusible menaquinone in the cytoplasmic membrane (Jankielewicz et al., 1995; Hedderich et al., 1999).

Properties of hydrogenase in *W. succinogenes* have been studied in detail. The enzyme is membrane-bound, contains nickel, and catalyzes the reduction of dimethylnaphthoquinone or benzylviologen with H_2 (Unden et al., 1982). It could be isolated from the membrane fraction of *W. succinogenes* and was found to consist of three subunits, HydA (30 kDa), HydB (60 kDa) and HydC (23 kDa). A deletion mutant without the hydrogenase (Δ*hydABC*) cannot grow with H_2 and either polysulfide or fumarate. The three subunits of hydrogenase are encoded by three adjacent genes, *hydABC*. The HydA subunit is a hydrophilic protein that is likely to be localized in the periplasm because the gene, *hydA*, contains a coding sequence for a leader peptide. The HydA subunit contains eight cysteine residues, some of which are possible ligands for [FeS] clusters. The C-terminus of HydA contains about 20 hydrophobic residues that could constitute a membrane anchor by forming a transmembrane helix and in this way a membrane anchor for the protein. The HydB protein, the catalytic subunit of hydrogenase, is hydrophilic and contains eight cysteine residues that are likely to coordinate [FeS] clusters. The Cys546 residue is possibly functioning in ligation of Ni. The catalytic subunit HydB of the intact hydrogenase is located in the periplasm as demonstrated with activity tests and western blot analyses of cell fractions. The HydA and HydB proteins are homologous to the corresponding subunits of other known Ni-hydrogenases. The HydC subunit is a hydrophobic protein with four putative transmembrane-spanning segments. Biochemical studies indicated that HydC represents a cytochrome *b*, with the two heme-B groups ligated by four His residues. Mutants created by substitution of the heme-ligating His residues no longer had the activity to reduce quinone with H_2 and to transfer electrons to polysulfide reductase. These results indicate that the menaquinone bound as a prosthetic group to the PsrC is the primary acceptor for electrons from cytochrome *b* of HydC. This finding supports the assumption that also hydrogenase has

to be associated with polysulfide reductase for electron transfer in the membrane (Dross et al., 1992; Gross et al., 1998b) as in the case of formate dehydrogenase.

The exact mechanism for the generation of the electrochemical proton gradient with formate or H_2 as electron donors is not known. Possibly PsrC couples electron transfer via bound menaquinone to polysulfide to a translocation of protons (Hedderich et al., 1999; Fig. 18).

Polysulfide and fumarate respiration in *W. succinogenes* differ not only with respect to the involvement of quinone. Also, the orientation of the two reductases is different. Whereas the substrate-binding site of polysulfide reductase is oriented toward the periplasm, that of fumarate reductase is localized on the cytoplasmic side of the membrane (Kröger et al., 1980). The substrate-binding sites of hydrogenase and of formate dehydrogenase both face the periplasm (Kröger and Winkler, 1981).

Regulation of Sulfur Respiration Growth cultures of *W. succinogenes* on sulfur and formate in medium that also contained nitrate or fumarate, reduced sulfur but neither of the other two electron acceptors. This indicated that the energetically less favorable electron acceptor, sulfur, represses the utilization of the more favorable electron acceptors. In contrast, cells that were grown with nitrate or fumarate could respire both of these electron acceptors. Polysulfide reductase activity in fumarate-grown cells was as high as in sulfur-grown cells, but rather low in nitrate-grown cells (Lorenzen et al., 1993). In conclusion, regulation of anaerobic respiration with alternative electron acceptors is not clearly in accordance with their energetic "hierarchy."

Electron Acceptors Other Than Sulfur *Wolinella succinogenes* also can grow with nitrate and fumarate as electron acceptors. Nitrate is reduced to ammonia and not to N_2 as in "true" denitrifying bacteria. A hexaheme cytochrome c_3 acting as nitrite reductase has been isolated from *Wolinella succinogenes* (Liu et al., 1983). Another nitrogen compound reduced by *Wolinella succinogenes* is N_2O; unlike nitrate (or nitrite), N_2O is reduced to N_2 (Yoshinari, 1980). Furthermore, spirilloid sulfur reducers closely related to *Wolinella succinogenes* were shown to reduce dimethylsulfoxide to dimethylsulfide (Zinder and Brock, 1978; Widdel, 1988). In connection with the initial characterization of *Wolinella succinogenes*, microaerobic growth has been reported (Wolin et al., 1961). Microaerobic growth was also shown in related spirilloid sulfur-reducing bacteria (Wolfe and Pfennig, 1977; Widdel, 1988).

Research on Desulfuromonas *and* Desulfurella Among the sulfur-reducing bacteria, the capacity for a complete oxidation of organic substrates occurs necessarily in those genera and species that grow on acetate (*Desulfuromonas* and *Desulfurella*).

Desulfuromonas (Pfennig and Biebl, 1976) and *Desulfurella* (Bonch-Osmolovskaya et al., 1990) were directly isolated with acetate and sulfur. Both are obligate anaerobes. They are members of the δ Proteobacteria, with a specific relationship of *Desulfuromonas* to completely oxidizing sulfate-reducing bacteria. In addition to acetate, *Desulfuromonas* species may utilize a number of simple organic compounds (Table 2).

Oxidation of Acetate via the Citric Acid Cycle The presence of all enzymes of the citric acid cycle could be demonstrated in *Desulfuromonas acetoxidans* and *Desulfurella acetivorans* (Gebhardt et al., 1985; Schmitz et al., 1990; for overview see Kröger et al., 1988; Thauer, 1988; Thauer, 1989a; Thauer et al., 1989b). [^{14}C]-labeling experiments demonstrated a functioning citric acid cycle in *Desulfuromonas acetoxidans* (Gebhardt et al., 1985). *Desulfuromonas* activates acetate like *Desulfobacter* via succinyl-CoA:acetate CoA transferase (Fig. 12). In *Desulfurella acetivorans*, however, the formation of succinate from succinyl-CoA is associated with the synthesis of one ATP; this amount is used again to activate acetate by acetate kinase (Schmitz et al., 1990; Fig. 12 C). Citrate formation in *Desulfuromonas acetoxidans* occurs with si-specificity, as in *Desulfobacter*, but without coupling to ATP formation. Malate in *Desulfuromonas* and *Desulfurella* is dehydrogenated with NAD^+, as in mitochondria and most bacteria; *Desulfurella* has in addition $NADP^+$-specific malate dehydrogenase. The reduction of $NAD(P)^+$ ($E_0' = -0.32$ V) with malate ($E_0' = -0.166$ V) in the sulfur reducers is understood in view of the way of citrate synthesis. By not being coupled to ATP formation, the reaction is exergonic and "pulls" the energetically unfavorable dehydrogenation of malate with pyridine nucleotides. Furthermore, an NADP:ferredoxin oxidoreductase has been detected (Kröger et al., 1988). A comparison of the modifications of the citric acid cycle found in sulfate- and sulfur-reducing bacteria is presented in Fig. 12.

Inasmuch as neither *Desulfuromonas* nor *Desulfurella* gain net ATP by substrate-level phosphorylation, energy conservation must be achieved by chemiosmosis. The electron transport from ferredoxin, which might accept electrons from 2-oxoglutarate and via (NADP), from

isocitrate, to the postulated electron donor for the sulfur reductase (ΔE' around 0.2 V) could pump $2H^+/2e^-$ or $4H^+$/acetate. As in *Desulfobacter*, succinate oxidation in *Desulfuromonas acetoxidans* to fumarate (E_0' = +0.033 V) with menaquinone (E_0' = –0.074 V) is endergonic from the viewpoint of standard potentials. It appears unlikely that the reaction is made feasible solely by shifting concentration ratios of involved redox couples, or by specific coupling to a favorable redox reaction (see *Desulfobacter*), because electrons from menaquinone have to be transported further to sulfur reductase (S/H_2S, E′ = –0.19 V for 10 mM H_2S; Fig. 14B). It is more likely that electron transport from succinate (oxidation) to sulfur (reduction) with a redox span of ΔE′ = –0.22V is driven by chemiosmosis (reversed electron transport). With the consumption of 2 H^+ for the energy-driven oxidation of 1 succinate, 2 H^+/acetate remain for ATP synthesis, yielding $^1/_2$ to $^2/_3$ mol ATP/mol acetate. This is in relatively good agreement with energetic considerations based on growth yields. The growth yield of *Desulfuromonas acetoxidans* growing on acetate and sulfur was 4.2 g dry mass/mol acetate at an average doubling time of 3.8 hours (Pfennig and Biebl, 1976). The yield of *Desulfovibrio vulgaris* at this doubling time with acetate as C-source (H_2 as electron donor) was 9.1 g/mol sulfate (Nethe-Jaenchen and Thauer, 1984). Assuming a similar maintenance and YATP, *Desulfuromonas acetoxidans* should have a net yield of around 0.6 mol ATP/mol acetate.

Cytochromes Sulfur-reducing bacteria of the genus *Desulfuromonas* contain large amounts of various cytochromes (Pfennig and Biebl, 1976; Bache et al., 1983). A triheme *c*-type cytochrome, referred to as cytochrome $c_{551\text{·}5}$ or c_7, has been characterized (Probst et al., 1977; Fiechtner and Kassner, 1979). A *c*-type cytochrome has been suggested to transport electrons to sulfur reductase in *Desulfuromonas* (Kröger et al., 1988). Indeed, the cytochrome $c_{551\wedge.5}$ was demonstrated to reduce polysulfide in *Desulfuromonas acetoxidans*, which is indicative of its function in terminal reduction (Pereira et al., 1997). In addition, the cytochrome $c_{551\wedge.5}$ from *Desulfuromonas acetoxidans* was shown to function in Fe(III) reduction (Roden and Lovley, 1993; Lojou et al., 1998); the final reduction of insoluble Fe(III)-minerals must occur in direct contact with the cell and therefore requires electron transport through the cytoplasm. The structural gene of cytochrome $c_{551\wedge.5}$ was cloned and heterologously overexpressed in *Desulfovibrio desulfuricans*. The purified recombinant cytochrome $c_{551\wedge.5}$ had the same biochemical and metal-reducing properties as the protein from *Desulfuromonas acetoxidans* (Aubert et al., 1998b). Structural analysis revealed strong analogies between the triheme cytochrome $c_{551\wedge.5}$ and the tetraheme cytochrome c_3. The region that harbors the heme II group in c_3 is not present in $c_{551\wedge.5}$. However, the orientation of the other three heme groups is very similar in the two cytochromes (Banci et al., 1996; Coutinho et al., 1996; Turner et al., 1997).

Recently two new *c*-type cytochromes were isolated from *Desulfuromonas acetoxidans*, a monoheme cytochrome *c* (M = 10 kDa) and a tetraheme cytochrome *c* (M = 50 kDa), both of which are located in the periplasm (Bruschi et al., 1997). Hexaheme and octaheme cytochromes also have been isolated from *Desulfuromonas acetoxidans* (Pereira et al., 1997).

Sulfur-Reducing Archaea The capacity to reduce elemental sulfur to sulfide is found in several genera of hyperthermophilic archaea (Stetter, 1996; Hedderich et al., 1999). If growth occurs with H_2 as electron donor (+CO_2), energy conservation can be only explained by a chemiosmotic process rather than by substrate-level phosphorylation. If such prokaryotes utilize alternatively organic compounds as electron donors (and carbon sources), one may assume that also the metabolism of these substrates involves chemiosmotic energy conservation during sulfur reduction. This section will primarily treat such sulfur-respiring or "true" sulfur-reducing archaea, as far as their biochemistry has been investigated. In addition, results from investigations on *Pyrococcus furiosus* are included; fermentative growth of this archaeon is stimulated by sulfur. Biochemistry of this species has been investigated intensively.

Reduction of Sulfur and Polysulfide Archaeons of the genus Pyrodictium grow chemolithotrophically by sulfur respiration at around 100°C (Fischer et al., 1983; Stetter et al., 1993). A H_2:sulfur-oxidoreductase complex was isolated from the membrane fraction of Pyrodictium abyssi isolate TAG11. This enzyme complex was shown to consist of nine polypeptides with an estimated total molecular mass of 520 kDa. The enzyme complex contains several uncharacterized [FeS] clusters, Ni and Cu ions, two cytochrome b and one cytochrome c. The enzyme complex is proposed to encompass hydrogenase- and sulfur-reductase activity as well as electron carrier components; the molecular arrangement is supposed to allow the coupling of S^0 reduction with H_2 to energy conservation (Dirmeier et al., 1998). The organization of the different components in such a large enzyme complex may allow stabilization of the interacting components and represent a strategy in hyperthermophiles to

perform sulfur respiration at temperatures of around 100°C.

Pyrococcus furiosus grows at 100°C by fermentation of carbohydrates to acetate, CO_2 and H_2. If sulfur is present in the medium, H_2S is produced in addition to H_2, and the growth yield increases (Fiala and Stetter, 1986; Schicho et al., 1993). There are doubts whether sulfur reduction is a respiratory, chemiosmotically coupled process. Sulfur may serve as an electron sink for certain dehydrogenations and render fermentation more effective. Growth with H_2 + sulfur as energy source has not been observed. An "H_2-evolving" hydrogenase was purified from *P. furiosus* which had a heterotrimeric (αβγ) structure and contained one [2Fe-2S] cluster and Ni (Bryant and Adams, 1989). Polysulfide can be reduced to H_2S by this hyperthermophile and is assumed to be the natural substrate during sulfur reduction (Blumenthals et al., 1990). Two different enzymes (sulfhydrogenase and sulfide dehydrogenase) were identified to catalyze reduction of sulfur in *P. furiosus*. The "bifunctional" sulfhydrogenase was isolated from the cytoplasm and shown to be identical with the aforementioned hydrogenase. Sulfhydrogenase can reduce both, sulfur and polysulfide, and oxidize H_2 (Ma et al., 1993). Isolation of the coding genes for sulfhydrogenase revealed that this enyzme actually consists of four subunits (β,γ,δ and α) encoded in a transcriptional unit, *hydBCDA*. Homology studies revealed a similarity of HydB and HydG with subunits of sulfite reductase from *Salmonella typhimurium* (Pedroni et al., 1995). Further biochemical and spectroscopic studies provided a more detailed insight into the molecular architecture of sulfhydrogenase and revealed that more [FeS] clusters were present in this enzyme than previously identified. The hydrogenase activity is localized at the αδ-subunits and the sulfur reductase activity at the βγ-subunits. Redox centers are proposed to be arranged as follows. Three [4Fe-4S] cubanes reside in the δ-subunit, two [4Fe-4S] cubanes in the β-subunit, one [2Fe-2S] cluster and one FAD in the γ-subunit and the NiFe center in the α-subunit (Arendsen et al., 1995; Silva et al., 1999a). Sulfide dehydrogenase, which was also identified in the cytoplasm, catalyzes the reduction of polysulfide to H_2S with NADPH as electron donor. This enzyme was found to have a heterodimeric structure and to contain flavin and four [FeS] centers (Ma and Adams, 1994). A possible physiological role of sulfhydrogenase and sulfide dehydrogenase is assumed to be that of an electron sink (Ma and Adams, 1994; Hedderich et al., 1999). During fermentative degradation of glucose to acetate, liberated electrons are transferred to ferredoxin by oxidoreductases (Schäfer and Schönheit, 1992; Mukund and Adams, 1991). Reoxidation of reduced ferredoxin could be directly achieved by sulfhydrogenase. Moreover, NADPH accumulating during glutamate fermentation (Robb et al., 1992) could be reoxidized by sulfide dehydrogenase.

The formation of high concentrations of H_2S from sulfur with H_2 or methanol has been observed in cultures of methanogens (Stetter and Gaag, 1983a). However, growth due to this reaction has not been demonstrated.

Metabolism of Organic Electron Donors

Carbohydrates Most of the archaeal sulfur-reducers grow either lithotrophically on H_2 or heterotrophically on complex substrates such as meat or yeast extracts. Only a few isolates like *Thermoproteus* species and *Pyrococcus* species were found to utilize defined carbohydrates (for review see Adams, 1994; Schönheit and Schäfer, 1995; Kengen et al., 1996; Hedderich et al., 1999).

The thermoacidophilic, sulfur-reducing archaeon *Thermoproteus tenax* utilizes, besides other substrates, glucose for growth by sulfur-respiration (Zillig et al., 1981; Fischer et al., 1983). Part of the glucose can be transiently stored as glycogen (König et al., 1982). Glucose in the energy metabolism is completely oxidized (Selig and Schönheit, 1994). Labeling experiments with [^{13}C]- and [^{14}C]-glucose and enzymatic studies demonstrated that *T. tenax* employs in parallel a modified Embden-Meyerhof-Parnas (EMP) pathway and the nonphosphorylated Entner-Doudoroff (ED) pathway to metabolize glucose (Siebers and Hensel, 1993; Selig et al., 1997). Of the two pathways, the EMP-pathway is used predominantly for glucose metabolism. It was suggested that the preference for one of the two pathways is regulated in response to physiological conditions (Schönheit and Schäfer, 1995). The key enzyme of the modified EMP-pathway in *T. tenax* is the PP_i-dependent phosphofructokinase (PP_i-PFK). In contrast to ATP-PFK, which is present in most organisms, the PP_i-PFK uses PP_i rather than ATP to phosphorylated fructose-6-phosphate. Purified PP_i-PFK from *T. tenax* was found to be a multimeric enzyme of ca. 100 kDa mass and not to be regulated by ATP, ADP or fructose-2,6-bisphosphate, the classical effectors of ATP-PFK (Siebers et al., 1998). Phylogenetic analysis of the PP_i-PFK encoding gene sequence demonstrated that the *T. tenax* PFK is of early descent (Siebers et al., 1997). Glucose dehydrogenase, which is the first enzyme of the ED pathway, was also purified from *T. tenax*. The active form of the enzyme is a homodimer with a total mass of 84 kDa and uses $NADP^+$ as cosubstrate for

glucose oxidation (Siebers et al., 1997). Decarboxylation of pyruvate to acetyl-CoA is catalyzed by pyruvate:ferredoxin oxidoreductase (Selig and Schönheit, 1994; Schönheit and Schäfer, 1995). This enzyme is also operative in *Pyrobaculum islandicum* and *Pyrococcus furiosus* (Schäfer and Schönheit, 1991). Further oxidation of acetate to CO_2 in *T. tenax* and *P. islandicum* involves the citric acid cycle (Selig and Schönheit, 1994).

The sugar metabolism in *Pyrococcus furiosus*, which has been intensively investigated, has many parallels to that in *Thermoproteus tenax* (Mukund and Adams, 1991; Schäfer and Schönheit, 1992; Kengen et al., 1994; Kengen et al., 1996; Schäfer et al., 1994). *P. furiosus*, in which sulfur reduction facilitates fermentation, has no capability for complete oxidation and forms acetate as an organic end product (Schönheit and Schäfer, 1995).

Peptides *Thermoproteus tenax* and *Pyrobaculum islandicum* (Huber et al., 1987) have been reported to utilize peptides with sulfur as electron acceptor. Considering the capacity of these two archaea to oxidize acetate completely to CO_2 via the citric acid cycle (Selig and Schönheit, 1994), it can be assumed that also peptides are completely oxidized to CO_2.

Autotrophic Carbon Assimilation *Thermoproteus neutrophilus* (Zillig et al., 1981; Fischer et al., 1983) is a facultative autotroph that can use either CO_2 or acetate as carbon source during growth on H_2 and sulfur. The pathway for CO_2 fixation was studied by ^{14}C-labeling experiments and measurement of enzyme activities. The key enzyme of the Calvin-cycle, ribulose-1,5-bisphosphate carboxylase (for summary see Watson and Tabita, 1997), was not detected in extracts of *T. neutrophilus* cells. Results rather suggested the presence of a reductive citric acid cycle (Schäfer et al., 1986). Enzyme activities corroborating this CO_2 fixation pathway, including the ATP citrate lyase, were subsequently demonstrated (Beh et al., 1993).

Acidianus is a genus of facultatively anaerobic Archaea that can grow aerobically by sulfur oxidation or anaerobically by sulfur reduction with H_2. In both cases, growth is autotrophic (Segerer et al., 1986; Segerer and Stetter, 1992). Enzyme studies with extracts of autotrophically grown *A. infernus* cells indicated that acetyl-CoA carboxylase and propionyl-CoA carboxylase function as the main CO_2-fixation enzymes. A 3-hydroxypropionate cycle is proposed for this organisms as route of CO_2 fixation (Menendez et al., 1999), where two moieties of CO_2 are fixed by the aforementioned enzymes, and glyoxylate is formed for further synthesis of organic compounds from malyl-CoA, while acetyl-CoA is concomitantly regenerated. This pathway has originally been detected in the phototrophic bacterium *Chloroflexus aurantiacus* (Holo, 1989; Strauß et al., 1992).

Detoxification of Superoxide Superoxide reductase (SOR), was purified from *Pyrococcus furiosus*, and proposed to function in scavenging superoxide via a net reduction to H_2O_2 rather than via dismutation to H_2O_2 and O_2 as is known from superoxide dismutase (Jenney et al., 1999). Reduced rubredoxin is suggested as the primary source of reducing power for SOR. Reduction of rubredoxin is catalyzed by NAD(P)H:rubredoxin oxidoreductase that has also been purified from *P. furiosus* (Ma and Adams, 1999).

Reductive scavenging of superoxide appears to be a widespread mechanism in anaerobes to protect against the deleterious superoxide species. Homologs of the SOR encoding gene have been identified in many complete genomes of anaerobic microorganisms, but not in those of aerobic organisms. In contrast, genes coding for superoxide dismutase are not generally present in anaerobic microorganisms. The SOR encoding gene also shows homology to the redox proteins desulfoferredoxin and neelaredoxin from *Desulfovibrio desulfuricans* and *D. gigas*, respectively (Jenney et al., 1999).

Microorganisms Reducing Sulfur Compounds Other Than Sulfate or Sulfur

Bacteria The capacity for the dissimilatory reduction of sulfur compounds other than sulfate and sulfur, especially sulfite and thiosulfite, has been frequently observed among sulfate-reducing microorganisms and also among some sulfur-reducing microorganisms. However, there are also prokaryotes that neither reduce sulfate nor elemental sulfur but instead utilize other sulfur compounds as electron acceptors.

There are several reports on tetrathionate and thiosulfate reduction in bacteria other than sulfate or sulfur reducers (Barrett and Clark, 1987). A reduction of these sulfur species seems to be abundant especially among enterobacteria. Often, the capacities to reduce tetrathionate and thiosulfate coincide, which is explained by one reductase for both compounds (Oltmann et al., 1975; Barrett and Clark, 1987). Tetrathionate is first reduced to thiosulfate. In *Citrobacter* and *Proteus*, there is evidence for an electron transport chain to tetrathionate allowing respiratory

energy conservation (Oltmann et al., 1975; Novotny and Kapralek, 1979); the growth substrates were sugars. A complete oxidation (viz. acetyl-CoA oxidation) associated with tetrathionate reduction has not been reported for the enterobacteria. Formed thiosulfate may be further reduced to sulfide and sulfite, the latter being often an end product that is not reduced further. For energetic reasons, such an incomplete reduction of thiosulfate probably does not allow a chemiosmotic process and thus appears to be a by-reaction. Suspensions of the phototroph, *Thiocapsa floridana*, reduced thiosulfate with endogeneous hydrogen donors in the dark (Trüper and Pfennig, 1966). A marine *Pseudomona*s-like strain grew anaerobically on lactate in the presence of thiosulfate or sulfite and formed sulfide. Lactate alone was not utilized (Tuttle and Jannasch, 1973). It is unknown whether sulfite reduction was of a respiratory type or just a facilitated fermentation. Reduction of thiosulfate or sulfite, probably as a mere hydrogen sink, has also been observed in mesophilic and thermophilic saccharolytic clostridia. The sulfite reductase in *Clostridium pasteurianum* was induced by sulfite and distinctive from the assimilatory enzyme (Harrison et al., 1984). Even yeast cells catalyzed a reduction of thiosulfate to sulfite and sulfide, and of sulfite to sulfide (Neuberg and Welde, 1914; Hollaus and Sleytr, 1972; McCready and Kaplan, 1974; Stratford and Rose, 1985); sulfite reduction was observed in an aerated culture. The reductions seemed to be by-reactions.

An organic sulfur compound used by several bacteria as electron acceptor is $(CH_3)_2SO$, dimethylsulfoxide (DMSO), which is reduced to dimethylsulfide (DMS). The utilization of DMSO was first shown in the phototroph *Rhodobacter capsulatus* that did not grow anaerobically on sugars in the dark unless the acceptor was added (Yen and Marrs, 1977). It was first assumed that DMSO serves merely as a H_2 sink allowing substrate-level phosphorylation (Madigan and Gest, 1978; Madigan et al., 1980). Later, however, *Rhodobacter capsulatus* and also *Rhodospirillum rubrum* were reported to grow by DMSO reduction most likely in a respiratory manner (Schultz and Weaver, 1982). Among other substrates, also acetate allowed anaerobic growth in the dark when DMSO was present. Earlier, a definitive respiratory DMSO reduction had been already shown with a *spirillum* isolated with lactate as electron donor (Zinder and Brock, 1978a). The *spirillum* grew with H_2 if acetate as a carbon source and some yeast extract as sulfur source for assimilation were present. In addition to DMSO, the organism reduced sulfur, sulfite and thiosulfate and resembled *Desulfospirillum delyianum* (*spirillum* 5175) isolated with sulfur (Wolfe and Pfennig, 1977). The latter also turned out to reduce DMSO (N. Pfennig, personal communication). Anaerobic growth due to DMSO reduction with H_2 was also observed with *Escherichia coli* (Yamamoto and Ishimoto, 1978). The obvious respiratory character of DMSO reduction was confirmed by measurements with H_2 and glycerol (Bilous and Weiner, 1985). However, as with nitrate (Thauer, 1988), there is no evidence that DMSO is an electron acceptor for acetyl-CoA oxidation in *E. coli*; the citrate cycle is not operative in *E. coli* under anoxic conditions, and acetate is an end product. Some other enterobacteria, *Pseudomonas aeruginosa* and *Bacillus subtilis*, reduced DMSO in complex glucose medium (Zinder and Brock, 1978b). However, DMSO reduction seemed to be not very effective (<0.5 mM/day). Bacteria reducing DMSO usually can also reduce trimethylamine-N-oxide (TMAO).

Archaea The archaeon *Archaeoglobus veneficus*, a phylogenetic relative of sulfate-reducing *Archaeoglobus* species cannot reduce sulfate but only sulfite (Huber et al., 1997). *A. veneficus* is the only hyperthermophile reported so far to grow with free acetate as electron donor and organic carbon source.

Genetic Systems

Approaches from molecular biology and genetics have been applied in the study of sulfate-reducing and sulfur-reducing microorganisms since the 1980s. These approaches included cloning and heterologous expression of genes for the analysis of sequences, operon structures and confirmation of gene products; in addition, several possibilities for genetic manipulation of sulfate- and sulfur-reducing bacteria have been established to study genes and gene products in homologous systems and thus in a natural, functional context.

Sulfate-Reducing Bacteria

Genome Sizes Bacterial and archaeal genomes range between 0.6 to 8.7 Mb, which are the sizes as determined for *Mycoplasma genitalium* and *Bradyrhizobium japonicum*, respectively (Cole and Girons, 1994). Early studies using two-dimensional electrophoresis to separate fragments of double-digested genomic DNA suggested genome sizes of 1.6 Mb for *Desulfovibrio gigas* and 1.7 Mb for *Desulfovibrio vulgaris* (Postgate et al., 1984c). Later on, however, application of pulsed-field gel electrophoresis (PFGE) of linearized, full-length chromosomal

DNA revealed genome sizes of 3.1, 3.6 and 3.7 Mb in *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* and *Desulfobulbus propionicus*, respectively (Devereux et al., 1997). Thus it appears that genomes of sulfate-reducing bacteria are considerably larger than the 2.2 Mb genome of the archaeal sulfate reducer *Archaeoglobus fulgidus* (Klenk et al., 1997).

Cloning, Sequencing and Expression of Genes The *hydAB* genes (coding for hydrogenase) from *Desulfovibrio vulgaris* (Hildenborough) were the first genes from a sulfate-reducing bacterium that were cloned, sequenced and expressed in *E. coli* (Voordouw and Brenner, 1985a; Voordouw et al., 1985b). Expression of the cloned *hyd* genes in *E. coli* yielded proteins, the N-terminal sequence of which corresponded to the sequence of the cloned gene. However, the expressed *Desulfovibrio* hydrogenase was inactive. Evidence that the transcriptional/translational machinery of *E. coli* can in principle produce functional enzymes from *D. vulgaris* genes was provided by complementation experiments with a gene from the biosynthetic machinery. The auxotrophic *E. coli* $PyrF^{-}$ mutant carries a disrupted orotidine-5'-phosphate decarboxylase and is therefore deficient in the biosynthesis of pyrimidines. Complementation of the *E. coli* $PyrF^{-}$ mutant with the structural gene from *D. vulgaris* abolished the auxotrophic phenotype (Li et al., 1986). Subsequently, many genes coding for catabolic enzymes, regulatory proteins or electron carriers were cloned, sequenced and expressed (Voordouw, 1993a). They have already been mentioned in the subsection on Physiology, Biochemistry and Molecular Biology of Sulfate-Reducing Bacteria. Genes cloned from sulfate-reducing bacteria are listed in Table 8.

Physiological and Practical Prerequisites for Genetic Studies in Sulfate-Reducing Bacteria Studies aiming at genetic manipulation of sulfate-reducing bacteria have mostly been carried out thus far with *Desulfovibrio desulfuricans* and *D. vulgaris* strains because various proteins in these species have been studied in great detail and because these species can be cultivated relatively easily. Other practical reasons for choosing these species were high plating efficiencies and antibiotic sensitivities for potential selection of mutants (Voordouw and Wall, 1993b; van Dongen et al., 1994).

In the application of plating techniques for sulfate-reducing bacteria, anoxic growth conditions have to be guaranteed. van den Berg et al. (1989) developed a plating technique with a recovery of 50 to 100%. Aerobically prepared agar plates were stored under an anoxic atmosphere until use. *Desulfovibrio vulgaris* was then plated under oxic conditions in an agar overlay of 3 ml. Immediately after solidification of the agar, the plates were incubated under argon $(Ar)/CO_2$ in the presence of $Na_2S_2O_4$ as O_2 scavenger in the incubation container. Hence, anoxic conditions were necessary during growth, but not during plating.

The presence of alternative modes of energy conservation is important for genetic studies of catabolic capacities. *Desulfovibrio* species can gain energy either from a respiratory metabolism with H_2 or organic electron donors and with sulfate or sometimes nitrate as electron acceptors (Table 1), or from fermentative degradation of pyruvate. This allows the genetic inactivation of genes involved in one process while other processes are still operative to sustain viability. A mutant of *Desulfovibrio desulfuricans* was generated by chemical mutagenesis that could no longer use H_2 for the reduction of sulfate, but could still grow with lactate and sulfate (Odom and Wall, 1987).

The use of DNA inserts with resistance markers and application of antibiotics is a common method of generating and selecting mutants. Even though sulfate-reducing bacteria are generally resistant to many antibiotics (Saleh et al., 1964), some antibiotics proved to be useful. Plasmid-borne resistance to streptomycin and sulfonamides was expressed in *Desulfovibrio desulfuricans* (Powell et al., 1989), and resistance to chloramphenicol in *D. vulgaris* (van den Berg, 1989).

Delivery Systems for DNA A prerequisite for defined genetic manipulation is the availability of tools to deliver genetic information into cells. All tools commonly used in genetic studies of other microorganisms have also been applied to sulfate-reducing bacteria.

Transduction Knowledge about the occurrence of bacteriophages in sulfate-reducing bacteria and their applicability for transduction is limited. Handley et al. (1973) presented first evidence for bacteriophages in sulfate-reducing bacteria. They observed bacteriophage-like particles in cultures of *Desulfovibrio vulgaris* that had been treated with mitomycin C. A defective bacteriophage, termed Dd1, was demonstrated to mediate transduction in *Desulfovibrio desulfuricans* strain ATCC (Rapp and Wall, 1987). Mixing and incubating *D. desulfuricans* strains resistent to either rifampicin or nalidixic acids resulted in the development of colonies simultaneously resistant to both antibiotics at higher rates than expected for spontaneous mutations. The phage Dd1 was identified as the transducing agent of

Table 8. Genes cloned and characterized from sulfate-reducing bacteria.[a]

Gene(s) product	Gene(s) designation	Organism[b]	References
[Fe]hydrogenase	*hydA, hydB*	*D. vuigaris* (Hildenborough)	Voordouw and Brenner, 1985
			Voordouw et al., 1985
		D. vulgaris subsp. *oxamicus* (Monticello)	Voordouw et al., 1989a
		D. desulfuricans	Hatchikian et al., 1999
		D. fructosovorans	Casalot et al., 1998
Protein, unknown function	*hydC*	*D. vulgaris* (Hildenborough)	Stokkermans et al., 1989
			Voordouw et al., 1989a
[NiFe]hydrogenase	*hynA, hynB*	*D. gigas*	Li et al., 1987
			Voordouw et al., 1989b
		D. vulgaris (Miyazaki F)	Deckers et al., 1990
		D. fructosovorans	Rousset et al., 1990
	hynC	*D. fructosovorans*	Rousset et al., 1993
[NiFeSe]hydrogenase	*hysA, hysB*	*Desulfomicrobium baculatum*	Menon et al., 1987, 1988
			Voordouw et al., 1989b
NADP-reducing hydrogenase	*hndA, B, C, D*	*D. fructosovorans*	Malki et al., 1995
Cytochrome c_3	*cyc*	*D. vulgaris* (Hildenborough)	Voordouw and Brenner, 1986
	CycD	*D. desulfuricans* (Norway)	Aubert et al., 1997
Acidic cytochrome c_3		*D. africanus*	Magro et al., 1997
Basic cytochrome c_3		*D. africanus*	Magro et al., 1997
Cytochrome *c*		*Desulfomonile tiedje* (DCB-1)	Louie et al., 1997
Cytochrome c_{553}		*D. vulgaris* (Hildenborough)	van Rooijen et al., 1989
High-molecular-mass cytochrome *c* (Hmc)	*hmc*	*D. vulgaris* (Hildenborough)	Pollock et al., 1991
Nonaheme cytochrome *c*	*ddE*	*D. desulfuricans* (Essex 6)	Fritz, 1999
The *hmc* operon, potential transmembrane redox protein complex	*hmc*, Orf2–6, Rrf1–2	*D. vulgaris* (Hildenborough)	Rossi et al., 1993
Flavodoxin	*fla*	*D. vulgaris* (Hildenborough)	Voordouw, 1988a
			Curley and Voordouw, 1988
			Krey et al., 1988
			Carr et al., 1990
		D. vulgaris (Miyazaki F)	Kitamura et al., 1998
		D. salexigens	Helms et al., 1990
		D. desulfuricans	Helms and Swenson, 1991
Ferredoxin		*D. gigas*	Chen et al., 1994b
Rubredoxin	*rub*	*D. vulgaris* (Hildenborough)	Voordouw, 1988a
			Brumlik and Voordouw, 1989
		D. vulgaris (Miyazaki F)	Kitamura et al., 1997
		Desulfoarculus baarsii	Pianzzola et al., 1996
Rubredoxin oxidoreductase	*rbo*	*D. vulgaris* (Hildenborough)	Brumlik et al., 1989
		Desulfoarculus baarsii	Piznzzola et al., 1996
Desulforedoxin	*dsr*	*D. gigas*	Brumlik and Voordouw, 1990
OMPase (orotidine-5′-phosphate decarboxylase)	*pyrF*	*D. vulgaris* (Hildenborough)	Li et al., 1986
Nitrogenase, Fe protein	*nifH*	*D. gigas*	Postgate et al., 1988
			Kent et al., 1989
*Dde*I, restriction endonuclease, methylase	*hsdM, hsdR*	*D. desulfuricans*	Howard et al., 1986
			Sznyter et al., 1987
Rubrerythrin	*rbr*	*D. vulgaris* (Hildenborough)	Prickril et al., 1991
Methyl-accepting chemotaxis proteins, DcrA-L	*dcrA-L*	*D. vulgaris* (Hildenborough)	Dolla et al., 1992
			Deckers and Voordouw, 1994a
			Deckers and Voordouw, 1996
APS reductase	*aprBA*	*D. desulfuricans* (Essex)	Fritz, 1999
Assimilatory sulfite reductase	*asr*	*D. vulgaris* (Hildenborough)	Tan et al., 1991
Cytochrome c oxidase-like protein		*D. vulgaris* (Miyazaki F)	Kitamura et al., 1995
Selenocysteine-inserting tRNA ($tRNA^{Sec}$)	*selC*	*Desulfomicrobium baculatum*	Tormay et al., 1994
MOP, molybdenum-containing aldehyde oxido-reductase,	MOP gene	*D. gigas*	Thoenes et al., 1994
FOR, pyruvate-ferredoxin oxidoreductase	*por*	*D. africanus*	Pieulle et al., 1997
Proline and leucine biosynthesis		*D. desulfuricans* (Norway)	Fons et al., 1987

[a]This table has been modified from Voordouw (1993)
[b]The genus *Desulfovibrio* is abbreviated with *D*.

the resistance markers and found to resemble morphologically the coliphages T7 and T3. A practical limitation to the use of bacteriophage Dd1 is its inability to transfer resistance to other strains of *Desulfovibrio desulfuricans* or to other *Desulfovibrio* species (Voordouw and Wall, 1993b). A different bacteriophage was isolated from marine sediment that could lyse cells of *Desulfovibrio salexigens*, as demonstrated by plaque formation. Morphological and molecular characteristics suggested a relation of the isolate to the lysogenic bacteriophage λ but the potential of this phage for genetic transfer was not investigated (Kamimura and Araki, 1989). Treatment with UV light allowed the induction of lytic bacteriophages in *Desulfovibrio vulgaris* (Hildenborough). Subsequent mapping studies indicated the presence of two prophages in *D. vulgaris* (Seyedirashti et al., 1991; Seyedirashti et al., 1992).

Conjugation Conjugation has been used several times to transfer broad host-range plasmids belonging to the incompatibility group Q (IncQ) from *Escherichia coli* to *Desulfovibrio* species. Derivatives of plasmid pSUP104 were transferred with a frequency of about 10^{-2} from *Escherichia coli* to *Desulfovibrio vulgaris*. Stable maintenance of the plasmids in *D. vulgaris* could be demonstrated (van den Berg et al., 1989). At the same time, Powell et al. (1989) reported on a retrotransfer of plasmid R300B from *Desulfovibrio* back to *E. coli*. The cytochrome c_3-encoding *cyc* gene from *Desulfovibrio vulgaris* (Hildenborough) was ligated into plasmid pJRDC800-1, transferred by conjugation from *E. coli* to *Desulfovibrio desulfuricans* G200 and then functionally expressed in the new host (Voordouw et al., 1990). Interestingly, broad host-range plasmids from the incompatibility group P and W (IncP and IncW) could not be transferred to *D. desulfuricans* G100A (Argyle et al., 1992). This finding pointed at the specificities in the ability of *Desulfovibrio* to receive or maintain broad host-range plasmids.

Endogenous Plasmids The presence of plasmids in several *Desulfovibrio* species was previously reported (Postgate et al., 1984a; Postgate et al., 1986). *Desulfovibrio gigas* (NCIMB 9332) carries two plasmids of the sizes 105 kb and 60 kb, and *D. vulgaris* (Hildenborough; NCIMB 8303) a single plasmid of 195 kb. *D. desulfuricans* strain Berre sol and *D. vulgaris* strain Wandle both carry a single plasmid. No plasmids could be detected in 10 other *Desulfovibrio* species, including *D. salexigens* and *D. africanus.*

A small 2.3 kb plasmid, designated pBG1, was isolated from *Desulfovibrio desulfuricans* strain G100A and sequenced (Wall et al., 1993). Plasmid pBG1 was present in about 20 copies per genome. This plasmid replicates in *D. desulfuricans* strain G100A and *D. fructosovorans,* but not in *Escherichia coli*. The analysis of the sequence of plasmid pBG1 allowed a replicon area to be assigned to a sequence. Integration of pBG1 fragments that included this small replicon into derivates of the IncQ plasmid RSF1010 generated composite plasmids that were stable and replicated in *E. coli*, *D. desulfuricans* strain G100A and *D. fructosovorans*. Several recombinant plasmids that are most effective in *Desulfovibrio* were constructed that carried genes for resistance to antibiotics, e.g., chloramphenicol. These plasmids could be transferred by either electroporation or conjugation (Rousset et al., 1998a).

Transformation The first successful transformation of a sulfate-reducing bacterium was reported by Rousset et al. (1991). A recombinant plasmid belonging to the IncQ group was transformed into *Desulfovibrio fructosovorans* by means of electroporation (Dower et al., 1988). Transformation via electroporation has also been successfully applied to *Desulfovibrio desulfuricans* (Aubert et al., 1998b).

Creation of Mutants Stability and function of recombinant DNA introduced into *Desulfovibrio* species or other sulfate-reducing bacteria could be hampered in principle by an endogenous restriction/modification system. Only little is known about these systems in sulfate-reducing bacteria. Two restriction endonucleases, DdeI and DdeII were discovered in *D. desulfuricans* (Norway). Genes coding for DdeI endonuclease and methylase were identified (Howard et al., 1986; Sznyter et al., 1987).

Chemical Mutagenesis Mutants of *Desulfovibrio desulfuricans* strain ATCC 27774 that were deficient in H_2 utilization but could still grow on lactate were generated by exposure to UV light (Odom and Wall, 1987).

Transposon Mutagenesis Transposons can insert principally near or in any gene on the chromosome and completely annihilate gene function as a result of gene disruption (Maloy et al., 1996). Wall et al. (1996) developed a transposon-based method for random mutagenesis in *Desulfovibrio desulfuricans* strain G20. No evidence was found for transposition of wild-type transposon Tn5, Tn9 and Tn10 in this sulfate reducer. The transposon Tn7 was found to insert into the genome of *D. desulfuricans* (G20) with a frequency of 10^{-4} to 10^{-3}. The transposon Tn7 is

target specific and inserts specifically into the attTn7 site. This directed insertion is mediated by the Tn7-encoded TnsD protein (Craig, 1991; Bainton et al., 1993). Southern blot analysis demonstrated that transposon Tn7 inserted also in *D. desulfuricans* strain G20, specifically into the attTn7 site. By inactivation of the *tnsD* gene, Wall et al. (1996) were able to create a mutated Tn7, designated Tn7-IN1, that transposed randomly in the chromosome at a frequency of 10^{-6}. An improved version of the transposon, designated Tn7K-IN1, carried a kanamycin-resistance cassette and mediated a Knr-phenotype after transposition. This transposon as part of gene constructs allows the integration of single copies of recombinant genes at neutral spots in the chromosome. Plasmids used for the delivery of Tn7, its derivatives and adjacent genes by conjugation are not maintained in *D. desulfuricans*.

The insertion element ISD1, which was discovered in *D. vulgaris* strain Hildenborough, is the first described transposable element detected in sulfate-reducing bacteria (Fu and Voordouw, 1998). This element is 1.2 kb in length, encodes a transposase and can actively transpose into different sites on the genome of *D. vulgaris* strain Hildenborough. Sequence analysis revealed a relation of ISD1 to the IS3 family. Members of the IS3 family have been isolated from Gram-negative and Gram-positive bacteria (Fayet et al., 1990). ISD1 may be used as a platform to generate artificial transposons for random mutagenesis in *Desulfovibrio* species.

Gene Deletion Deletion of the *hydN* gene (coding for the [NiFe]-hydrogenase) in *Desulfovibrio fructosovorans* by marker-exchange mutagenesis was the first report of a successful gene replacement in sulfate-reducing bacteria (Rousset et al., 1991). The cloned *hydN* gene was replaced by a kanamycin-resistance cassette while the conserved flanking regions were maintained to allow homologous recombination. In the applied procedure, an IncQ plasmid was used as shuttle vector and transferred to *D. fructosovorans* by electroporation. Replacement of the chromosomal *hydN* gene with the marker was verified by expression of kanamycin resistance and a 90% decrease of hydrogenase activity. Furthermore, analysis by southern hybridization confirmed the gene replacement. In subsequent studies, single and double mutants of the $NADP^+$-reducing hydrogenase were generated in *D. fructosovorans* by this method of marker exchange mutagenesis (Malki et al., 1997).

Fu and Voordouw (1997) developed a method for gene replacement based on a suicide plasmid in *Desulfovibrio vulgaris* strain Hildenborough to study the oxygen sensor DcrA. A suicide-integration plasmid (pΔDcrA2CTB) was constructed from an IncQ plasmid carrying the cloned *dcrA* gene. The vector carried a *dcrA* allele disrupted by the cat gene (conferring resistance to chloramphenicol) and contained the counterselectable marker *sacB* (coding for levansucrase; Gay et al., 1983) from *Bacillus subtilis*. Plasmid pΔDcrA2CTB was transferred from *E. coli* strain S17-1 to *D. vulgaris* by conjugation. Integration of plasmid pΔDcrA2CTB into the chromosomal *dcrA* gene by the first event of homologous recombination was selected for by the presence of chloramphenicol. Addition of sucrose to the growth medium then selected for the second homologous recombination which resulted in excision of the plasmid from the chromosome and the replacement of the *dcrA* gene. In the presence of sucrose, the gene product of *sacB* is toxic for *E. coli* and other Gram-negative bacteria (Gay et al., 1983) and has therefore widely been used as a counterselectable marker for the rare second recombination event which yields clones cured from plasmid (Ried and Collmer, 1987; Blomfield et al., 1991). The genes of the *hmc* operon (coding for the high-molecular-mass cytochrome redox protein complex, the Hmc-complex) and the *rbo* gene (like *dcrA* related to oxygen sensitivity), both of *D. vulgaris*, were also deleted employing the aforementioned method (Keon et al., 1997; Voordouw and Voordouw, 1998).

Site-Directed Mutagenesis Site-directed mutagenesis was applied to study the signal-peptide consensus box of [NiFe] hydrogenase of *Desulfovibrio vulgaris* (Hildenborough) in a fusion protein with β-lactamase from *E. coli* (Nivière et al., 1992). Exchange of an arginine residue in the consensus box for a glutamate prevented export of the fusion protein from the cytoplasm of *E. coli*.

Sulfur-Reducing Bacteria

Genome Sizes, Genomic Libraries and Cloning of Genes The genome sizes of *Desulfurella acetivorans* and *D. multipotens* were reported to be around 1.9 Mb (Pradella et al., 1998).

Genomic libraries of *W. succinogenes* were constructed using the bacteriophage (EMBL-3 (Frischauf et al., 1983; Lauterbach et al., 1987). From these, subcloning of several genes was possible. These were *frd* genes coding for fumarate reductase (Lauterbach et al., 1987); *fdh* genes coding for formate dehydrogenase (Bokranz et al., 1991); *psr* genes coding for polysulfide reductase (Krafft et al., 1992); and *sud* gene coding for the periplasmic sulfide dehydrogenase (Kreis-Kleinschmidt et al., 1995).

Physiological and Practical Prerequisites for Genetic Studies in Sulfur-Reducing Bacteria The sulfur-reducing bacterium *Wolinella succinogenes* can grow on agar plates when anoxic conditions are maintained. In addition, antibiotics can be used as selection markers, because *W. succinogenes* is sensitive to, for instance, kanamycin. Transformation is accomplished by means of electroporation and has to be performed under anoxic conditions like plating. The broad host-range plasmid pBR322 (Bolivar et al., 1977), which is commonly used as shuttle vector in Gram-negative bacteria (Maloy et al., 1996), can be used to transfer recombinant DNA from *E. coli* back to *W. succinogenes*.

Gene Deletion For gene deletion, plasmid-hosted genes from *W. succinogenes* are disrupted by antibiotic-resistance cassettes (e.g., kanamycin) leaving homologous regions to both sides of the marker. Deletion of the chromosomal target genes is then accomplished by homologous recombination. Several genes have been deleted in *W. succinogenes* by this procedure: *psr* genes coding for polysulfide reductase (Krafft et al., 1995); the *sud* gene coding for periplasmic sulfide dehydrogenase (Kotzian et al., 1996); fdh genes coding for formate dehydrogenase (Lenger et al., 1997); *frd* genes coding for fumarate reductase (Simon et al., 1998); and hyd genes coding for hydrogenases (Gross et al., 1998a; Gross et al., 1998b).

Sulfur-Reducing Archaea

At present, only initial steps in the establishment of systems for genetic manipulation of sulfur-reducing archaea have been undertaken. In contrast, a variety of genetic tools have already been developed for halophilic archaea (e.g., Cline and Doolittle, 1987). Nevertheless various genetic elements have been discovered in archaeal sulfur reducers and new, composite shuttle vectors are being developed.

Several types of viruses were discovered in *Thermoproteus* species. A plasmid, termed pDL10, was found to be present in *Desulfurolobus* species (Zillig et al., 1996). Plasmid pGT5 was isolated from Pyrococcus abyssi (Erauso et al., 1996). A mobile intron from *Desulfurococcus mobilis* could be transferred to and established in *Sulfolobus acidocaldarius* (Aagaard et al., 1995).

A new hybrid shuttle vector, designated pAG1, was constructed by combining portions of the archaeal plasmid pGT5 with the bacterial plasmid pUC19. The plasmid pAG1 was stably maintained and propagated both in bacteria and archaea (Aravalli and Garrett, 1997). A different strategy to create a new shuttle vector is to incorporate the mobile intron from *Desulfurococcus mobile* into the bacterial vector pUC18 (Aagaard et al., 1996).

Acknowledgements and Contact. This work was supported by the Max-Planck-Gesellschaft and the Fonds der Chemischen Industrie.

We thank Heribert Cypionka (Oldenburg), Christine Dahl (Bonn), Günter Fritz (Konstanz), Achim Kröger (Frankfurt), Peter Kroneck (Konstanz), Bernhard Schink (Konstanz) and Rolf Thauer (Marburg) for providing informative material.

We would very much appreciate any type of comments and further information that may help to improve future releases of this article. Comments and information may be sent via email to Dr. Rabus or to Dr. Widdel.

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Prokaryotes (2006) 2:769–792
DOI: 10.1007/0-387-30742-7_23

CHAPTER 1.23

The Denitrifying Prokaryotes

JAMES P. SHAPLEIGH

Introduction

One of the hallmarks of bacterial physiology is the ability to use a wide variety of substrates as oxidants or terminal oxidants for respiration. Because of its high redox potential and prevalence, oxygen is generally the preferred reductant for respiratory bacteria. However, bacteria can utilize many other compounds as terminal respiratory oxidant. One of these compounds is nitrate. Nitrate respiration occurs via two dissimilar pathways that utilize the same initial substrate but produce different end products. One of these pathways, termed ammonification, is carried out by bacteria such as *Escherichia coli*, and is marked by reduction of nitrate to nitrite and then to ammonia.

The second pathway of nitrate respiration is denitrification, which is the reduction of nitrate to gaseous nitrogen oxides, principally nitrogen gas (Fig. 1). The initial step in denitrification is the reduction of nitrate to nitrite, as occurs in ammonification. In the next step, the defining reaction, nitrite is reduced to nitric oxide, a gaseous nitrogen oxide. This conversion of a fixed, non-gaseous form of nitrogen to gaseous forms has led this respiratory process to be termed "denitrification" because biologically preferred forms of nitrogen are lost. Once nitric oxide is produced, it is further reduced to nitrous oxide and then to nitrogen gas. The production of nitrogen gas connects denitrification to the nitrogen cycle via nitrogen fixation. The ammonia produced by nitrogen fixation can be converted by nitrifying bacteria to nitrite and nitrate, the substrates of denitrification. This series of reductions and oxidation reactions constitute the nitrogen cycle (Fig. 2).

Gayon and Dupetit carried out the first systematic study of nitrate conversion to gaseous forms of nitrogen in 1882 (Gayon, 1882). Noting the loss of nitrate from decomposing sewage, they called it "denitrification" and were the first to isolate denitrifying bacteria (Gayon, 1886), which they dubbed *Bacterium denitrificans* α and β. In the early stages of the study of denitrification, it was erroneously assumed that nitrate was releasing and thus supplying elemental oxygen to organisms that subsequently carried out a reaction equivalent to oxygen respiration. The observation of denitrification, although biologically significant, was disquieting to agronomists who soon realized that the addition of organic matter to soils could lead to the loss of fixed nitrogen. The agricultural importance of the process provided the impetus for much of the early work on denitrification, and by the end of the 19th century, denitrification had been reasonably well-defined.

In the 20th century, a significant interest in the agricultural consequences of denitrification has continued. However, with the realization that nitric oxide and nitrous oxide play important roles in atmospheric and biological chemistry, research emphases have shifted to the environmental consequences of denitrification and the molecular mechanisms of enzymes and gene regulation.

Defining the Denitrifiers

Prokaryotes (mostly Bacteria, but a few Archaea) constitute the vast majority of organisms capable of denitrification. A number of fungal isolates carry out reduction of nitrate to nitrous oxide, but the contribution of this reduction to cell growth is variable (Usuda, 1995). In prokaryotes and a few filamentous fungi, the reduction of nitrate to gaseous intermediates is a respiratory process. That is, reduction of nitrate is coupled to ATP synthesis via electron transport chains. With one or two exceptions, denitrifiers can also respire with oxygen as the terminal electron acceptor and, because it is usually available at higher concentrations, oxygen is typically the preferred electron acceptor. However, when oxygen becomes limiting, the capacity to utilize nitrate as a terminal oxidant allows denitrifying bacteria to continue respiration using an alternative electron acceptor.

The reduction of nitrate to nitrogen gas is a multi-step process (Fig. 1). The redox couple for each reduction step is greater that 0.35 V, making

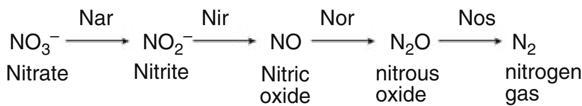

Fig. 1.

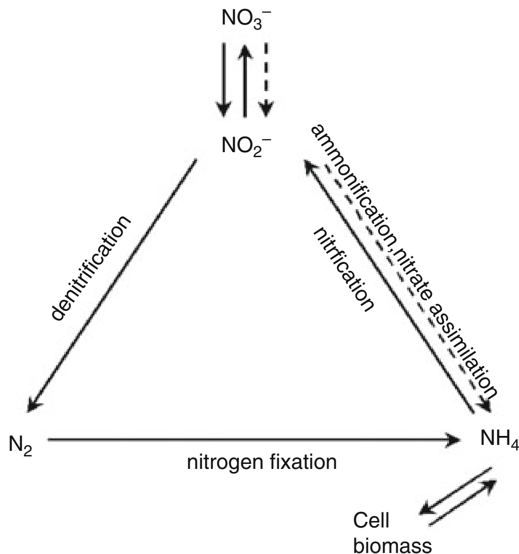

Fig. 2.

denitrification energetically comparable to oxygen reduction. Because each nitrogen oxide reduction has a positive redox couple, every step in denitrification need not be carried out to achieve a net conservation of energy. In fact, it is quite common to isolate bacteria that express only portions of the denitrification electron transport chain. Those prokaryotes that contain partial denitrification chains will be included in this chapter provided these organisms can metabolize the gaseous intermediates in denitrification, nitric oxide and nitrous oxide. So, a bacterium such as *E. coli*, which can reduce nitrate and nitrite, is not considered a denitrifier because it reduces nitrite to ammonia. Conversely, a bacterium such as *Wolinella succinogenes*, even though it also reduces nitrate to ammonia, is included in this review because it also uses nitrous oxide as sole terminal oxidant (Yoshinari, 1980).

Denitrification is a widely dispersed metabolic pathway of prokaryotes. Table 1 shows a list of prokaryotic genera, some of which are denitrifiers. A list of prokaryotic genera suggested to include dentrifying strains is shown in Table 1. This has probably led to an underestimate of the number of denitrifying strains. It is frequently concluded that, if one strain does not denitrify, neither will its close relatives. The essential criterion for inclusion in this list is the metabolism of gaseous nitrogen oxides. In most cases, this means the production of nitrous oxide or nitrogen gas from reduction of nitrate. However, all the strains in this list have not been shown to grow as denitrifiers. Several strains appear to have the capacity to reduce only nitrite or nitric oxide, making them difficult to culture under laboratory conditions.

Archaea

Only a few Archaea capable of denitrification have been isolated. With one exception, all the known archaeal species that have been isolated are also capable of aerobic respiration. Aerobic respiration is relatively frequent among some of the halophilic Archaea, which includes most of the denitrifying Archaea. The denitrification components of these halophilic bacteria have not been characterized extensively. A copper-containing nitrite reductase has been purified from *Haloferax denitrificans* and was shown to be spectroscopically similar to related eubacterial nitrite reductases; but antiserum to the copper-type nitrite reductase from the denitrifier "*Achromobacter cycloclastes*" did not react with the archaeal nitrite reductase (Inatomi, 1996). More recently, other non-halophilic Archaea have been found which are capable of denitrification. One of these is *Pyrobaculum aerophilum*, a hyperthermophile (Volkl, 1993). The genome of this organism is currently being sequenced and information from this effort will be of greatest interest since no genes whose products are required for dentrification have ever been sequenced from an Archaea or a thermophile. The other potential denitrifier from among the Archaea is the strict anaerobe *Ferroglobus placidus* (Hafenbradl, 1996). This bacterium can couple Fe^{++} oxidation to nitrate reduction. Studies on this bacterium have shown cell extracts can reduce nitrite to nitrous oxide, with nitric oxide as an intermediate (Vorholt, 1997). Even though this evidence strongly suggests this organism is a denitrifier, the capacity of whole cells to reduce nitrate to nitrite and small amounts of nitric oxide is puzzling. This result could be due to nitrite toxicity. However, further work should be done to confirm that *F. placidus* is a denitrifier inasmuch as this is one of only two reportedly strictly anaerobic denitrifiers. While denitrification is limited to a few archaeal genera, identification of more archaeal strains capable of denitrification seems likely.

Eubacteria

Denitrification ability is widespread amongst the eubacteria, and almost exclusively in those strains that are capable of aerobic growth. Also,

Table 1. Listing of microbial genera that are suggested to include denitrifiers.

Archaea
Haloarcula
Halobacterium
Haloferax
Ferroglobus
Pyrobaculum

Bacteria
Gram-negative

Aquifex
Flexibacter (formerly *Cytophaga*)
Empedobacter
Flavobacterium
Sphingobacterium
Synechocystis sp. PCC 6803

Purple Bacteria
α subdivision

Agrobacterium
Aquaspirillum
Azospirillum
Blastobacter
Bradyrhizobium
Gluconobacter
Hyphomicrobium
Magnetospirillum
Nitrobacter
Paracoccus
Pseudomonas (G-179)
Rhizobium
Rhodobacter
Rhodoplanes
Rhodopseudomonas
Roseobacter
Sinorhizobium (formerly *Rhizobium*)
Thiobacillus

β subdivision

Achromobacter
Acidovorax
Alcaligenes
Azoarcus
Brachymonas
Burkholderia
Chromobacterium
Comamonas
Eikenella
Hydrogenophage
Janthinobacterium
Kingella
Microvirgula
Neisseria
Nitrosomonas
Ochrobactrum
Oligella
Ralstonia (formerly *Alcaligenes*)
Rubrivivax
Thauera
Thermothrix
Thiobacillus
Vogesella
Zoogloea

γ subdivision

Acinetobacter
Alteromonas
Azomonas
Beggiatoa
Deleya
Halomonas
Marinobacter
Moraxella
Pseudoalteromonas
Pseudomonas
Rugamonas
Shewanella
Thiopioca
Thiomargarita
Xanthomonas

δ subdivision

None

ε subdivision

Wolinella
Campylobacter
Thiomicrospira

Others
Gram-positive

Bacillus
Corynebacterium
Frankia
Dactylosporangium
Dermatophilus
Gemella
Jonesia (formerly *Listeria*)
Kineosporia
Micromonospora
Microtetraspora
Nocardia
Pilimelia
Propionibacterium
Saccharomonospora
Saccharothrix
Spirrilospora
Streptomyces
Streptosporangium

it is found rarely in those bacteria that carry out fermentation. Therefore, genera having strictly respiratory bacteria are likely to have denitrifying strains. Most of the characterized denitrifiers belong to the proteobacteria. However, a number of denitrifiers are in other eubacterial genera.

GRAM-POSITIVE BACTERIA Even though the majority of denitrifiers are Gram-negative, denitrifying bacteria are well represented among Gram-positive bacteria. For example, there have been a number of denitrifying *Bacillus* species described. However, because *Bacillus subtilis*

(the type strain of the most commonly studied strain of the genus) is not a denitrifier, denitrification in *Bacillus* species is often overlooked. It should be pointed out that a strain shown by rDNA analysis to be closely related to *B. subtilis*, *Bacillus azotoformans*, is a denitrifier (Mahne, 1995) and that strains designated as *B. subtilis* have been found to denitrify (Sakai, 1996; Sakai, 1996). However, these *B. subtilis* strain need to be described in more detail. Work done on characterizing denitrification enzymes in *Bacillus* has been limited (Denariaz, 1991). Other Gram-positive bacteria capable of denitrification include strains of *Propionibacterium* (Swartzlander, 1993) and *Jonesia* (originally *Listeria*) *denitrificans* (Rocourt, 1987). These strains are somewhat unusual because they seem to be denitrifying strains in groups of bacteria that are primarily non-denitrifiers. More typical is *Frankia*, in which a screen for denitrification found a number of denitrifying strains (Lensi, 1990). Recently, it has been shown that a number of actinomycetes, including *Streptomyces*, *Dermatophilus* and *Nocardia*, are capable of nitrous oxide evolution from nitrate or nitrite (Shoun, 1998). This work significantly expands the list of denitrifying Gram-positive bacteria and demonstrates that, once denitrification is observed in a group of bacteria, further characterization of its members and close relatives will uncover additional denitrifiers, even if denitrification had never been ascribed previously to any member of the group.

GRAM-NEGATIVE BACTERIA Among the Gram-negative bacteria that are not proteobacteriaare, denitrifying strains are found in the genera *Aquifex* (Huber, 1992), *Flexibacter* (Jones, 1990), and *Flavobacterium* (Coyne, 1989). *Aquifex pyrophilus* is a thermophile that constitutes one of the deepest (earliest) branches of the eubacteria. This may indicate that denitrification represents one of the first forms of respiration. Denitrifying strains of the related *Hydrogenobacter* have not been isolated as yet, but this may be due to incomplete characterization. The gliding bacterium *Flexibacter canadensis* is unique in that its nitrous oxide reductase is apparently insensitive to acetylene, unlike nitrous oxide reductases from other bacteria (Jones, 1990).

Nearly all of the bacteria defined as denitrifiers are identified because of their ability to reduce nitrate or nitrite to gaseous end products. Recently, genomic sequencing efforts indicated that *Synechocystis* sp. strain PCC 6803 is also a denitrifier, but with a truncated electron transport chain (Kaneko, 1996). Analysis of the genome sequence of PCC 6803 showed that it encodes a nitric oxide reductase and a transcriptional regulator that may regulate expression of this enzyme. This strain does not encode a nitrite or nitrous oxide reductase, making it an extreme example of an organism with a truncated denitrification electron transport chain. It will be interesting to see if genomic sequencing finds additional bacteria with truncated denitrification chains.

The majority of currently characterized denitrifiers are found in the group known as the proteobacteria (purple bacteria). The proteobacteria have been subdivided into five subdivisions, α, β, γ, δ and ε. Denitrifiers have been found in four of these. The δ subdivision, which contains a number of strict anaerobes, has not been found to contain any strains that denitrify, as yet. The α subdivision contains a number of well-characterized denitrifiers including *Paracoccus denitrificans*, various *Rhodobacter* strains, and several rhizobia. Even in the α subdivision, in which it is well established, denitrification may be overlooked if the first strains characterized do not denitrify. A good example of this problem is *Rhodobacter sphaeroides*. The original isolates of *R sphaeroides* were not robust denitrifiers, so little effort was made to determine if other strains of *R sphaeroides* were denitrifiers. However, a number of denitrifying isolates were eventually isolated (Michalski, 1988). Further work has indicated that all strains of *Rhodobacter* probably encode nitric oxide and nitrous oxide reductase, but that most have lost the ability to reduce nitrite (Kwiatkowski, 1997).

Denitrifiers of note in the α subdivision include *Hyphomicrobium*, budding bacteria often found in waste-water treatment facilities (Fesefeldt, 1998). *Hyphomicrobium* can be used to help rid water supplies of high nitrate concentrations while growing on inexpensive feedstocks such as methanol. Denitrification among the rhizobia, which are best known for their roles as nitrogen-fixing symbionts, is also noteworthy because these strains have the seemingly contradictory capacity for both nitrogen fixation and denitrification (O'Hara, 1985). This ability to both fix and "unfix" nitrogen is fairly widespread among denitrifiers: *Rhodobacter*, *Hyphomicrobium*, *Frankia*, *Azospirrilum*, *Azoarcus* and some pseudomonad strains can both fix nitrogen and reduce nitrates to nitrogen gas. Some members of the genus *Nitrobacter*, which are nitrifying α-proteobacteria, reportedly produce nitric oxide from nitrite. Even though a putative nitrite reductase has been purified from *Nitrobacter vulgaris* (Ahlers, 1990), other studies have not revealed production of nitric oxide or nitrous oxide in cultures of *Nitrobacter* species (Baumgartner, 1991; Goreau, 1980). Further studies are required to determine if these nitrifying bacteria are also denitrifiers.

Although a number of members of the β-proteobacteria denitrify, they are not as well studied, on the whole, as members of the α or γ subdivisions. However, new developments suggest this situation is likely to be only temporary. One new area of interest is the anaerobic degradation of aromatic organic compounds. In recent years, it has become evident that denitrifying bacteria can metabolize aromatic compounds under denitrifying conditions. A number of aromatic compound-degrading denitrifiers have been isolated, and many belong to the genera *Azoarcus* or *Thauera*, which are both β-proteobacteria (Fries, 1994; Springer, 1998; van Schie, 1998; Anders, 1995). Given the interest in anaerobic degradation of aromatic compounds, there is likely to be more in-depth studies on denitrification in *Azoarcus* or *Thauera*. One strain, *Azoarcus anaerobius*, is a completely anaerobic denitrifier (Gorny, 1992) and is unusual in that it cannot fix nitrogen, a trait common to all other strains of *Azoarcus*.

Another reason to expect that denitrification in members of the β subdivision will receive more attention is that both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are denitrifiers. Even though it has been well documented that several other species of *Neisseria* can denitrify (in fact, one species is named *Neisseria denitrificans*), denitrification in *N. gonorrhoeae* and *N. meningitidis* was originally overlooked because of their apparent sensitivity to nitrite. However, recently it has been shown that the gene *aniA* in *N. gonorrhoeae* encodes a copper-containing nitrite reductase (Mellies, 1997). Ongoing sequence analysis of the genomes of *N. gonorrhoeae* (Roe, 1999) and *N. meningitidis* (Sanger Centre, 1999) has revealed that both organisms encode a nitrite reductase and nitric oxide reductase, but only *N. gonorrhoeae* is known to encode nitrous oxide reductase; whether *N. meningitidis* also has this gene is not determined as yet. Little is known about the regulation of these proteins or what contributions they make to the physiology of these bacteria. Nitrite reductase is apparently not required for pathogenicity (Mellies, 1997). However, because reactive nitrogen oxide is an important part of the host defense response in humans, the role of the denitrification enzymes in these pathogenic *Neisseria* will undoubtedly receive more attention.

Also, among the β subdivision denitrifiers are species of the genus *Nitrosomonas*, a genus defined by the ability to oxidize ammonia to nitrite. It has been known for some time that nitrous oxide is a product of ammonia oxidation (Goreau, 1980; Poth, 1985). Subsequent studies have confirmed these observations and a copper-containing nitrite reductase has been purified from *Nitrosomonas europaea* (Miller, 1985; Dispirito, 1985). This strongly indicates that *Nitrosomonas* species are nitrifying denitrifiers. However, the role of denitrification in *Nitrosomonas*, whether for detoxification or for energy conservation, has not been established as yet.

Denitrification in members of the γ subclass of proteobacteria has been well studied in *Pseudomonas stutzeri* and *Pseudomonas aeruginosa*. Recent characterizations of the pseudomonads has resulted in the reclassification of rRNA group II bacteria to the genus *Burkholderia*, which is in the γ subdivision (Yabuuchi, 1992). Members of the rRNA group I of the pseudomonads are tightly clustered, and *P. stutzeri* and *P. aeruginosa* are in this group.

Some very unusual nitrate respirers are found in the γ group. Both *Beggiatoa* and *Thioploca* species have the unique capacity to accumulate nitrate in internal vacuoles until they contain several thousandtimes the external concentration (McHatton, 1996; Fossing, 1995). The organisms then use this accumulated nitrate as an oxidant and sulfide in the surrounding environment as a reductant. They also make sheaths in which they move between the nitrate-rich waters and sulfide-rich sediments, allowing them to commute from one environment to another depending on their physiological requirements. A unique relative of *Thioploca* and *Beggiatoa*, *Thiomargarita*, has been described recently (Schulz, 1999). *Thiomargarita* accumulates high concentrations of nitrate in a central vacuole. This very large vacuole is the reason *Thiomargarita* is the largest known bacterium. All of these bacteria are presumed to be denitrifiers, however, there is little direct evidence for this. Some evidence suggests *Beggiatoa* species are denitrifiers (Sweerts, 1990), but more work needs to be done to show that these strains contain a nitric-oxide-producing nitrite reductase. The use of reduced sulfur compounds as electron donors is known to occur in several denitrifiers including *Aquifex* (Huber, 1992), *Paracoccus* (Friedrich, 1981), and *Thiobacillus* (Schedel, 1980).

A few denitrifiers have been found in the ε subdivision. Some exhibit truncated denitrification chains. For example, both *Wolinella succinogenes* and some *Campylobacter* species have the capacity to grow with nitrous oxide as sole terminal oxidant (Yoshinari, 1980; Payne, 1982). However, neither bacterium is able to reduce nitrite to nitrous oxide. *Thiomicrospira denitrificans*, which is closely related to the *Campylobacter* group (Muyzer, 1995), can reduce nitrite to gaseous end products (Timmer-ten Hoor, 1975). Also, *T. denitrificans* can use sulfur compounds as reductants.

Enzymology of Denitrification

As shown in Figure 1, complete denitrification is a multi-step process, requiring four separate enzymes for the reduction of nitrate and three intermediate nitrogen oxides, and ending in the evolution of nitrogen gas. The basic arrangement of the nitrogen oxide reductases is shown in Fig. 3. A basic description of the nature of these proteins and ancillary proteins is presented here. Additional information can be obtained from two recent reviews (Berks, 1995; Zumft, 1997).

Nitrate Reductase

The first step in denitrification, the two-electron reduction of nitrate to nitrite, is catalyzed by nitrate reductase. Early studies on nitrate reductase activity in cells demonstrated the existence of at least two types of nitrate reductase: a soluble assimilatory enzyme, used when nitrate is the nitrogen source, and a membrane-associated respiratory enzyme. The situation became more complicated when nitrate reductase activity was found in the periplasm of *R. sphaeroides* IL 106 (Sawada, 1980). Further work has demonstrated that this periplasmic enzyme is found in a wide variety of bacteria, including denitrifiers and *E. coli*.

Most denitrifiers contain more than one type of nitrate reductase. The enzyme typically associated with nitrate respiration is a membrane-bound three-subunit complex, whose membrane-anchoring subunit (γ or NarI) is sometimes lost during purification. A significant amount of the research on the respiratory nitrate reductase has made use of non-denitrifiers, particularly *E. coli* (Berks, 1995). The largest of the three subunits (α or NarG) contains molybdenum, bound by the cofactor molybdopterin guanine dinucleotide and a [4Fe-4S] center. The remaining subunit (β or NarH) contains several [4Fe-4S] centers and a [3Fe-4S] center. NarG and NarH are exposed on the cytoplasmic side of the inner membrane. The membrane anchoring subunit typically contains a b-type heme. The direct electron donor of the respiratory nitrate reductase is quinol. The electrons from quinol are thought to be transferred through the heme in the membrane-anchoring subunit to the [Fe-S] centers in NarH and then to the molybdenum center in NarG where nitrate reduction occurs.

The periplasmic nitrate reductase (Nap) is a heterodimer with prosthetic groups similar to those found in the membrane-bound nitrate reductase. The largest subunit (NapA) binds molybdopterin guanine dinucleotide and a [4Fe-4S] center. The smaller subunit binds heme (NapB) that is required for transfer of electrons to the active site. Electrons are transferred from the membrane-associated quinol pool to the Nap complex by a membrane-bound tetra-heme c-type cytochrome, NapC. Insertional inactivation of NapC in *R. sphaeroides* resulted in the loss of nitrate reductase activity (Reyes, 1996). NapC is related to a larger family of proteins, which apparently bind heme via a bis-His ligation (Roldan, 1998).

Recently the crystal structure of the periplasmic nitrate reductase from the non-denitrifying bacterium *Desulfovibrio desulfuricans* has been reported (Dias, 1999). This enzyme contains a single subunit containing the active site and related cofactors. This subunit has significant similarity to NapA from denitrifiers and *E. coli*. Comparisons with other known structures revealed the enzyme is structurally related to formate dehydrogenase and dimethylsulfoxide reductase, both of which contain molybdenum cofactors. Comparisons of these various struc-

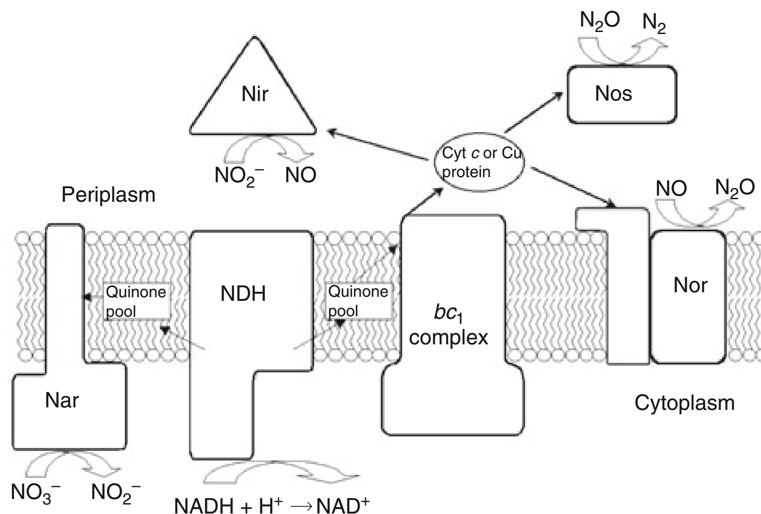

Fig. 3.

tures will be very useful in determining the structural constraints required for nitrate binding and reduction.

The periplasmic and membrane-bound enzymes can be distinguished in several ways. First, the membrane-bound enzyme is sensitive to micromolar levels of azide, whereas the periplasmic form is not (Bell, 1990). Second, the membrane-bound enzyme can reduce chlorate but the periplasmic enzyme is limited to nitrate, a result that led to the development of a useful method to select nitrate reductase mutants. Third, because the active sites of the two enzymes are on different sides of the inner membrane, the differential membrane solubilities of methyl viologen and benzyl viologen can be used to differentiate activities (Carter, 1995). Methyl viologen, which is membrane permeant, can be used as an electron source for both enzymes in whole cell assays. Benzyl viologen, which is membrane impermeant, will act as an electron source only for the periplasmic enzyme in whole cell assays. By comparing the nitrate reductase activity determined with each viologen, the relative levels of activity of each form of nitrate reductase can be estimated. Lastly, the regulation of the two enzymes is different.

While the function of the respiratory nitrate reductase in denitrification is obvious the physiological role of the periplasmic enzyme is unclear. It seems unlikely that the periplasmic enzyme is used by most denitrifiers for energy conservation inasmuch as bacteria such as *R. sphaeroides* 2.4.1 have this enzyme but can not grow anaerobically with nitrate as the sole terminal electron acceptor. Moreover, expression of the periplasmic nitrate reductase is repressed during denitrification in *P. denitrificans* and *R. eutropha* (Warnecke-Eberz, 1993; Sears, 1993). It seems likely that the major role of the periplasmic enzyme is to aid dissipation of excess reductant. This role is supported by the observation that the periplasmic nitrate reductase of *P. denitrificans* GB17 is expressed at a higher level on reduced substrates (that is, those that would likely cause a buildup of reductant) than on more oxidized substrates (Richardson, 1992). However, there are two examples of Nap being the principal nitrate reductase in a denitrifier. Inactivation of the genes encoding Nap in both *Pseudomonas* G-179 (Bedzyk, 1999) and *R. sphaeroides* resulted in a loss of nitrate reductase activity under anaerobic conditions.

The assimilatory nitrate reductase is a single-subunit enzyme. As with the other nitrate reductases the enzyme binds molybdopterin guanine dinucleotide and an [Fe-S] center. The activity of this enzyme is differentiated from the other enzymes, principally because it is only expressed when nitrate is used as a sole source of nitrogen.

Nitrite Reductase

Nitrite reduction is the defining reaction of denitrification—the step that differentiates denitrification from other forms of nitrate metabolism. Nitrite reductase catalyzes the one electron reduction of nitrite to nitric oxide. There are two types of nitrite reductases, but, unlike the different types of nitrate reductase, the nitrite reductases are not structurally related and contain different prosthetic groups. They are, however, both located in the periplasm. They also appear to be functionally redundant (Glockner, 1993). One type of nitrite reductase utilizes copper as a redox active metal (CuNir), and the other utilizes heme-bound iron (cd_1). There is no obvious phylogenetic distribution of the two enzymes. Moreover, both types of enzyme can be found within members of a single genus but have not been found in a single bacterium. In studies assessing the frequency of occurrence of either enzyme in the environment, the cd_1 type was found in a greater number of organisms (Coyne, 1989).

The CuNir has been studied extensively, and much is known about its structure and the nature of the copper centers. Enzymes from several different denitrifiers have been crystallized under different conditions and their high-resolution structures determined (Dodd, 1998; Adman, 1995; Kukimoto, 1994; Godden, 1991). These studies have revealed that the enzyme is a homotrimer with each monomer containing two copper atoms. The copper atom in type-1 centers is attached to Cys, Met and two His residues. Type-1 centers are often referred to as blue copper centers and are found in electron transfer proteins, such as azurin, or in proteins with multiple copper centers, such as laccase (Solomon, 1996). In multi-copper enzymes, including CuNir, the type-1 copper is involved in electron transfer to the active site. The other copper atom in CuNir is bound by three His residues making it a type-2 copper center. Type-2 centers are found in many multi-copper enzymes and are frequently sites of substrate binding. In CuNir, the type-2 center has been shown to be the site of nitrite binding (Adman, 1995). In the CuNir-nitrite cocrystals, the nitrite binds to the type-2 center with its oxygens and upon binding displaces a bound water (Adman, 1995). The copper centers in the cocrystal are in an oxidized state, however, which may influence how the nitrite binds. Studies of synthetic copper centers have suggested that the nitrite nitrogen binds to a reduced copper center (Halfen, 1996).

Less is known about how nitrite reduction proceeds. The redox potential of the type-1 center is higher than that of the type-2 center (Olesen, 1998). This suggests that the type-1 center acts as

a gate, by holding on to an electron until nitrite binds to the type-2 center. Nitrite binding to the type-2 center will raise its potential, permitting electron flow from the type-1 to the type-2 center. Little is known about how nitrite reduction proceeds once the type-2 center is reduced. It has been suggested that the nitrite is reduced and protonated, transiently forming a copper (II)-nitric oxide complex (Murphy, 1997). Because the nitric oxide is bound to oxidized copper, the complex is relatively unstable and nitric oxide can dissociate and diffuse away. It is critical that nitric oxide not bind to the reduced type-2 center, as such a complex would be stable and prevent enzyme function. This may explain how the type-1 center can function as a one-electron gate (Olesen, 1998).

The homodimer cd_1-type nitrite reductase contains a single c-type heme and d_1 heme molecule per monomer. The d_1 heme is a modified tetrapyrrole ring that is partly reduced and has oxo, methyl and acrylate sidegroups (Chang, 1986). The high-resolution structures of the cd_1 type enzymes from *P. denitrificans* GB17 (*Thiosphera pantotropha*) (Fulop, 1995) and *P. aeruginosa* (Nurizzo, 1997) have recently been determined. The c-heme of the oxidized form of the enzyme from GB17 enzyme is bound to two His residues. However, upon reduction, the enzyme refolds causing one of the His ligands to be lost and replaced by a Cys, a more common ligand of c-type cytochromes. This unexpected ligand shuffling does not occur in the *P. aeruginosa* enzyme because both the oxidized and reduced forms have the same His and Cys ligand (Nurizzo, 1998).

The d_1 heme in GB17 enzyme is bound by a Tyr and a His residue. The attachment of the d_1 residue is unusual in that the Tyr residue (identified as the ligand) is not conserved in other cd_1 reductases, nor does a potentially equivalent Tyr in other cd_1 reductases play a role in heme ligation (Cutruzzola, 1997). The high-resolution structure of the *P. aeruginosa* enzyme revealed that the sixth ligand of the d_1 heme is a hydroxide ion. In both the *P. aeruginosa* and GB17 enzymes, the sixth ligand is lost when substrate binds to the d_1 heme. The high-resolution structures of the reduced forms of both enzymes are very similar.

In addition to static structures, a time-resolved structural study of the catalytic cycle of the GB17 enzyme has been carried out (Williams, 1997). This work revealed the large-scale structural changes discussed above. It also indicated that the nitrite nitrogen binds to the reduced enzyme, in contrast with the oxidized CuNir. The ligand shuffling in the GB17 enzyme was postulated to change redox potentials of the heme groups to prevent formation of a Fe(II)-NO complex. The end product (Fe(III)-NO) is ensured by limiting the number of electrons available to the d_1 heme during each nitrite reductase step. Then, Fe(III)-NO can dissociate spontaneously or by the return of the Tyr ligand in the GB17 enzyme.

Both the CuNir and cd_1 enzymes also can reduce oxygen. Early studies often designated the cd_1 enzyme a cytochrome oxidase (Wharton, 1976). The product of oxygen reduction by the cd_1 enzyme is water (Lam, 1969), although it is not clear how four electrons are passed to the oxygen in this process, inasmuch as a one-electron reduction is normally carried out. The oxidase activity of these enzymes is potentially significant because, if activated oxygen species are also produced by CuNir, they can react rapidly with nitric oxide to produce reactive and toxic products such as peroxynitrite (Stamler, 1992).

Nitric Oxide Reductase

The *P. stutzeri* nitric oxide reductase has been the most intensively studied of these enzymes. It is purified as a heterodimer with subunits NorB and NorC being integral membrane proteins (Kastrau, 1994). NorC is c-type cytochrome, proposed to accept an electron from a periplasmic donor and then to pass the electron to NorB. NorB contains two b-type hemes and a non-heme iron. Spectroscopic analysis indicates one b-heme is low spin, whereas the other is high spin and capable of binding carbon monoxide. The low-spin heme is likely to be the direct acceptor of electrons from NorC. Metal analysis revealed that more iron was present than could be accounted for by heme content, indicating non-heme iron. As additional nitric oxide reductases have been purified, similar metal stoichiometry has been observed (Dermastia, 1991; Girsch, 1997). There is no evidence for nitric oxide reductase containing an [Fe-S] center.

The isolation of the genes encoding the nitric oxide reductase in *P. stutzeri* provided significant insight into the structural organization of the enzyme (Zumft, 1994). Examination of the deduced primary sequence indicated that the nitric oxide reductase is related to the heme-copper family of cytochrome oxidases (van der Oost, 1994; Saraste, 1994). Although the overall identity of nitric oxide reductases and cytochrome oxidases is low, a set of six His residues is conserved in pairwise alignments of subunit I of cytochrome oxidase and NorB of nitric oxide reductase. The conservation of these residues is significant because they have been shown to serve as metal center ligands in cytochrome oxidase. In cytochrome oxidase, these His residues bind a six-coordinate heme, five-coordinate heme, and copper—the latter two metal centers

constituting a binuclear center that is the site of oxygen binding and reduction (Iwata, 1995). By comparison, the equivalent His residues in NorB, ligate a six-coordinate heme, a five-coordinate heme and, because there is no copper in nitric oxide reductase, a non-heme iron.

Recent spectroscopic studies on the enzymes purified from *P. denitrificans* (Girsch, 1997) and *P. stutzeri* (Cheesman, 1998) support the structural similarity of nitric oxide reductase and heme-copper oxidases. The active site of nitric oxide reductase is most likely a five-coordinate heme and non-heme iron in close proximity. The two metal centers are close enough to permit interaction of the electron orbitals, as also observed in heme-copper oxidases. NMR, Raman, and FTIR analyses have demonstrated that the local molecular environment of the binuclear center in nitric oxide reductase is distinct from any type of heme-copper oxidase (Moenne-Loccoz, 1998; Mitchell, 1998). The differences in the structure are also manifested in the primary sequences. For example, in the aa_3-type cytochrome oxidases, one of the most highly conserved regions is within membrane spanning helix six, which contains the His residues that bind the five-coordinate heme. Comparison of the aa_3-type consensus sequence of the helix-six region with sequences of the similar region in nitric oxide reductases reveals significant differences in the primary sequences (Fig. 4). Of particular note is the absence of a Glu in nitric oxide reductase that may be involved in proton pumping in cytochrome oxidase and in an overall increase in polarity of the nitric oxide reductase sequence (Verkhovskaya, 1997). The sequence differences also make it possible to differentiate members of this diverse family of proteins. This is important in those bacteria, such as *N. gonorrhoeae* and *N. meningitidis*, where nitric oxide reductase has not been demonstrated, but sequence information indicates the gene encoding this enzyme is present along with other members of the heme-copper oxidase superfamily.

A variant on the *P. stutzeri* prototype structure has recently been identified in the bacterium

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Psaer PENLTRDKFYWWWVVHLWVEGVWELIMGAILAFVLAFITG
Psstu PENLSRDKFYWWWVVHLWVEGVWELIMGAILAFVLIKVTG
Phalo PSNLAVDKLYWWWVVHLWVEGVWELIMASILGYLLIKMTG
Pdeni PANLVLDKQYWWWVIHLWVEGVWELIMAAILAFLMIKLTG
Brjap PANLALDKMYWWWVVHLWVEGVWELIMASVLAYLMIKLNG
Rspha PDNLGLDKMYWWYIVHLWVEGTWELVMAAVLGYLMIKLTG
G-179 PANLALDKMYWWYVVHLWVEGVWELIMASVLSFLMIKLNG
Synec TRISVAEYWRWW-VVHLWVEGFFEVFATVAIAYLCSELGF
Reutr TSITVMEYWRWW-VVHLWVEGFFEVFATVALAFIFSTLGL
Ngono SPIAVMEYWRWW-VVHLWVEGFFEVFATVAFAFVFYNMGF
Nmeni SPIAVMEYWRWW-VVHLWVEGFFEVFATAAFAFVFYNMGF
Sub I GGDPVLYQHLFWFFGHPEVYILILPAFGIISEVISTFSRK
CcoN  VFSGVQDAMVQWWYGHNAVGFFLTAGFLGMMYYFVPKQAE
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Fig. 4.

Ralstonia eutropha (*Alcaligenes eutrophus* H16) (Cramm, 1997). This bacterium contains two nitric oxide reductases, one designated NorZ, a product of genes on the chromosome, and the other designated NorB, a product of genes located on a plasmid. The products of these genes have significant identity (>90%) and are functionally redundant. They have significant similarity with NorB of other nitric oxide reductases such as that of *P. stutzeri*, with the exception of an N-terminal extension of about 300 residues. These extra residues of the *R. eutropha* enzymes likely add two additional membrane-spanning regions and a large hydrophilic loop. The primary sequence of the N-terminal extension does not have similarity with other known proteins. Part of its function may relate to the observation that there is no evidence for a gene encoding a NorC equivalent in *R. eutropha*. This suggests that the amino terminal extension is functionally equivalent to NorC, but no direct evidence has been provided in support of this conclusion.

A gene whose product is similar to the NorB and NorZ products has turned up in the genome of *Synechocystis* strain PCC 6803 (Kaneko, 1996). Interestingly, genes encoding nitrite and nitrous oxide reductase were not identified in this bacterium. Because PCC 6803 can not reduce nitrite, it has never been considered as capable of gaseous nitrogen oxide metabolism. This makes the presence of nitric oxide reductase unexpected. However, as discussed above, other strains have recently been characterized which have nitric oxide reductase but lack nitrite reductase, suggesting this mode of truncation of the denitrification electron transport chain may be fairly prevalent. Ongoing genome sequencing efforts in *N. meningitidis* (Sanger Centre, 1999) and *N. gonorrhoeae* (Roe, 1999) have also identified genes whose products are similar to the *R. eutropha* nitric oxide reductases. *Neisseria* and *R. eutropoha* are both in the β-subgroup of the proteobacteria (Table 1), but it is unclear if this class of enzymes will be preferentially found in one taxonomic group. Alignment of nitric oxide reductases also demonstrates that the *R. eutropoha*-type nitric oxide reductases have other unique sequence motifs not found in the *P. stutzeri* type reductases (Fig. 4).

The reduction of nitric oxide to nitrous oxide occurs at the binuclear center. Current models suggest that two nitric oxide molecules bind at the active site, although it is not clear if nitric oxide binds to both metal centers or if a dinitrosyl complex is formed (Ye, 1994; Moenne-Loccoz, 1998). Electrons enter the Nor complex through the cytochrome c in NorC and then flow to the six-coordinate heme in NorB. Electrons are then transferred to the binuclear center. The high affinity of reduced heme for nitric oxide

makes it unclear if reduction of the catalytic site involves formation of a ferrous heme-nitric oxide species. Irrespective of the exact electron transfer steps, it is clear that proximity of the two nitric oxide molecules is critical in the formation of the N-N bond.

Nitrous Oxide Reductase

The presence of nitrous oxide reductase can be identified independently of the other reductases because many organisms can grow with nitrous oxide as sole terminal oxidant. This has led to the identification of nitrous oxide reductase in several bacteria that are not complete denitrifiers (Yoshinari, 1980; Payne, 1982). The purification and characterization of nitrous oxide reductase was difficult because its activity is lost in cell extracts. Nutritional studies had identified copper as an essential nutrient for nitrous oxide reductase activity (Matsubara, 1982) and further work led to the isolation of a soluble copper protein which, under the proper conditions, had nitrous oxide reductase activity (Zumft, 1982). Additional studies have demonstrated that enzyme purified anaerobically and assayed using reduced viologens as electron donor has the highest specific activity. The latter observation is somewhat puzzling because the natural electron donors to nitrous oxide reductase are likely to have much higher redox potential.

Nitrous oxide reductases from several complete denitrifiers have been extensively characterized (Riester, 1989; SooHoo, 1991; Hulse, 1990; Snyder, 1987). These enzymes are all related and are multi-copper periplasmic proteins. The protein appears to be homodimeric in most preparations, with four coppers per subunit. Current data suggest there are two binuclear copper centers in the active enzyme. These centers undergo spectroscopic shifts depending on their redox state, and this has resulted in the characterization of different colored forms of the enzyme.

One of the copper centers has been structurally defined as a CuA site. The CuA center was described originally in the heme copper oxidases. However, the exact nature of this site was unclear until the related site in nitrous oxide reductase was characterized. Analysis of the deduced primary sequence of the nitrous oxide reductase identified a structural motif found otherwise only in the CuA-containing subunit of cytochrome oxidase (Viebrock, 1988). Additional spectroscopic studies provided further evidence of the similarities between one of the sites in nitrous oxide reductase and the CuA site of cytochrome oxidase (Farrar, 1991; Scott, 1989; Antholine, 1992). A more precise understanding of the structure of the CuA center has been obtained with the determination of the high-resolution structure of two cytochrome oxidases (Tsukihara, 1995; Ostermeier, 1997). In both cytochrome oxidase and nitrous oxide reductase, the CuA site has a role in transferring electrons from external electron donors to the active site. The other copper center in nitrous oxide reductase is, by exclusion, assumed to be the site of nitrous oxide binding and reduction. This site has been designated CuZ. Initial studies suggested both CuZ and CuA sites were bis-thiolate-bridged dinuclear copper sites (Farrar, 1991). Isolation of spectral signals arising from the CuZ center has proved difficult. Spectroscopic analysis of the CuZ signals has relied on poising the enzyme in specific oxidation states to make the CuA center Electron Paramagnetic Resonance (EPR) or optically silent. However, it has been suggested that the signals assigned to the CuZ site are actually different redox states of the CuA center (Farrar, 1998). In this latter work, it was shown that both the CuA site and the putative CuZ site were bis-thiolate-bridged dinuclear copper sites. This would require, if these were separate sites, four conserved Cys residues for formation of the two binuclear centers. Alignment of deduced nitrous oxide reductase sequences does not identify four conserved Cys residues. Instead, there are only two absolutely conserved Cys residues in nitrous oxide reductase. It is possible that the position of a Cys is shifted in one sequence relative to others accounting for the deficiency of conserved residues. However, mutation of one of the Cys residues outside the CuA domain does not lead to a loss of nitrous oxide reductase activity (Dreusch, 1996). Taken together, these data are not consistent with assuming that there are two bis-thiolate-bridged dinuclear copper sites in nitrous oxide reductase.

If conserved residues do not bridge the catalytic center, what then is its structure? Alignment of available nitrous oxide sequences does indicate there are a number of completely conserved His residues. The catalytic center might therefore be a His-ligated structure similar to the Type 3 copper centers found in enzymes such as laccase (Solomon, 1996). However, there is currently no direct evidence for the presence of such a center.

Given the uncertainty in the structure of the catalytic center, it is difficult to develop a useful model of the nitrous oxide reductase catalytic cycle. Nitrous oxide is chemically inert and also a poor ligand, making its reduction an interesting problem in transition metal-ligand chemistry (Kroneck, 1990). Other enzymes, including nitrogenase (Jensen, 1986) and carbon monoxide dehydrogenase (Lu, 1991), also have been found to have nitrous oxide reductase activity, indicat-

ing that it is possible that transition metals other than copper can reduce nitrous oxide.

Only copper-containing nitrous oxide reductases have been purified from complete denitrifiers. However, there appear to be variants of this structure present in other bacteria. The nitrous oxide reductase from *W. succinogenes* has been purified and shown to contain both copper and iron (Teraguchi, 1989; Zhang, 1991). The iron is attributed to an associated cytochrome c, though EPR suggests the presence of a CuA site. Because the primary structure of the *W. succinogenes* enzyme is unknown, the relationship of this enzyme to other nitrous oxide reductases is unknown. Another novel nitrous oxide reductase has been suggested to occur in *Flexibacter canadensis* (Jones, 1990). Most nitrous oxide reductases are inhibited by acetylene (Balderston, 1976; Yoshinari, 1976). However, the nitrous reductase from *F. canadensis* is insensitive to this compound (Jones, 1990). Preliminary characterization of this enzyme suggests it is membrane-associated, further differentiating it from other reductases (Jones, 1992). The nitrous oxide reductase from *R. sphaeroides* IL106 was also thought to be divergent because it was reported to contain Zn and Ni in addition to copper (Michalski, 1986). However, a more rigorous characterization of this enzyme suggests it is similar to typical copper-containing enzymes (Sato, 1998).

Genetics of Denitrification

Gene Organization

Analysis of the structure and organization of denitrification genes has been investigated in denitrifiers that are primarily members of the proteobacteria. The most extensive characterizations have been carried out with genomes of the pseudomonads as well as that of *P. denitrificans* strains. Although no true denitrifier has had its chromosome sequenced, there are several projects to sequence organisms that are partial or complete denitrifiers.

NITRITE AND NITRIC OXIDE REDUCTASE The most extensive examinations of denitrification gene organization have involved *P. aeruginosa* and *P. stutzeri* in which the nitrite reductase and nitric oxide reductase structural genes are about 10 kb apart (Arai, 1995; Braun, 1992). This tight linkage is found also in *P. denitrificans* (Baker, 1998). In general, such tight linkage of the nir and nor gene clusters is not observed in those denitrifiers containing a copper-type nitrite reductase. In *R. sphaeroides* strains IL106 and 2.4.3, the genes for nitrite reductase are not closely linked to the genes for nitric oxide reductase (Schwintner, 1998; Tosques, 1997). This is also the case in *N. gonorrhoeae* (Roe, 1999) and *N. meningitidis* (Sanger Centre, 1999). One exception is *Pseudomonas* G-179 (which is apparently a member of the α proteobacteria subclass) where it has been shown that the gene encoding the copper-type nitrite reductase is in a cluster with the genes for nitric oxide reductase and the periplasmic nitrate reductase (Bedzyk, 1999).

Genes encoding proteins required for assembly of a particular reductase are typically found clustered with the structural gene for that particular reductase. For example, in those bacteria encoding a cd_1-type nitrite reductase, several genes whose products are involved in the synthesis of d_1 heme will be required for the production of an active enzyme. Systematic inactivation of genes closely linked to the nitrite reductase structural gene has identified a number of genes whose products are involved in heme d_1 biosynthesis (Kawasaki, 1997; Palmedo, 1995). Although this has led to the identification of a set of genes uniquely required for heme synthesis during denitrification, the details of the biosynthesis of heme d_1 are not elucidated as yet.

The organization of the genes for assembly of the cd_1 protein is dissimilar in every denitrifier. In *P. aeruginosa*, eleven adjacent genes have been identified whose products are suggested to be involved in nitrite reductase activity (Arai, 1994). These genes are transcribed in the same direction and are postulated to be transcribed in a single transcript (Kawasaki, 1997). In *P. stutzeri*, this cluster of genes has been rearranged so that the genes are no longer adjacent and at least three different transcripts are produced (Palmedo, 1995).

The total number of proteins that is required for expression of an active copper-containing nitrite reductase would be expected to be less than with the heme-type nitrite reductase. Examination of sequence flanking the structural gene encoding the copper-type nitrite reductase in *N. gonorrhoeae* (Roe, 1999), *N. meningitidis* (Sanger Centre, 1999), *R. sphaeroides* 2.4.3 and *Pseudomonas* G-179 (Bedzyk, 1999) revealed only one other conserved gene. In *R. sphaeroides* 2.4.3, this undesignated gene is located about 200 bp downstream of the putative translation stop of nirK (the nitrite reductase structural gene). Preliminary evidence suggests it is transcribed from the nirK transcription start. The role of the product of this gene is unclear. The difference in the amount of DNA required to produce an active copper-containing nitrite reductase versus the heme-containing nitrite reductase is notable. In *R. sphaeroides* 2.4.3, about 2.5 kb are required to encode the copper-

containing nitrite reductase and the accompanying gene of unknown function. In *P. aeruginosa*, about 10 kb appear to be required to code for proteins necessary for assembly of an active nitrite reductase.

The structural genes for the heterodimeric form of nitric oxide reductase, designated norC and norB, have been sequenced from a number of denitrifiers. In every case, the transcription start precedes norC and the gene order is norCB. The complete nor operon consists of norCB and one or two additional genes. For example, in *P. denitrificans* and *R. sphaeroides* norQ and norD follow norB (Bartnikas, 1997; De Boer, 1996). In *P. aeruginosa* and *P. stutzeri*, norD follows norB (Arai, 1995; Zumft, 1994). In both these pseudomonads norQ (designated nirQ) is present, but it is immediately upstream, and divergently transcribed from the structural gene for nitrite reductase, nirS (Arai, 1994; Jungst, 1992). Inactivation of norQ or norD leads to a loss of nitric oxide reductase activity but does not appear to inhibit assembly of nitric oxide reductase (Jungst, 1992; Mitchell, 1998; De Boer, 1996). This suggests the likelihood that both NorQ and NorD are accessory proteins required for the assembly of nitric oxide reductase. One possible role would be insertion of non-heme iron.

Neither norQ nor norD has been found in those denitrifiers encoding the single subunit type of nitric oxide reductase. There are no obvious orthologs of either norQ or norD present in the chromosome of *Synechocystis* sp. strain PCC 6803 (Kaneko, 1996). Nor have norQ or norD orthologs been identified in the ongoing genome sequencing efforts in *N. gonorrhoeae* or *N. meningitidis*. The absence of these proteins in these denitrifiers is somewhat surprising given the sequence similarity of the single subunit and heterodimeric nitric oxide reductases. The sequence of the *R. eutropha* norB is preceded by an open reading frame (ORF) encoding a protein containing a high percentage of His residues (Cramm, 1997). It is possible the product of this gene might play a role in assembly of an active nitric oxide reductase.

R. eutropha is unusual in that it contains two nitric oxide reductase structural genes (Cramm, 1997). One of these, norB, is located on a megaplasmid while the other, norZ, is located on the chromosome. This is the only bacterium described in which functionally redundant terminal N-oxide oxidoreductases have been observed. It is also interesting that in *R. eutropha* norZ does not appear to be tightly linked to the nitrite reductase structural gene. *R. eutropha* contains a cd_1-type nitrite reductase. This genetic organization is different from those bacteria containing a heterodimeric nitrite oxide reductase and a cd_1-type nitrite reductase.

In *P. denitrificans*, there are two additional genes, designated norE and norF, whose products appear to be required for nitric oxide reductase activity (De Boer, 1996). These genes are located immediately downstream of the norCBQD cluster. Insertional inactivation of either norE or norF reduces nitric oxide reductase activity but does not significantly affect its expression. Orthologs of norE have been found in other denitrifiers, for example, the ORF175 protein in *P. stutzeri* (Glockner, 1996) and in *Pseudomonas* sp. G-179 (Bedzyk, 1999). However, no norE ortholog has been identified in *R. sphaeroides* or in those bacteria encoding the single subunit nitric oxide reductase. No obvious norF orthologs have been identified, but genes whose products have some similarity to norF have been described in *P. stutzeri* (ORF82) and in *Pseudomonas* sp. G-179 (Bedzyk, 1999). The role of norE and norF remain undetermined. However, because of its similarity to the subunit III of cytochrome oxidases, it has been suggested NorE is a third subunit of the nitric oxide reductase protein complex (De Boer, 1996). Experimental conformation for NorE as a part of an active nitric oxide reductase complex is lacking.

Nitrous Oxide Reductase While the nir and nor gene clusters are often linked, the location of the nos gene cluster (nos refers to genes related to nitrous oxide reductase and not to genes for nitric oxide synthase) relative to other denitrification genes is more variable. In *P. stutzeri*, the genes encoding nitrous oxide, nitrite and nitric oxide reductase are within a stretch of about 30 kb (Jungst, 1991). In *P. aeruginosa*, the nitrous oxide reductase gene cluster is not as tightly linked to the nitrite and nitric oxide reductase genes (Vollack, 1998). However, the genes for all four nitrogen oxide reductases are located within the 20- to 36-min segment of the *P. aeruginosa* chromosome. In several denitrifiers, including *R. eutropha* (Zumft, 1992), *Sinorhizobium meliloti* (Holloway, 1996) and *R. capsulatus* (Rhodobacter, 1999), nos genes are found on plasmids. By comparison, all denitrifiers characterized in detail have both nir and nor located on the chromosome. The variable location of the nos cluster may reflect nitrous oxide's lack of toxicity, and therefore the accumulation of nitrous oxide that would follow the loss of nitrous oxide reductase activity has only limited consequences.

Gene organization within the nos cluster is much more conserved than is that in the nir or nor clusters. In almost every sequence, nosR is immediately upstream of nosZ, and nosZ is typically followed by nosDFYL. Undefined genes clustered with nosZ are likely to be involved in assembly of an active nitrous oxide reductase,

perhaps in copper incorporation. Inactivation of nosF, nosD or nosY leads to production of an inactive nitrous oxide reductase (Zumft, 1990). Sequence analysis indicates that nosY encodes a membrane-bound protein, nosD a periplasmic protein, and nosF a cytoplasmic nucleotide-binding protein. It has been suggested these proteins form a complex involved in copper processing and insertion into nitrous oxide reductase (Zumft, 1997).

In *S. meliloti*, a gene that has been designated nosX follows nosL. Inactivation of nosX causes a loss of nitrous oxide reductase activity in *S. meliloti* (Chan, 1997). Possible nosX orthologs have been found in other denitrifiers including "*A. cycloclastes*" (McGuirl, 1998) and *B. japonicum* (Genbank accession number {AJ002531}). A gene encoding a product similar to the nosX product has also been identified in *P. denitrificans*, but since it is part of the nir gene cluster it has been designated nirX (Genbank accession number AJ001308). A nosX ortholog has not been identified as yet in the peudomonads. The role of nosX is unclear but current data do not suggest a role in copper processing.

Isolation of mutants of *P. stutzeri* unable to use nitrous oxide as sole terminal oxidant led to the isolation of an additional gene whose product is required for nitrous oxide reductase activity. This gene was designated nosA, and it was shown to encode an outer membrane protein that is required for copper transport into the cell (Lee, 1991; Lee, 1989). Inactivation of nosA results in expression of the nitrous oxide reductase apoprotein. Putative nosA orthologs have been found in other denitrifiers, including *P. aeruginosa* (Yoneyama, 1996), but nosA is not found in the nos cluster of *P. stutzeri*. This organization may be because the nosA product is playing a more general role in cell physiology.

Nitrate Reductase

Of the various nitrate reductases, the respiratory and periplasmic forms have been studied in the most detail in the context of denitrification. Genes encoding the respiratory nitrate reductase, designated nar, have been completely sequenced in *P. aeruginosa* (Genbank accession number {Y15252}) and partially sequenced in *P. denitrificans* (Berks, 1995) and *Pseudomonas fluorescens* (Philippot, 1997). The nar genes are not clustered with other genes required for denitrification in *P. aeruginosa* (Vollack, 1998). The relatively limited interest in the nar genes in denitrifiers is due to the extensive study of nar genes in *E. coli* and other non-denitrifiers.

Genes encoding the periplasmic nitrate reductase have been characterized in several denitrifiers including *P. denitrificans* GB17 (Berks, 1995) and *R. sphaeroides* (Reyes, 1996). The structural genes for this nitrate reductase are napA and napB: napA encodes the molybdopterin cofactor and napB encodes the cytochrome c containing subunit. These two genes are clustered with other genes that have been designated napCDE. The napC gene encodes a membrane-bound cytochrome c, which is the likely electron donor for the periplasmic nitrate reductase; napD encodes a cytoplasmic protein and napE a small membrane protein. The function of these genes' products is unknown. The nap genes of *P. aeruginosa* are present on the chromosome but are not tightly linked to nar or other denitrification-related gene clusters (Vollack, 1998). In *R. eutropha* (Siddiqui, 1993)and *R. sphaeroides* strain 2.4.1 (Schwintner, 1998), the nap genes are localized on plasmids. In *R. sphaeroides* IL106, the nap genes are apparently located on the chromosome (Schwintner, 1998). In *Pseudomonas* G-179, the nap genes are part of a large cluster which includes nir and nor genes (Bedzyk, 1999).

Additional Genes Required for Denitrification

A few other genes frequently associated with nitrogen oxide gene clusters deserve mention. One is hemN, which encodes an oxygen-independent coproporphyrinogen oxidase (Gibson, 1992). *R. sphaeroides* encodes two hemN paralogs, the second of which is designated hemZ (Zeilstra-Ryalls, 1995). The *R. sphaeroides* hemN is clustered with the genes encoding nitric oxide reductase (unpublished). Inactivation of hemN (which was originally designated hemF) inhibits the ability to grow anaerobically under any conditions tested (Gibson, 1992). Aerobic growth is not affected because there is an oxygen-dependent form of this enzyme. The role of the hemZ product is unclear. Interestingly, hemZ is adjacent to fnrL whose product is an important regulator of anaerobically expressed genes (Zeilstra-Ryalls, 1995). These two genes are clustered with the ccoN operon that encodes the cbb_3-type oxidase. The cbb_3-type oxidase is a heme-copper enzyme and is the oxidase with the highest level of similarity to nitric oxide reductase (Saraste, 1994). It is notable then that both hemZ and hemN are clustered with related terminal oxidoreductases and regulatory proteins important in maintaining anaerobic physiology. It is possible that this gene arrangement may have occurred by gene duplication providing further evidence for the evolutionary link between aerobic respiration and denitrification.

The hemN gene is clustered with denitrification genes in *Pseudomonas* sp. G-179 (Bedzyk, 1999) and is clustered with the ccoN genes in both *P. denitrificans* (van Spanning, 1997) and *P. aeruginosa* (Zumft, 1997). A hemN ortholog has not been found in the nor cluster in *P. denitrificans* or *P. aeruginosa*. The regulation of hemN in *P. aeruginosa* (discussed in more detail below) further emphasizes the importance of the activity of the hemN product during denitrification (Rompf, 1998).

Sequencing of the nor cluster in *R. sphaeroides* (Bartnikas, 1997) and nos region in *P. stutzeri* (Glockner, 1996) has revealed a gene present in both regions whose product may be important to the physiology of denitrification. This gene has been designated nnrS in *R. sphaeroides* and orf396 in *P. stutzeri* (Fig. 5). The product of the genes from both bacteria is a membrane protein probably containing twelve membrane-spanning regions. In *R. sphaeroides*, nnrS is expressed only during denitrification and is regulated by NnrR, which also regulates nirK and nor (unpublished). Inactivation of nnrS has no obvious effect on growth under any conditions (unpublished). Though nnrS orthologs have not been found in the denitrification gene clusters of other well studied denitrifiers, examination of ongoing genomic sequencing efforts reveal the presence of nnrS orthologs in every denitrifier. In *R. capsulatus*, a nnrS ortholog is present on a plasmid and is closely linked to the nos genes (Rhodobacter, 1999). There is also an nnrS ortholog located on the chromosome. Copies of genes encoding products similar to nnrS have been identified in the *N. gonorrhoeae* (Roe, 1999), *N. meningitidis* (Sanger Centre, 1999) and *P. aeruginosa* chromosome (Pseudomonas, 1999), although none of these are clustered with denitrification genes. Significantly, nnrS orthologs have not been found in non-denitrifying bacteria. The function of nnrS has not been determined, but it is obviously not essential for denitrification or it would likely have been isolated in mutant screens. However, work in *R. sphaeroides* and its distribution among the bacteria indicates nnrS is a denitrification-associated gene. It seems likely that as more work is done on the genetics of denitrification, many genes that are not essential to, but whose products are physiologically important for, denitrification will be described.

Regulation of Genes Required for Denitrification

This section will focus primarily on the regulation of those genes encoding respiratory nitrogen oxide reductases and genes required for their assembly. As denitrification depends on the presence of nitrogen oxides, it is natural to describe denitrification genes as part of a stimulon, a term that refers to operons responding together to a particular environmental stimulus (Neidhardt, 1990). In general, the regulators of denitrification can be differentiated into the nitrate, nitric oxide, and nitrous oxide stimulons. The organization of denitrification genes roughly reflects the organization of the stimulons. The nitrate and nitrous oxide stimulons are primarily made up of the nar and nos gene clusters, respectively. These two, independently regulated, gene clusters are not linked to each other and are frequently distant from other denitrification-related clusters. The nitric oxide stimulon is made up of both the nir and nor clusters. These two gene clusters are the most strongly linked of any of the denitrification gene clusters.

As denitrification is, in most cases, an anaerobic process, other stimulons and regulons required for anaerobic growth overlap the denitrification-related stimulons. This overlap can make it difficult to differentiate regulatory factors that directly modulate gene expression from those that indirectly affect gene expression. This discussion will focus on those proteins that current data suggest are directly involved in regulation of the nitrogen oxide reductases. It is important to note that a particular stimulon will likely include genes whose products are not directly required for denitrification and, consequently, not covered in this review. However, this does not minimize the usefulness of organizing denitrification genes into stimulons.

One other important consideration is the relationship of denitrification and oxygen respiration. Denitrification is primarily an alternative form of respiration inasmuch as oxygen is a preferred oxidant. However, this does not imply that all denitrifiers have the same set point at which oxygen respiration is switched to nitrogen oxide respiration. Available data suggest that the onset of expression of denitrification genes occurs over a wide range of oxygen concentrations. The best example of an aerobic denitrifier is *P. denitrificans* GB17, which was originally described as an aerobic denitrifier (Robertson, 1984). In addition, other strains have been described as aerobic denitrifiers (Bonin, 1991; Patureau, 1994; van Niel, 1992; Frette, 1997; Robertson, 1995; Ka-Jong, 1997). None of the intensively studied model denitrifiers activate gene expression, even at moderate concentrations of oxygen. Therefore, the molecular mechanisms that permit aerobic denitrification are not currently understood.

Nitrate Stimulon The presence of nitrate and a reduction in oxygen tension stimulates

```
NmNnrS          -------------------MKFT-KHPVWAMA---FRPFYSLAALYGALSVLLWGFGYT-
NgNnrS          -------------------MKFT-KHPVWAMA---FRPFYSLAALYGALSVLLWGFGYT-
PsORF396        ------------MQUIDRRKALS-IAPIWRLA---FRPFFLAGSLYALLAIPBWVAWWTG
PaNnrS          ---------------------MA-IPPIWRLG---FRPFFLGGALFAVLAIALWLAALAG
RsNnrS          -------------MASDRPRTYT-GPALLSYG---FRPFFLLSALFAAGAUPUWLAUWS-
RcNnrS          MAHPHLDSPAAGRHHPDIPAAGAPMTALLRLLSDSFRUFFLLASLWAAAAMALWLWWLWQ
                                                   ** *:    *       :*

NmNnrs          ---G-THELSGFY----WHAHEMIWGYAGLUUIAFLLTAUATWTGBGHQPPTRGGULTIF
NGNNRS          ---G-THELSGFY----WHAHEIWGYAGLUUIAFLLTAUATWTGQPPPTRGGULUGLTAF
PsORF396        LWPG-FQPTGGWLA---WHRHEMLFGFAMAIUAGFLLTAUQTWTGQTAPSGNRLUGLAAU
PsNnrS          LWSG-FQPTGGWLA---WHRHEMLFGFGUAIIAGFLLTAUQTWTGUPGLQGRPLALLAGL
RcNnrS          ---G-RIGLAGPFSPIDWHIHEMLFGYTSAUIAGFLFTAIPNWTGRMPRRGLPLAALAAL
RcNnrS          NQIGPGGDLPNALAPSHWWHAHELIFGFGMAATAGFFLTAAPNWTGKPUAGPRFIALMAGL
                   *             ** **:::*:  :: :*::**:  ***        ::  :  :

NmNnrs          WLAARIAA-FIPGWGASASGILGTLFFWYGAUCMALPUIRSQNQRNYUAUFALFULGGTH
NGNNRS          WLAARIAA-FIPGWGAAASGILGTLFFWYGAUCMALPUIRSQNRRNYUBAFAIFULGGTH
PsORF396        WLAARLG--WLFGLPAAWLAPLDLLFLUALUWMMAQMLWAUPQKRNYPIUUULSLMLGAD
PsNnrS          WLAARLA--WLFDAPLALLLULQLSFLPLLAWAIGRSLWRURQKRNYPUUGLLLLLTLAD
RcNnrS          WIAGRFAUAGAFGTDPLLULUIDAGFLLAUTLMAUIEIAAGKNWKNLMUUGPUGLYLAAN
RcNnrS          WLAGRGAULLWGSUPPULAAGUULAFPALLTERMARQLIRRPASSEGLYLALLGLITLAE
                *:* *                ::   *    -      :           :  : :

NmNnrs          AAFHUQLHNGNLGGLLSGLQSGLUMUSGFIIGLIGTRISFFTSKRLN-----UPQIPSPK
NGNNRS          AAFHUQLHNGNLGGLLSGLQSGLUMUSGFIGLIGMRIISFFTSFFLN-----UPQIPSPK
PsORF396        ULILTGLLQGNDALQRQGULAGLWLUAALMALIGGRUIPFFTQRGLGK----UDAUKPWU
PsNnrS          ALULLGLFEGNDDWQRRASIAALWLIAGMMNLIGGRUIPFFTQRGLGR----QQQUPAIA
RcNnrS          ULFHLEAMQ--QGESDIGRPLGFATUTFLIMLIGGRIIPSFTRNWLAKG--GPGPLPUPF
RcNnrS          ARULLDWLDLPPGDAAAGLRGGLAALUALUAULGGRITPQFTRNALARAGAPPAALPRSF
                : :   :               ::       ::* *: _ **   *         :

NmNnrs          -WUAQASLWLPMLTAMLMAHGU----MPWLSAAFAFAAGUIFTUQUYRWWYKPULKEPML
NGNNRS          -WUAQASLWLPMLTAILMAHGU----MPWLSAAFAFAAUUIFTVQVYRWWYKPVLKEPML
PsORF396        -WLDUALLUGTGUIALLHAFGUAMRPQPLLGLLFU-AIGVGHLLRLMRWYDKGIWKVGLL
PsNnrS          -WLDNGILLGCULUALLTAAGUTTQPTPWLAGLFA-ALGGAQLWRLWRWRDRILWQVPLL
RcNnrS          -GRFDGASLLUAUGALLCUTLA---PDAILTAALLALAAALHUURLURWRGHLUWRSPLL
RcNnrS          PWLDRSVAGCACLAALAAUFPL----SGALAGAAALALGAGQLARMGFWRSRKVLGNPLL
                            : *::           :*  ::: ::       :  *    :    :*

NmNnrs          WILFAGYLFTGLGLIAUGASYFKPAFLN-LGUHLIGUGGIGULTLGMMARTALGHTGNPI
NGNNRS          WILFAGYLFTGLGLIAUGASYFKPAFLN-LGUHLIGUGGIGULTLGMMARTALGHTGNSI
PsORF396        WSLHUAMLWLUUAAFGLALWHFGLLAQSSPSLHALSUGSMSGLILAMIARUTLGHTGRPL
PsNnrS          WSLHLAYFWIAUAPLGMALWSLGLALAPSQSLHALAUGGMGGLILAMLARUTLGHTGRAL
RcNnrS          LMMHUAYGFLPLGLAATAAAAUGWASAP-AGLHLLGIGAIGGMTLAUMMRASLGHTGRPL
RcNnrS          AALHLAMLGTGIGALLQGFAAFGRGEEI-GALHVSAGUGGMILAUMADSRATLAQTGRAL
                : *        :  :      :         :*   :  :  : * :: *  * :**   :

NmNnrs          UPPPKAUPUAFWLMMAATAURMUARFSSGTAYTH---SIRTSSVLFALALLVYAWKYIPW
NGNNRS          YPPPKAUPUAFWLMMAATAURMUAUFSSGTAYTH---SIRTSSULFALALLUYAWKYIPW
PsORF396        QLP-AGIIGAFULFNLGTAAR---UFLSUAWPUG---GLWLAAVCWTLAFALYUWRYAPM
PsNnrS          QPP-AAMPWAFALLNLGCAAR---UFLPSLLPANW--ALPLAGGLWALAFLLFAWFYAPM
RcNnrS          EAG-RALSAGFACIUAAAAAR--TULAATDIGGID--GYSLAAAFWTMGFAIYVGEIGPC
RcNnrS          VAP-RPVVLAYLLLPLAALAR----FAAATWPETYVAAMLSAAALWVLAFALFALSSAPA
                      :   :  :  :  ::*    :                   : : ::::     *

NmNnrs          LIRPRSDGRP--G
NGNNRS          LIRPRSDGRP--G
PsORF396        LUAARUDGHP--G
PsNnrS          LCRPRUDGHP--G
RcNnrS          LLRPSLGRTPSKG
RcNnrS          FLGPR--QTP---
                :        *
```

Fig. 5.

expression of the nar genes. Because nitrate respiration leads to the production of the other denitrification intermediates, it can be difficult in wild type cells to demonstrate that nitrate is the effector for only a limited set of genes. Evidence for a nitrate stimulon was then demonstrated by experiments using nitrite reductase-deficient cells. These experiments showed that nitrate alone was not sufficient to activate expression of genes whose products are required for reduction of the other nitrogen oxides (Tosques, 1997; Korner, 1993). Nor does nitrite alone cause significant induction of nitrate reductase. However, there is evidence of cross talk between the nitrate and nitrous oxide stimulons in *P. stutzeri* and *P. denitrificans* (Baumann, 1996; Korner, 1989), although it is difficult to rationalize why this occurs. Experiments monitoring gene expression in relation to oxygen concentration show that the nar genes and nitrate reductase are expressed at higher oxygen levels than the nir and nor genes (Baumann, 1996). Expression of nos occurs at similar oxygen levels.

The molecular mechanisms required for nar activation have not been extensively studied in a denitrifying bacterium. A pair of two component sensor-regulators is responsible for regulating nar in *E. coli*. The function of these proteins has been extensively studied and has been reviewed in Darwin (1996). A nitrate sensor (NarL) and its response regulator (NarX) have been characterized in *P. stutzeri*. However, their deletion did not affect denitrification (Hartig, 1999). This has led to the suggestion that there is a nitrate-responsive system that functions independently of the NarXL system.

Nitric Oxide Stimulon Even before isolation of the proteins regulating the genes encoding the nitrite and nitric oxide reductases, it had been observed that the expression of nitric acid reductase in nitrite reductase mutants was negatively affected. This dependence of nitric oxide reductase expression on the activity of nitrite reductase has been demonstrated in many denitrifiers (Ye, 1992; Tosques, 1997; de Boer, 1994; Zumft, 1994). The decrease in nor gene expression in nitrite reductase mutants appears indirect, as any mutation that leads to a loss of nitrite reductase activity has a negative affect on nor expression (de Boer, 1994; Zumft, 1994). The expression of nir genes is also dependent on nitrite reductase activity (Tosques, 1997). The expression of nir is not directly dependent on nitric oxide reductase, but the accumulation of nitric oxide in nitric oxide reductase mutants probably affects nitrite reductase activity (Kwiatkowski, 1996; Zumft, 1994).

The observation that it is nitrite reductase activity not nitrite reductase per se that is required for the expression of nir and nor genes suggests that a product of nitrite reduction is required for gene expression. An obvious candidate for the likely effector is nitric oxide, a possibility consistent with the observation that addition of nitric oxide generators to nitrite reductase-deficient strains results in expression of both nir and nor genes (Kwiatkowski, 1996). Moreover, trapping of nitric oxide by hemoglobin decreases expression of nir and nor genes (Kwiatkowski, 1996). The accumulated evidence strongly indicates that it is the production of nitric oxide that stimulates expression of those genes in the nitric oxide stimulon. However, it is also possible that a derivative of nitric oxide may be the actual signal.

The role of nitric oxide as a signal molecule in humans is well known (Schmidt, 1994). The first use of nitric oxide as a signal molecule by a living organism, however, was likely by denitrifying bacteria. It is easy to rationalize why nitric oxide or some derivative serves as a signal molecule for denitrifiers. Denitrifying bacteria must keep the steady state levels of nitric oxide low to minimize potential cytotoxic reactions. As nitric oxide acts to regulate expression of the genes whose products are responsible for establishing the steady state levels of nitric oxide, the possibility nitric oxide might accumulate to cytotoxic levels is abated. Also, as it reacts rapidly with oxygen, nitric oxide will accumulate to the levels required to activate gene expression only when oxygen tension is low. This permits a single molecule to be used as an indicator of both oxygen and nitrogen oxide concentrations in the environment. The direct control of nitric oxide levels by oxygen may explain why nitrite reductase is expressed at lower oxygen concentrations than nitrate and nitrous oxide reductase.

In several denitrifiers, a gene has been isolated whose product directly regulates nir and nor expression but not nar or nos genes. The regulation of nir and nor by a single regulatory protein not involved in regulating expression of the other nitrogen oxide reductases is consistent with studies of gene expression indicating three separate stimulons. Not surprisingly, this gene has been given a different name in each bacterium. In *P. denitrificans*, it is designated nnr (van Spanning, 1995), nnrR in *R. sphaeroides* (Tosques, 1996), dnr in *P. aeruginosa* (Arai, 1995) and dnrD in *P. stutzeri* (Hartig, 1999). The family of proteins encoded by these genes will be referred to as the Nnr family in this review. Recent sequencing efforts have also identified likely orthologs in *Synechocystis* strain PCC 6803 (Kaneko, 1996), *Pseudomonas* sp. G-179 (Bedzyk, 1999) and *R. capsulatus* (Rhodobacter, 1999). All of these proteins are members of the

Fnr/CRP family of transcriptional regulators (Spiro, 1994).

Comparison of the sequences of these various proteins reveals little about how they might interact with an effector. Significantly, there are no obvious metal binding motifs in any of these proteins. Fnr and CooA, which are also members of the Fnr/CRP family, have metal centers that are targets for effector interaction. Fnr from *E. coli* binds an iron-sulfur center that apparently undergoes structural changes as the oxygen concentration in the cell changes (Popescu, 1998). CooA from *Rhodospirillum rubrum* binds a heme protein that acts as a carbon monoxide sensor (Shelver, 1997). Current data is not consistent with members of the Nnr family containing any type of metal center.

Phylogenetic analysis of the Nnr family reveals that the relatedness of the proteins does not coincide with relatedness predicted by 16S rRNA analysis. For example, *R. sphaeroides* is closely related to both *R. capsulatus* and *P. denitrificans*. The Nnr from *P. denitrificans* and the putative Nnr ortholog identified in *R. capsulatus* have significant identity but have only limited similarity to NnrR from *R. sphaeroides* (Fig. 6). This suggests species relatedness is not the major factor controlling the degree of relatedness of the members of the Nnr family.

Based on available data, a model can be presented in which nitrite produced by nitrate reductase activity is reduced by nitrite reductase, and some compound (most likely nitric oxide or a nitric oxide derivative) activates the transcriptional regulator resulting in expression of genes in the nitric oxide stimulon. Even though this model may be generally correct, differences appear in the regulation of the expression of various members of the Nnr family. In *R. sphaeroides*, NnrR appears to be constitutively expressed but may be negatively autoregulated (Tosques, 1996). There is no evidence of negative autoregulation of Nnr in *P. denitrificans* (van Spanning, 1995). Expression of Dnr in *P.* may be regulated by another member of the Fnr/Crp family, Anr, an apparent ortholog of Fnr in *E. coli* (Arai, 1995). This suggests a regulatory hierarchy where under low oxygen tension Anr activates expression of dnr, whose product can then activate expression of nir and nor genes under appropriate conditions. This type of regulatory hierarchy is not present in *P. stutzeri* (Hartig, 1999).

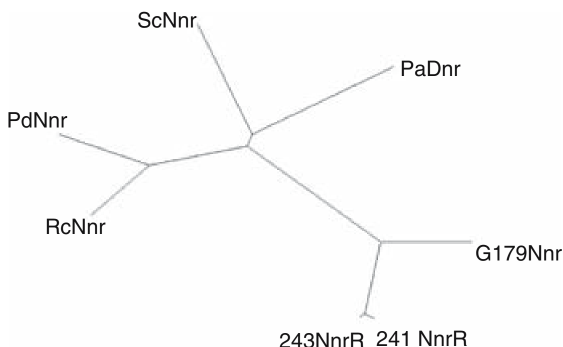

Fig. 6.

In *P. aeruginosa*, Dnr and Anr both regulate hemN, which encodes an oxygen-independent coproporphyrinogen oxidase required for heme synthesis (Rompf, 1998). The hemN gene is also coregulated by NnrR and FnrL in *R. sphaeroides* (Shapleigh, unpublished). Both FnrL and NnrR regulate the gene encoding pseudoazurin in *R. sphaeroides* (Jain and Shapleigh, unpublished). This dual regulation of selective genes by both a global regulator such as Anr/Fnr and a regulator of a limited set of genes such as Dnr/NnrR suggests that transcriptional activation by the global regulator alone is not sufficient for optimal growth under denitrification conditions. Therefore, denitrifiers have developed mechanisms to ensure sufficient levels of expression of genes whose products are in heavy demand during denitrification and other modes of anaerobic growth. Further, in *R. sphaeroides* nitrite accumulation negatively affects expression of FnrL regulated genes (Shapleigh, unpublished). This may provide another explanation as to why FnrL and NnrR are dual regulators of specific genes. It seems likely such dual regulation will be observed in other denitrifiers and will probably encompass a larger set of gene targets.

NITROUS OXIDE STIMULON Evidence for a nitrous oxide stimulon was initially provided by the observation that growth on nitrous oxide strongly stimulates nitrous oxide reductase expression, modestly stimulates expression of the nitrate reductase, and does not stimulate expression of nitrite or nitric oxide reductase (Korner, 1989). The observation that inactivation of the regulator of genes in the nitric oxide stimulon did not affect growth at the expense of nitrous oxide provided additional support for existence of a set of genes whose transcription depends solely on the presence of nitrous oxide.

The nature of the genes responsible for regulating nitrous oxide expression is not well defined. One gene suggested to play a role in regulation of nos genes is nosR. Inactivation of nosR in *P. stutzeri* inhibited expression of nosZ, the nitrous-oxide-reductase structural gene (Cuypers, 1992). NosR is a putative membrane protein containing a cytoplasmic C-terminal domain with two motifs that resemble [Fe-S] containing motifs. This unusual combination of a membrane bound, [Fe-S] protein involved

in expression of a copper-containing protein increases interest in this protein. It seems unlikely NosR is involved in copper metabolism, as inactivation of nosR results in the inhibition of nosZ transcription (Cuypers, 1992). In contrast, inactivation of nosFDY, suggested to be involved in copper metabolism, does not cause inhibition of nosZ transcription (Zumft, 1990).

Other Regulatory Proteins Searches for regulatory proteins required for expression of denitrification genes are only just beginning to identify possible regulators and to determine their physiological roles. Although the majority of the genes discussed in the preceding sections have been found in most denitrifiers, other putative regulatory proteins have been identified in only a single bacterium. One example is nirI, a gene implicated in regulation of expression of nirS, the structural gene for nitrite reductase in *P. denitrificans* (Genbank accession number AJ001308). Interestingly, the nirI product is similar to NosR, which is required for expression of nos genes. The involvement of NirI in nitrite reductase expression makes it less likely that NosR is involved in nitrous oxide reductase assembly. Instead, NosR and NirI are likely members of a family of proteins involved in regulation of nitrogen oxide metabolism in denitrifiers. Other regulatory proteins have been found clustered with denitrification genes, but their role in denitrification has not been defined. Clearly, our understanding of the regulation of denitrification genes is very limited. The list of the various proteins involved in regulation will expand, no doubt, as additional denitrifiers are characterized in greater detail.

Metabolism of Related Nitrogen Oxides

Because of its reactivity, nitric oxide will react with many compounds generating a wide range of different nitrogen oxide containing molecules. Some of these derivatives are more toxic than nitric oxide. For example, nitric oxide can react with the thiol of glutathione to generate S-nitrosoglutathione (GSNO), which has been shown to be more toxic for *Salmonella typhimurium* than nitric oxide (De Groote, 1995). Another very toxic nitric oxide derivative, peroxynitrite, is generated from the reaction of nitric oxide and superoxide (Squadrito, 1998). There has been a great deal of interest in the interaction of these types of compounds with pathogenic bacteria inasmuch as they are generated as part of the host defense mechanism during infection. These compounds could possibly be generated during denitrification as well. If so, denitrifiers have probably developed mechanisms to mitigate the potentially cytotoxic effects of such derivatives.

Despite the paucity of work on the metabolism of nitric oxide derivatives by denitrifiers, some data suggest that denitrifiers are useful models for understanding how cells mitigate nitric oxide toxicity. In experiments assessing the sensitivity of *R. sphaeroides* strains to GSNO, those strains with nitrite reductase activity were completely resistant to levels of GSNO to which *S. typhimurium* exhibited sensitivity (Wu, 1998). Comparable assays on a naturally occurring nitrite reductase-deficient strain of *R. sphaeroides* demonstrated that its sensitivity to GSNO was similar to that exhibited by *S. typhimurium*. There was no indication that the resistant strain had any special capacity to degrade GSNO suggesting the likely modification of a GSNO-sensitive target in the resistant strain. The results of this study need to be extended to other denitrifiers to determine whether general resistance to GSNO is intrinsic to denitrifiers. Also, it will be interesting to determine if denitrifying bacteria have any resistance mechanisms to other toxic nitric oxide derivatives such as peroxynitrite. Probing the molecular mechanisms denitrifiers have developed for resistance to nitric oxide derivatives is of broad scientific interest and is further justification for the study of the diverse group of bacteria linked by their shared capacity to reduce nitrate and nitrite to gaseous nitrogen oxides and nitrogen gas.

Acknowledgments. I would like to thank W. J. Payne for critically reading the manuscript. I would also like to thank him for nurturing my interest in microbiology and introducing me to denitrification. I would also like to thank my colleagues working in this field for their contributions. The work in my laboratory is funded by the U.S. Department of Energy.

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Prokaryotes (2006) 2:793–817
DOI: 10.1007/0-387-30742-7_24

CHAPTER 1.24

Dinitrogen-Fixing Prokaryotes

ESPERANZA MARTINEZ-ROMERO

Introduction

Dinitrogen fixation, the biocatalytic conversion of gaseous nitrogen (N_2) to ammonium, is an exclusive property of prokaryotes. The enzymes responsible for this reaction are nitrogenases. Proof that bacteria associated with leguminous plants can fix atmospheric N_2 (making it available to the plants for growth) was first reported in 1888 (reviewed in Quispel, 1988). Nitrogen fixation is the most important way N_2 from air enters biological systems and therefore it is a key step in the nitrogen cycle. From a practical point of view, the importance of the process rests with its ability to reduce the chemical fertilization of crops, even under conditions of environmental stress (Bordeleau and Prévost, 1994; Zahran, 1999). Indeed, agronomically important crops such as soybean, alfalfa, pea, clover and bean obtain substantial amounts of their nitrogen from bacterial N_2 fixation. One of the long-term goals of N_2 fixation research is to select or engineer major cereal crops such as rice, maize and sugarcane so they can satisfy the bulk of their nitrogen requirements, either indirectly by association with N_2-fixing bacteria, or directly by insertion of N_2-fixing genes into the plant.

Many diazotrophs (*di* = two, *azo*te = nitrogen; *trophs* = eaters: dinitrogen fixers) are found to be associated with the roots of plants where they exchange fixed nitrogen for the products of photosynthesis. Plants associated with N_2 fixers can grow in very poor soils and swamps (Koponen et al., 2003) and be used successfully for soil remediation.

Nowadays industrial fixation of atmospheric N_2 exceeds the amount estimated to be produced by biological nitrogen fixation each year (Karl et al., 2002) and increased nitrogen (N) deposition seems to be responsible for loss of biodiversity and plant species extinction (Stevens et al., 2004). Biological N_2 fixation is still the main source of nitrogen in soil, marine environments such as oligotrophic oceanic waters (where dissolved fixed-nitrogen content is extremely low; Zehr et al., 1998; Staal et al., 2003), subtropical and tropical open ocean habitats (Karl et al., 2002), and hydrothermal vent ecosystems (Mehta et al., 2003). N_2 fixation in coastal marine environments may diminish because of habitat destruction and eutrophication (Karl et al., 2002). Dinitrogen fixation may be a major nitrogen source for supporting primary and secondary production of biomass in Antarctic freshwater and soil habitats (Olson et al., 1998) and has been reported to occur in moss carpets of boreal forests (DeLuca et al., 2002) and in woody debris (Hicks et al., 2003). Dinitrogen fixation by bacteria inside insect gut helps to compensate termites for their nitrogen-poor diet (Kudo et al., 1998; Nardi et al., 2002).

N_2-fixing prokaryotes inhabit a wide range of exterior environments (including soils, seas, and the oceans) and interior environments (including insects, cow rumena, human intestines, and feces; Bergersen and Hipsley, 1970), and even printing machines and paper-making chemicals (Vaisanen et al., 1998). Nevertheless, the presence of a N_2-fixing bacterium is not evidence for the occurrence of N_2 fixation. On the basis of N balance analyses, N_2 fixation seemed to account for excess N in humans with a low N diet, and N-fixing bacteria were obtained from their guts (Bergersen and Hipsley, 1970; Oomen and Corden, 1970).

Dinitrogen fixers are encountered in Bacteria and in some groups of Archaea. The number of nitrogen-fixing phyla or lineages within the domain Bacteria increased from 5 to 6 when nitrogen-fixing bacteria were discovered within the Spirochaetes (Lilburn et al., 2001). The inventory of the phyla containing nitrogen-fixing bacteria is probably still far from complete but enlarging, as with the report of a strain of *Verrucomicrobium* that is reported to have nitrogen fixation genes (Rodrigues et al., 2004). Lists of N_2-fixing prokaryotes have been published (Young, 1992; Phillips and Martínez-Romero, 2000), and new nitrogen-fixing species are continuously being described (Chen et al., 2001; Moulin et al., 2001; Distel et al., 2002; Von der Weid et al., 2002; Bianciotto et al., 2003; Rosenblueth et al., 2004). Nevertheless knowledge of

N_2 fixers is limited, and some not yet identified N_2 fixers could be found among the novel bacterial divisions that are mostly unculturable (Rappé and Giovannoni, 2003). The distribution of N_2 fixers among the prokaryotes is patchy (Young, 1992). They constitute restricted groups within larger bacterial clusters. The existence of non-fixers that are closely related to fixers has been explained by the loss of N_2 fixation genes or by the lateral transfer of these genes among bacterial lineages (Normand and Bousquet, 1989; Vermeiren et al., 1999). Nitrogen fixation is an energy costly process, which may explain why nitrogen fixation was lost in many bacterial lineages when not needed. The possession of N_2-fixing genes does not confer a selective advantage to bacteria in nitrogen-rich environments, as is the case where fixed nitrogen is added to agricultural fields. Application of ammonium sulfate reduced the number of *Azotobacter* in the plant rhizosphere, and when compared with plants fertilized with both nitrogen and phosphorus, maize treated with phosphate alone had increased nitrogenase activity (Döbereiner, 1974).

Similarly, very few or no *Gluconacetobacter diazotrophicus* microorganisms were isolated from sugarcane plants from heavily fertilized areas (Fuentes-Ramírez et al., 1993; Muthukumarasamy et al., 1999), and, perhaps as a result of chemical nitrogen fertilization, the bacterial population had very limited genetic diversity (Caballero-Mellado and Martínez-Romero, 1994; Caballero-Mellado et al., 1995). Subsequently, sugarcane colonization by *A. diazotrophicus* was found to be inhibited in plants supplied with chemical nitrogen fertilizer (Fuentes-Ramírez et al., 1999). Another effect of adding fixed nitrogen (diminished genetic diversity of *Rhizobium* from *Phaseolus vulgaris* bean nodules) was observed when the plants were treated with the recommended level of chemical nitrogen (Caballero-Mellado and Martínez-Romero, 1999).

The complete genome sequence of the Archaeon *Methylobacterium thermoautotrophicum* was reported in 1997 revealing the presence of *nif* genes (Smith et al., 1997), but N_2 fixation could not be demonstrated in this strain (Leigh et al., 2000). The sequences of the genomes of the legume-nodulating bacteria belonging to the genera of *Mesorhizobium* (Kaneko et al., 2000), *Sinorhizobium* (Galibert et al., 2001) and *Bradyrhizobium* (Kaneko et al., 2002) revealed contrasting chromosome sizes and highly diverging genomes. A common ancestor of *Mesorhizobium* and *Sinorhizobium* was deduced to exist nearly 400 million years ago (Morton, 2002). One of the most novel areas in nitrogen fixation research is genomics, and for sure many N_2-fixing bacteria will be used for the determination of their whole genome sequence in the near future. Post-genomic studies are already on course as well.

Diazotroph Isolation and Conditions for N_2 Fixation

N_2-fixing bacteria are normally isolated in N-free media. Whether a microorganism is a N_2 fixer is not easy to determine. In the past, claims for many fixers were shown to be erroneous, mainly because fixers were recognized by their ability to grow in nitrogen-free media. However, traces of fixed nitrogen in the media sometimes accounted for the bacterial growth. At other times, oligotrophic bacteria and fungi, which can grow on nitrogen-free media, have been incorrectly reported to be N_2-fixing organisms. These microorganisms appear to meet their nitrogen requirements by scavenging atmospheric ammonia (Postgate, 1988). Photosynthetic bacteria have been known for more than 100 years, but the capacity of some of these bacteria to fix N_2 was not recognized until much later. Microorganisms may fix N_2 under special conditions that may not be readily provided in the laboratory. For example, nitrogenases are inactivated in the presence of oxygen, and different levels of oxygen seem to be optimal for different N_2-fixing organisms. Also, some bacteria (e.g., some *Clostridium*) fix N_2 only in the absence of oxygen. In other cases, fixation may require specific nutritional conditions or a differentiation process or both. A remarkable case is the differentiation process of *Rhizobium* to form N_2-fixing bacteroids (Bergersen, 1974; Glazebrook et al., 1993) inside plant root or stem nodules. *Bradyrhizobium* species can fix N_2 both in plant nodules and in vitro, when provided with succinic acid and a small amount of fixed nitrogen (Phillips, 1974). To fix N_2, bacteria belonging to the genus *Azoarcus* (obtained from Kallar grass and more recently also from rice plants) require proline, undergo differentiation, and form a structure called a "diazosome" (Karg and Reinhold-Hurek, 1996). Stimulated by plants, cyanobacteria differentiate into N_2-fixing heterocysts that protect nitrogenase from oxygen (Wolk, 1996). Light was found to induce circadian rhythms of N_2 fixation in the cyanobacterium *Synechococcus* (Chen et al., 1993). Wheat germ agglutinins were found to stimulate N_2 fixation by *Azospirillum*, and a putative receptor of this agglutinin was found in the *Azospirillum* capsule. The stimulus generated from the agglutinin-receptor interaction led to elevated transcription of both structural and regulatory nitrogen-fixation genes (Karpati et al., 1999).

Methods for Detecting Nitrogen Fixation

The methods used to measure biological N_2 fixation include the quantification of the total nitrogen difference from Kjeldahl analysis, acetylene reduction, and ^{15}N incorporation or dilution. The acetylene reduction assay has been used for over 30 years to measure nitrogenase activity and as an indicator of N_2 fixation (Hardy et al., 1968). These methods have been used both in the laboratory and the field, and improvements of the methods especially for field evaluations have been proposed, including double labeling using ^{34}S as a control reference (Awonaike et al., 1993). The ^{15}N-based techniques have been thoroughly reviewed (Bergersen, 1980; Hardarson and Danso, 1993).

Nitrogenases may reduce other substrates in addition to N_2 and this has been the basis for the acetylene reduction assay, which measures N_2 fixation activity indirectly. However, the nitrogenase described by Ribbe et al. (1997) does not have the ability to reduce acetylene. In *Paenibacillus*, N_2 fixation has been demonstrated in some cases by the increase in nitrogen measured by the microKjeldahl method but not by acetylene reduction (Achouak et al., 1999).

To circumvent the problems of estimating N_2 fixation under laboratory conditions, a strategy to detect nitrogenase genes has been successfully followed. This strategy was made possible by identification of conserved signatures (useful as primers for the synthesis of the nitrogenase reductase gene by means of polymerase chain reaction [PCR] amplification) in the structural *nif* gene sequences, namely *nifH*, found in many microorganisms (Dean and Jacobson, 1992; Ueda et al., 1995). In other cases, homologous or heterologous probes have been used in hybridization experiments to detect N_2 fixers. With some *nifH* primers containing conserved sequences, alternative nitrogenases may also be amplified but not the nitrogenase (superoxide) that is structurally unrelated to the classical nitrogenase (Ribbe et al., 1997). Thus a search for N_2-fixing organisms using a procedure based only on the classical *nifH* gene would be incomplete. Nevertheless, with nitrogenase DNA primers and PCR synthesis, novel N_2-fixing genes may be found. Eight *nifH* gene types corresponding mainly to those of diazotrophic Proteobacteria were detected in rice root from endophytic or rhizoplane-borne bacteria (Ueda et al., 1995). Remarkably, none of the sequences amplified corresponded to previously described *nifH* sequences. The nucleotide sequence of one of the types was found to resemble those of the *Azoarcus nif* genes. Some bacteria in the gut of termites also have *nifH* sequences similar to those obtained from rice roots (Ohkuma et al., 1999). *nif* genes were found in human and bovine treponemas (Lilburn et al., 2001) but not in the completely sequenced genomes of the spirochetes *Treponema pallidum* or *Borrelia burgdorferi*.

Few N_2-fixing organisms from the oceanic environment have been cultivated and it is estimated that less than 10% of marine diazotrophs are cultivable. Nevertheless, on the basis of the amplification of nitrogenase *nifH* genes, new N_2-fixing organisms have been detected in oligotrophic oceans. Nitrogenase genes characteristic of cyanobacteria and of Alpha- and Betaproteobacteria were obtained, whereas *nifH* sequences from samples associated with planktonic crustaceans were found to be clustered with the corresponding sequences from either sulfate reducers or clostridia (Zehr et al., 1998). Since knowledge of the nitrogenase gene diversity has improved (over 1500 sequences were available at the time this manuscript was being written), different sets of primers have been designed (Bürgmann et al., 2004) to better amplify *nifH* genes directly from DNA extracted from various samples including environmental samples. More diverse diazotrophic populations have been revealed with this approach than with classical microbiological techniques that require culturing of the bacteria (Zehr et al., 1998; Bürgmann et al., 2004).

A different method of N_2-fixation detection involves the growth of indicator non-N_2-fixing organisms in a co-culture with putative N_2-fixing bacteria. Such an approach has the additional advantage of identifying bacteria that not only fix N_2 but also can release fixed nitrogen into the environment and thereby have potential use in agriculture. *Gluconacetobacter diazotrophicus* (Yamada et al., 1997), a N_2-fixing isolate from sugarcane, was cultured with the yeast *Lipomyces kononenkoae* on nitrogen-free medium, and yeast growth was shown to be proportional to the amount of N_2 fixed (Cojho et al., 1993).

Distribution of Dinitrogen-Fixing Ability among Prokaryotes

Archaea and Bacteria nitrogenases are phylogenetically related (Leigh, 2000), and supposedly the last common ancestor was a N_2-fixing organism (Fani et al., 1999). Alternatively, nitrogen fixation could have evolved in methanogenic archaea and subsequently transferred into the bacterial domain (Raymond et al., 2004). Nowadays, only 6 out of 53 currently identifiable major lineages or phyla within the domain Bacteria

have nitrogen-fixing members, namely: Proteobacteria, cyanobacteria, Chlorobi (green nonsulfur), spirochetes and the Gram-positives (Firmicutes and Actinobacteria; Fig. 1).

Dinitrogen-fixing organisms have an advantage over non-fixers in N_2-deficient but not in N_2-sufficient environments where the N_2 fixers are readily outcompeted by the bulk of microorganisms. The *nif* genes may be expected to disappear from bacteria that become permanent inhabitants of environments with available fixed N_2; this may explain why some non-N_2 fixers emerged and are closely related to N_2 fixers in phylogenetic trees of bacteria. Even within species of N_2 fixers, some strains do not fix N_2 perhaps because of the loss of this unique capacity, as is evident in *Azotobacter*, *Beijerinckia* (Ruinen, 1974) and *Frankia* (Normand et al., 1996). In *Rhizobium*, *nif* genes and genes for nodule formation may be easily lost concomitantly with the symbiotic plasmid (Segovia et al., 1991). Similarly, nonsymbiotic *Mesorhizobium* strains are found in nature that lack a symbiotic island (Sullivan et al., 1996). N_2-fixing species seem to be dominant in Rhodospirillaceae (Madigan et al., 1984), and within the methanogens (in Archaea), nitrogen fixation is widespread (Leigh, 2000). While all *Klebsiella variicola* isolates were N_2-fixing bacteria (Rosenblueth et al., 2004), only 10% of its closest relatives (*K. pneumoniae* from clinical specimens) had this capacity (Martńez et al., 2004).

The N_2-fixing capability is unevenly distributed throughout prokaryotic taxa, and N_2-fixing bacteria are in restricted clusters among species of non-N_2-fixing bacteria. Only a subset of cyanobacterial species are able to fix N_2. *Gluconacetobacter diazotrophicus* and a couple of other N_2-fixing species are the only diazotrophs in a larger group comprising *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* (Fuentes-Ramŕez et al., 2001). Similarly, among aerobic endospore-forming Firmicutes (Gram-positive

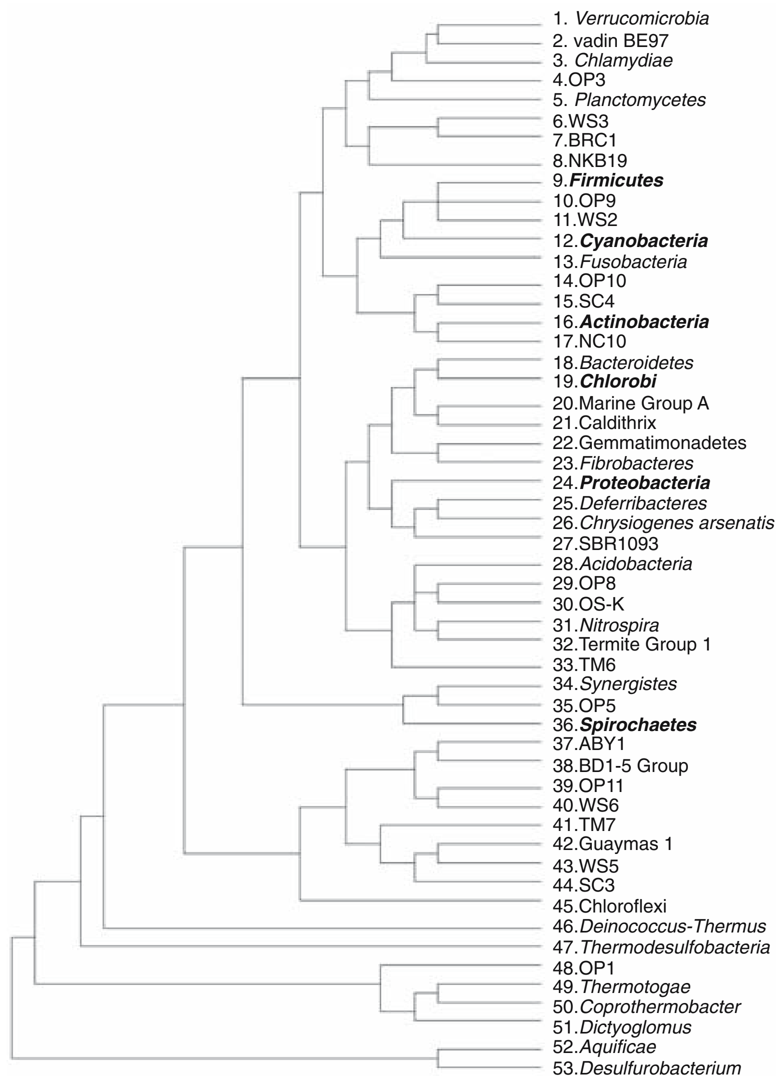

Fig. 1. Relatedness of *nifH* genes from different organisms according to DNA sequence (after Hurek et al., 1997a; Ueda et al., 1995; Young et al., 1992; Zehr et al., 1995). In parentheses, the Proteobacteria subclass.

bacteria), N_2 fixers are encountered mainly in a discrete group (defined by cluster analysis from 16S rRNA gene sequences) corresponding to *Paenibacillus* (Achouak et al., 1999). Among the actinomycetes, N_2-fixing *Frankia*, represented by a diversity of phenotypes from different habitats, are grouped by their 16S rRNA gene sequences (Normand et al., 1996). In Archaea, N_2-fixing organisms are found in the methanogen group and in the halophile group within the Euryarchaeota but not in the sulfur-dependent Crenarchaeota (Young, 1992).

Pseudomonas spp. were considered unable to fix N_2, but recently new isolates have been recognized as N_2 fixers. Some isolates, closely related to fluorescent pseudomonads, possess in addition to the FeMo nitrogenase an alternative molybdenum-independent nitrogenase (Loveless et al., 1999; Saah and Bishop, 1999). Dinitrogen-fixing *Pseudomonas stutzeri*, (previously designated *Alcaligenes faecalis*) (Vermeiren et al., 1999), is widely used as a rice inoculant in China (Qui et al., 1981). Following rice inoculation, *P. stutzeri* aggressively colonize the roots, and the *nifH* gene is expressed in these root-associated bacteria (Vermeiren et al., 1998). Other reports list different N_2-fixing *Pseudomonas* species that have been isolated from sorghum in Germany (Krotzky and Werner, 1987), from *Capparis* in Spain (Andrade et al., 1997), and from *Deschampsia caespitosa* in Finland (Haahtela et al., 1983). The sporadic occurrence of *nif* genes in *Pseudomonas* may be explained by the acquisition of these genes by lateral transfer (Vermeiren et al., 1999). *Pseudomonas stutzeri* strains are known to be naturally competent for DNA uptake (Lorenz and Wackernagel, 1990). Other *nifH* gene sequences obtained from rice-associated bacteria were in the same cluster as the *P. stutzeri nifH* gene (Ueda et al., 1995; Vermeiren et al., 1999).

The phylogenetic relationship of N_2-fixing organisms inferred from the comparative analysis of *nif* and 16S rRNA gene sequences led Hennecke et al. (1985) to propose that the *nifH* genes may have evolved in the same way as the organisms that harbor them; a similar conclusion was obtained by Young (1992) from the analysis of a larger number of diazotrophs. Ueda et al. (1995) and Zehr et al. (1995), using different reconstruction methods, reported *nifH* gene phylogenies in general agreement with the phylogenetic relationships derived from 16S rRNA gene sequences, with some exceptions. A more recent comparison of *nif*H and 16S rRNA phylogenies has been performed with a very short fragment of the *nifH* gene. An early possible duplication of *nifH* and paralogous comparisons make interpretations difficult (see Fig. 3 in Zehr et al., 2003). Four major clusters of *nifH* are recognized and functional nitrogenases are found in three of them (Zehr et al., 2003). The phylogenies of *nifH* genes are continuously revised and updated with novel sequences (including environmental ones) and more robust reconstruction methods. *nifH* genes from Gammaproteobacteria are found in different groups, as well as those from Betaproteobacteria (Bürgmann et al., 2004). Anomalies in the phylogenetic position of Betaproteobacteria have been reported as well (Hurek et al., 1997; Minerdi et al., 2001).

Ecology of Dinitrogen-Fixing Prokaryotes

The communities of dinitrogen-fixing bacteria in natural environments may be studied with approaches such as the amplification by PCR of the nitrogenase reductase gene (*nifH*) with *nifH* primers using environmental DNA, with subsequent analyses by cloning and sequencing, by terminal restriction fragment length polymorphism (T-RFLP; Ohkuma et al., 1999; Tan et al., 2003), or by denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993). Hybridization to macro- and microarrays may reveal the presence and frequency of different N_2-fixing prokaryotes (Jenkins et al., 2004; Steward et al., 2004).

The ecology of the symbiotic N_2-fixing soil bacteria that are collectively designated rhizobia, has been comprehensively reviewed by Bottomley (1992), and ecogeographic and diversity reviews of these bacteria have been reported (Martínez-Romero and Caballero-Mellado, 1996; Sessitsch et al., 2002). Additional aspects of *Rhizobium* ecology in soil also have been reviewed (Sadowsky and Graham, 1998). *Frankia* symbiosis including some ecological aspects has been reviewed by Baker and Mullin (1992) and by Berry (1994). New molecular approaches have recently enhanced our perception of microorganisms in their natural habitats. By using PCR primers targeted to nitrogenase genes, the description and natural histories of communities of N_2-fixing microorganisms may be established more accurately than with traditional microbiological techniques. The fluctuations of marine diazotroph populations have been analyzed with these approaches. The bulk of N_2 fixation appears to shift from cyanobacterial diazotrophs in summer to bacterial diazotrophs in fall and winter (Zehr et al., 1995). The heterocystous cyanobacteria are not as efficient fixing nitrogen as the nonheterocystous cyanobacteria at the high temperatures of the tropical oceans (Staal, 2003). The diversity of marine N_2 fixers in benthic marine mats was determined from the sequences of *nifH* genes. The *nifH*

sequences obtained were most closely related to those of anaerobes, with a few related to Gammaproteobacteria including *Klebsiella* and *Azotobacter* species (Zehr et al., 1995).

The role of N_2 fixation was examined in microbial aggregates embedded in arid, nutrient-limited and permanent ice covers of a lake area in the Antarctic, and also in mats in soils adjacent to the ice border. Molecular characterization by PCR amplification of *nifH* fragments and nitrogenase activity measured by acetylene reduction showed a diverse and active diazotrophic community in all the sites of this environment. Nitrogenase activity was extremely low, compared to temperate and tropical systems. Diazotrophs may be involved in beneficial consortial relationships that may have advantages in this environment (Olson et al., 1998). Nitrogen fixation, observed in moderately decayed wood debris, was shown to be stimulated by warm temperatures (Hicks et al., 2003).

The diversity of the N_2-fixing microorganisms within the symbiotic community in the gut of various termites was studied without culturing the symbiotic microorganisms. Both small subunit (ss) rRNA (Kudo et al., 1998) and *nifH* genes (Ohkuma et al., 1999) were amplified in DNA extracted from the mixed microbial population of the termite gut. The analysis of the *nif* clones from diverse termites revealed different sequences in most of the individual termite species. Whereas the *nif* groups were similar within each termite family, they differed between termite families. Microorganisms from termites with high levels of N_2-fixation activity could be assigned to either the anaerobic *nif* group (clostridia and sulfur reducers) or to the alternative *nif* methanogen group. Highly divergent *nif* gene sequences (perhaps not even related to nitrogen fixation) were found in termites that showed low levels of acetylene reduction (Ohkuma et al., 1999). Expression of the N_2 fixation gene *nifH* was evaluated directly by amplifying *nifH* cDNA from mRNA by reverse transcription (RT)-PCR (Noda et al., 1999). Only the alternative nitrogenase (from *anf* gene) was preferentially transcribed in the gut of the termite *Neotermes koshunensis*. The levels of expression of the *anf* gene were related to the N_2 fixation activity recorded for the termites. The addition of Mo (molybdenum) to the termite diet did not repress the expression of the *anf* genes; however, Mo repression of other *anf* genes has been described (Noda et al., 1999). Estimates are that the contribution of insect-borne nitrogen-fixing bacteria in insects may be up to 30 kg of N/hectare (ha)/year (Nardi et al., 2002).

Endosymbionts from marine bivalve species, located in the shipworm gills, are cellulolytic and N_2-fixing. They provide cellulolytic enzymes to the host. They are a unique clade in the Gammaproteobacteria related to *Pseudomonas* and were designated as a new genus and species *Teredinibacter turnerae*, which fixes nitrogen in microaerobic in vitro conditions (Distel et al., 2002).

The arbuscular mycorrhizal fungus (*Gigaspora margarita*) has been shown to harbor a viable and homogeneous population of endosymbiotic bacteria that has been designated as "*Candidatus Glomeribacter gigasporarum*" (Bianciotto et al., 1996) related to Betaproteobacteria such as *Ralstonia* (Bianciotto et al., 2003). In the genomic library of total DNA from the fungal spores, clones carrying the bacterial genes *nifD* and *nifK* were identified. Both of these genes were arranged in a similar manner to the corresponding genes in archaea or bacteria and were similar to nitrogenases from different diazotrophs (Minerdi et al., 2001; Minerdi et al., 2002). mRNAs for the *nif* genes were detected, but whether these endosymbionts fix nitrogen is unknown.

Dinitrogen-fixing cyanobacteria form symbioses with diverse hosts such as fungi, bryophytes, cycads, mosses, ferns, and an angiosperm, *Gunnera* (Bergman et al., 1992). The genome of the cyanobacteria *Nostoc* (which is a symbiont of cycads, *Gunnera* and others) may be the largest among those from Prokaryotes, with nearly 10 Mb (Meeks et al., 2001).

New symbionts capable of forming nodules in the leguminous plant *Lotus corniculatus* were obtained in agricultural fields after the lateral transfer of genetic material to native nonsymbiotic soil mesorhizobia (Sullivan et al., 1995; Sullivan et al., 1996). Nonsymbiotic soil rhizobia, which outnumber symbiotic bacteria in some cases (Segovia et al., 1991; Laguerre et al., 1993), have been considered to be potential recipients of symbiotic plasmids. Molecular analyses (including the sequence of DNA fragments of 16S rRNA genes, the fingerprints of digested genomic DNA, and the hybridization patterns to cloned fragments) clearly demonstrated that a large segment of genetic material was acquired by soil *Mesorhizobium* bacteria (Sullivan et al., 1995) and that the original *Mesorhizobium loti* strain applied to the soil as an inoculant was the donor of these symbiotic genes. The mobilizable 500-kb DNA fragment has been designated a symbiosis island and it encodes genes for symbiotic N_2 fixation (*fix* genes) as well as those for the synthesis of vitamins (Sullivan et al., 2002). The symbiotic island was integrated into the phenylalanine-tRNA gene (Sullivan and Ronson, 1998). Interestingly, pathogenicity islands in other bacteria range up to 190 kb in size and most are either found adjacent to or integrated

within tRNA genes or flanked by insertion sequences (Cheetham and Katz, 1995; Kovach et al., 1996). In *M. loti*, the symbiotic genes are chromosomally located as in most *Mesorhizobium* and *Bradyrhizobium* sp. A similar symbiotic chromosomal region was identified in *M. loti* (Kaneko et al., 1999) that was later classified as *M. huakuii* (Turner et al., 2002). Only a few *Mesorhizobium* species such as *M. amorphae* possess symbiotic plasmids (Wang et al., 1999b), which are a common characteristic of *Rhizobium* and *Sinorhizobium* species (Martńez et al., 1990). The great chromosomal diversity, mainly based on 16S rRNA sequence (Wang and Martńez-Romero, 2000) and on glutamine synthetase (GSII) genes (Wernegreen and Riley, 1999) encountered in *M. loti*, may be ascribed to the natural occurrence of genetic transfer of symbiotic genes in *Mesorhizobium* (Sullivan et al., 1996).

The range of nodulating bacteria has enlarged. Nodulating *Methylobacterium* have been reported from *Crotalaria* nodules (Sy et al., 2001). Surprisingly, some Betaproteobacteria in the genera *Burkholderia* (Moulin et al., 2001) and *Ralstonia* (Chen et al., 2001) are capable of nodulating legumes. These bacteria have been classified as *Burkholderia phymatum*, *B. tuberum* (Vandamme et al., 2002), *B. caribensis* (Chen et al., 2003) and *Wautersia taiwanensis* (previously designated *Ralstonia taiwanensis*) (Chen et al., 2001; Vaneechoutte et al., 2004). Like *Rhizobium* and *Sinorhizobium* spp., these Betaproteobacteria possess symbiotic plasmids that carry nodulation genes (Chen et al., 2003). The similarity of these *nod* genes to those of the Alphaproteobacteria suggested that lateral transfer of *nod* genes occurred, most probably from Alpha- to Betaproteobacteria (Moulin et al., 2001; Chen et al., 2003). Similarly the lateral transfer of *nod* genes has been implied as a possible explanation for the nodulation capacity in *Devosia*, and a new species has been identified that carries *nodD* and *nifH* genes similar to those of *R. tropici* (Rivas et al., 2002).

Dinitrogen-Fixing Prokaryotes in Agriculture

The first industrial production of *Rhizobium* inoculants began at the end of the nineteenth century. In the absence of nitrogen fertilization, spectacular increases in plant and seed yield may be obtained by inoculation of legumes where the specific rhizobia for the legumes are absent or scarce (Singleton and Tavares, 1986). Factors affecting nodule occupancy by rhizobia inoculants were reviewed by Vlassak and Vanderleyden (1997). Inoculation of soybean is a common practice in Brazil (Hungria et al., 2000) or in the United States where production of soybean inoculants is a top priority for inoculant-producing companies (Paau, 1989), and inoculation of cash crops with nitrogen-fixing inoculants is considered a realistic alternative to the ever increasing use of fertilizers. High quality inoculants (whose characteristics were discussed by Maier and Triplett, 1996) as well as the improvement of management systems, are useful not only for agriculture but also for reforestation of devastated areas. Leguminous trees with their corresponding rhizobia have been recommended for many and diverse uses including reforestation, soil restoration, lumber production, cattle forage, and for human food. The so-called "actinorhizal plants" that associate with *Frankia* are also of great value for reforestation; actinorhizal plants belong to eight families (Baker and Mullin, 1992; Berry, 1994).

A high impact goal of nitrogen fixation research has been to extend nitrogen fixation to non-legumes and this has promoted the search for nitrogen fixing bacteria that are associated with agriculturally valuable crops. From a basic research perspective this has increased our knowledge of their diversity. The impact on agriculture and potential as a substitute for the high levels of fertilizer used in intensive agriculture is debatable, and a critical review of the actual contributions of N_2 fixation to the amount of fixed N present in cereals and other grasses finds that N_2-fixing bacteria in agriculture provide only a limited amount of fixed N. Careful long-term N balance studies would be required to accurately estimate these contributions (Giller and Merckx, 2003). Levels of fixed nitrogen (as low as 5–35 kg N/ha per year) that contribute over the long term to sustain fertility in nonagricultural areas (Stevens et al., 2004) are neglible for present modern intensive agricultural needs but may be of use in traditional, low input small farming systems. Legumes may fix over 200 kg N/ha per year and this is a significant contribution of nitrogen. Conservative values for bacterial fixation in nonlegumes are 20–30 kg N/ha per year, but higher, substantial values have been also estimated (see below). The rate of fixation of the tree *Acacia dealbata* is considered sufficient to replace the estimated loss due to timber harvesting (May and Attiwill, 2003).

Sugarcane and rice are the Gramineae most extensively studied with regard to N_2 fixation, but other crops are being studied as well (see below). Sugarcane has been grown for more than 100 years in some areas of Brazil without nitrogen fertilization or with very low nitrogen inputs, and removal of the total harvest has not led to decline in yield and soil nitrogen levels. This observation suggested that N_2 fixation may have been the source for a substantial part of the

nitrogen used by this crop (Döbereiner, 1961). Alternatively, irrigation water has been implicated as a possible source of N (Giller and Merckx, 2003).

From 25–55% (Urquiaga et al., 1989; Yoneyama et al., 1997) or perhaps as much as 60–80% (Boddey et al., 1991) of the plant N could be derived from associative dinitrogen fixation, but scepticism about the occurrence of high levels of nitrogen fixation has been expressed (Giller and Merckx, 2003). The problems of estimating sugarcane N_2 fixation, discussed by Boddey et al. (1995), include different patterns of nitrogen uptake by different sugarcane varieties (Urquiaga et al., 1989), declining ^{15}N enrichment of soil mineral nitrogen, carryovers of nitrogen from one harvest to the next, and differential effects on control plants during the three-year study (Urquiaga et al., 1992). The mean estimates of fixed N_2 for two sugarcane hybrids grown in concrete tanks ranged from 170–210 kg N_2 fixed/ha (Urquiaga et al., 1992). Correction for micronutrient soil deficiencies and high soil moisture seem to be key conditions that promote N_2 fixation in sugarcane plants (Urquiaga et al., 1992). The evidence of large differences in N_2 fixation among different sugarcane cultivars is compelling.

Dinitrogen-fixing bacteria isolated from the rhizosphere, roots, stems and leaves of sugarcane plants include *Beijerinckia*, *Azospirillum*, *Azotobacter*, *Erwinia*, *Derxia*, *Enterobacter* (reviewed in Boddey et al., 1995), *Gluconacetobacter* (Cavalcante and Döbereiner, 1988), and *Herbaspirillum* (Baldani et al., 1986). *Gluconacetobacter diazotrophicus* has the capacity to fix N_2 at low pH and in the presence of nitrate and oxygen. A *G. diazotrophicus nifD* mutant that cannot fix N_2 has been tested on plants derived from tissue cultures. Plant height was significantly increased by the wildtype strain and not by the mutant strain inoculants, suggesting a positive effect of N_2 fixation by *G. diazotrophicus* on sugarcane (Sevilla et al., 1998). Beneficial effects of *G. diazotrophicus* inoculation in experimental fields also have been reported (Sevilla et al., 1999), but global N balances were not analyzed. Selected strains of *Herbaspirillum* were reported to stimulate plant development (Baldani et al., 1999). *Gluconacetobacter diazotrophicus* (James and Olivares, 1997), *Herbaspirillum seropedicae* and *H. rubrisubalbicans* (Olivares et al., 1996) have been clearly shown to colonize sugarcane plants internally. Colonization by *G. diazotrophicus* was inhibited by nitrogen fertilization (Fuentes-Ramírez et al., 1999). Probably N_2 fixation in sugarcane is performed by a bacterial consortium.

Several studies have been carried out on nitrogen balance in lowland rice fields in Thailand (Firth et al., 1973; Walcott et al., 1977), in Japan (Koyama and App, 1979), and at the experimental fields of the International Rice Research Institute (IRRI) in the Philippines (App et al., 1984; Ventura et al., 1986). These studies report a positive balance with estimates of around 16–60 kg of nitrogen fixed per ha per crop (App et al., 1986; Ladha et al., 1993). In a nitrogen-balance study carried out on 83 wild and cultivated rice cultivars (6 separate experiments, each with 3 consecutive crops), large and significant differences between cultivars were found (App et al., 1986). But other assays showed only a small or nonsignificant contribution of fixed N_2 in rice (Watanabe et al., 1987b; Boddey et al., 1995).

Many different N_2-fixing bacteria have been isolated from rice roots. These include *Azotobacter*, *Beijerinckia* (Döbereiner, 1961), *Azospirillum* (Baldani and Döbereiner, 1980; Ladha et al., 1982), *Pseudomonas* (Qui et al., 1981; Barraquio et al., 1982; Barraquio et al., 1983; Watanabe et al., 1987a; Vermeiren et al., 1999), *Klebsiella*, *Enterobacter* (Bally et al., 1983; Ladha et al., 1983), *Sphingomonas* (described as *Flavobacterium* in Bally et al., 1983), *Agromonas* (Ohta and Hattori, 1983), *Herbaspirillum* spp. (Baldani et al., 1986; Olivares et al., 1996), sulfur-reducing bacteria (Durbin and Watanabe, 1980; reviewed in Barraquio et al. [1997] and in Rao et al. [1998]), *Azoarcus* (Engelhard et al., 1999) and methanogens (Rajagopal et al., 1988; Lobo and Zinder, 1992). The nitrogenase genes of *Azoarcus* are expressed on rice roots (Egener et al., 1998), and *Herbaspirillum seropedicae* expresses *nif* genes in several gramineous plants including rice (Roncato-Maccari et al., 2003).

Cyanobacteria have long been used to fertilize agricultural land throughout the world, most notably rice paddies in Asia. Increases in rice plant growth and increases in nitrogen content in the presence of cyanobacteria have been documented by many investigators. Plant promotion may also be related to growth-promoting substances produced by the cyanobacteria (Stewart, 1974). *Azolla* is a small freshwater fern that grows very rapidly on the surface of lakes and canals. Extensive employment of *Azolla-Anabaena* as a green manure in rice cultivation has been documented. *Anabaena*, a representative filamentous cyanobacterium, establishes symbioses with a diversity of organisms including *Azolla*. Unfortunately, various cyanobacteria also produce highly poisonous toxins and some of them are related to the high incidence of human liver cancer in certain parts of China. Highly toxic strains have been found in *Anabaena* and in other genera of cyanobacteria, and identification of such strains requires sophisticated biochemical tests (Carmichael, 1994).

Alternatively, other bacterial species are being tested to promote rice growth, such as the N_2-fixing *Burkholderia vietnamiensis* (Gillis et al., 1995). In some agriculture sites in Vietnam, this species has been isolated as the dominant N_2-fixing bacterium in the rice rhizosphere (Trâ Van et al., 1996). *Burkholderia vietnamiensis* inoculation has resulted in significant increases (up to 20%) in both shoot and root weights in pots and its use in rice inoculation seems highly promising (Trâ Van et al., 1994). However, a note of caution has been raised with a proposed moratorium on the agricultural use of *B. vietnamiensis*, which has a close genetic relationship to human pathogens implicated in lethally infecting patients with cystic fibrosis (Holmes et al., 1998). Detailed molecular analysis may allow for the distinction of pathogenic and environmental isolates (Segonds et al., 1999).

For over seven centuries, rice rotation with clover has significantly benefited rice production in Egypt. Clover is normally associated with *Rhizobium leguminosarum* bv. *trifolii* that forms N_2-fixing nodules in the root of this plant. Surprisingly, strains of this bacterium also were encountered inside the rice plant with around 10^4–10^6 rhizobia per gram (fresh weight) of root. These values are within the range of other bona fide endophytic bacteria (Yanni et al., 1997). Promotion of rice shoot and root growth was dependent on the rice cultivar, inoculant strain, and other conditions. Inoculation of rice with a selected strain gives best results in presence of low doses of nitrogen fertilizer. A number of investigators have reported growth stimulation of crops such as wheat and corn inoculated with a *R. leguminosarum* bv. *trifolii* strain, but these effects may not be related to N_2 fixation (Holflich et al., 1995).

In non-legumes (such as *Arabidopsis thaliana* [a model plant]), penetration of rhizobial strains has been found to be independent of nodulation genes that are normally required for bacterial entry into the legume root (Gough et al., 1996; Gough et al., 1997; Webster et al., 1998; O'Callaghan et al., 1999). This process probably requires cellulases and pectinases (Sabry et al., 1997). *Azorhizobium caulinodans*, in addition to forming nodules on *Sesbania rostrata*, has been found to colonize the xylem of its host (O'Callaghan et al., 1999) as well as to colonize wheat (Sabry et al., 1997). In wheat, *A. caulinodans* promotes increases in dry weight and nitrogen content as compared to uninoculated controls; acetylene reduction activity was also recorded. The interaction between azorhizobia and wheat root resembles the invasion of xylem vessels of sugarcane roots by *G. diazotrophicus* (James and Olivares, 1997) and *Herbaspirillum* spp. (Roncato-Maccari et al., 2003) and of wheat by *Pantoea agglomerans* (Ruppel et al., 1992). The xylem vessels may be the site of N_2 fixation because they provide the necessary conditions (carbohydrates and low oxygen tension), although the nutrient levels in the xylem have been considered as too low to maintain bacterial growth and N_2 fixation (Fuentes-Ramŕez et al., 1999; Welbaum et al., 1992). In acreage cultivated using *Sesbania rostrata*-rice rotation, *A. caulinodans* survives in the soils and rhizosphere of wetland rice (Ladha et al., 1992). *Azorhizobium caulinodans* can colonize the rice rhizosphere (specifically around the site of lateral root emergence), penetrate the root at the site of emergence of lateral roots, and colonize subepidermally intercellular spaces and dead host cells of the outer rice root cortex (Reddy et al., 1997).

The application of green manure has been an agronomic practice for increasing rice production, and legumes also can be used because of their symbiosis with N_2-fixing rhizobia. A large number of species are used both before and after rice culture including *Macroptilium atropurpureum*, *Sesbania* and *Aeschynomene* spp. (Ladha et al., 1992). Owing to their high N_2-fixing capacity and their worldwide distribution, flood-tolerant legumes such as *Sesbania rostrata* have been the focus of research. *Sesbania herbacea* nodulated by *R. huautlense* is also a flood-tolerant symbiosis (Wang and Martńez-Romero, 2000).

Nitrogen fixation in non-legumes is conditioned more by the plant than by the bacteria. Interestingly, aluminum-tolerant plants are more capable of maintaining bacterial nitrogen fixation than plants that are not tolerant (Christiansen-Weniger et al., 1992), maybe because they excrete dicarboxylics that are adequate to support bacterial N_2-fixation.

N_2-fixing bacteria associated to maize include: *Azospirillum*, *Herbaspirillum*, *Klebsiella* (Chelius and Triplett, 2001), *Burkholderia vietnamiensis* (Trâ Van et al., 1996), *R. etli* (Gutiérez-Zamora and Martńez-Romero, 2001), and the newly described species (*Paenibacillus brasilensis*; [Von der Weid et al., 2002] and *Klebsiella variicola* [Rosenblueth et al., 2004]). *Klebsiella variicola* was also found associated with banana plants (Martńez et al., 2003). Soil type instead of the maize cultivar determined the structure of a *Paenibacillus* community in the rhizosphere (Araujo de Silva et al., 2003).

Sweet potato (*Ipomoea batatas*) may grow in poor N-soil and associated N-fixation has been considered to contribute N to these plants. By a cultivation-independent approach, bacteria similar to *Klebsiella*, *Rhizobium* and *Sinorhizobium* were inferred to be present as sweet potato endophytes (Reiter et al., 2003).

Perspectives of Application of Nitrogen Fixation Research

The transgenic plants that will herald a revolution in agriculture are those with functional nitrogenase genes that, when expressed, will satisfy all the plant's nitrogen needs. The source of these genes will be prokaryotic. Research efforts are directed towards the ambitious goal of transforming rice plastids (Potrykus group in Zürich discussed in Rolfe et al., 1998) and plastids of the alga *Chlamydomonas reinhardtii* (Dixon et al., 1997; Dixon, 1999). Introduction of additional genes into plants to protect nitrogenase from oxygen damage will be needed. Such approaches could only be based on a profound understanding of N_2 fixation biochemistry, gene regulation and organization, as well as the structure and function of nitrogenases. Whether such a goal is feasible is difficult to predict.

The identification and selection of plant-associated microorganisms and their genetic improvement is an alternative strategy for obtaining agricultural crops that benefit from prokaryotic N_2 fixation. N_2 fixation (N_2 fixation without nodules) from associated bacteria is being considered as a suitable mode to exploit N_2 fixation in non-legumes (Triplett, 1996). Rhizosphere N_2 fixation by *Rahnella aquatilis* has been reported to occur in maize and wheat (Berge et al., 1991), and in other plants (Heulin et al., 1989). Mycorrhiza associate with most plants, and interestingly, bacteria-like organisms with nitrogenase genes have been found to be natural endosymbionts of the mycorrhiza (Minerdi et al., 2002). This association may be exploited to transfer N_2 fixation to non-legumes. The genetic improvement of mycorrhiza and bacterial symbionts may constitute a highly efficient system for the provision of fixed nitrogen to the plants.

The usefulness of N_2-fixing bacteria in bioremediation is also being recognized (Suominen et al., 2000; Prantera et al., 2002). Increased transformation of contaminating polychlorinated biphenyls was obtained with alfalfa inoculated with *Sinorhizobium meliloti* at 44 days after planting (Mehmannavaz et al., 2002). Dinitrogen fixation may decrease the need for nitrogen required by bacterial consortia used to degrade diesel fuel (Piehler et al., 1999).

Novel N_2 fixers may be found if the enrichment conditions for their isolation are more varied so as to include aerobic, anaerobic or microaerobic conditions, a variety of carbon sources at varying concentrations (copiotrophic and oligotrophic conditions; Kuznetsov et al., 1979), and media formulations that include or exclude Mo or V. The discovery of a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase from *Streptomyces thermoautothrophicus* (Ribbe et al., 1997) opens a new avenue in N_2 fixation research. Undoubtedly, other microorganisms containing this nitrogenase have yet to be identified. This nitrogenase may prove to be more amenable for introduction into plants because of its lower energy requirements and its higher tolerance to oxygen.

Elevated CO_2 levels provided to legumes were found to stimulate N_2 fixation indicating that N_2 fixation was limited by the availability of photosynthate (Zanetti et al., 1996). Environmental and management constraints to legume growth (basic agronomy, nutrition, water supply, diseases, and pests) are the major limiting factors of N_2 fixation in many parts of the world. Crop production on 33% of the world's arable land is limited by phosphorus availability (Sánchez and Vehara, 1980). Efforts to maximize the input of biologically fixed nitrogen into agriculture will require concurrent approaches, which include the alleviation of phosphorus and water limitation, the enhancement of photosynthate availability, as well as sound agricultural management practices.

Biochemistry and Physiology of Dinitrogen Fixation

Although the chemical nature of the primary product of N_2 fixation was the subject of debate for many years, the issue was clarified with the use of ^{15}N. All diazotrophs were thought to use the same two-component nitrogenases (consisting of an iron and an molybdenum-iron protein). Alternative nitrogenases were reported subsequently (Hales et al., 1986; Robson et al., 1986) and found in very different bacteria including *Anabaena variabilis*, *Azospirillum brasilense*, *Clostridium pasteurianum*, *Heliobacter gestii*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and bacteria corresponding to Gammaproteobacteria such as *Pseudomonas* (Saah and Bishop, 1999). *Azotobacter vinelandii*, an aerobic soil bacterium, was the first diazotroph shown to have three distinct nitrogenases: the classical molybdenum (Mo)-containing nitrogenase (nitrogenase 1), the vanadium (V)-containing (nitrogenase 2), and the iron-only nitrogenase (nitrogenase 3; Maynard et al., 1994). The alternative nitrogenases (nitrogenase 2) use V instead of Mo, and this substitution is advantageous under conditions where Mo is limiting (Jacobitz and Bishop, 1992). Similarly, the iron nitrogenase (nitrogenase 3) is expressed only in Mo- and V-deficient, nitrogen-free media. The V-containing nitrogenase produces around three times more hydrogen than the Mo-nitrogenase (Eady, 1996).

A Mo-dinitrogenase and a manganese-superoxide oxidoreductase have been found to couple N_2 reduction to the oxidation of superoxide. This nitrogenase is more efficient than the classical enzyme, which requires a fourfold greater input of ATP. This N_2-fixing system, which is not sensitive to oxygen, has only been described in *Streptomyces thermoautotrophicus* (Ribbe et al., 1997), and the genomic DNA of this bacterium does not hybridize to DNA probes for the classical *nif* genes. Although the overall reactions catalyzed by *S. thermoautotrophicus* are similar to those of previously characterized nitrogenases (e.g., the production of H_2), it is the subunit structure, polypeptides, and inability to reduce acetylene that distinguishes the nitrogenase of this system from other nitrogenases (Ribbe et al., 1997). The currently known dinitrogenase reductases are ca. 63-kDa γ_2 dimeric iron proteins that contain 4 Fe and 4 S^{-2} per dimer. In contrast, the St2 protein of *S. thermoautotrophicus* has been identified as a member of the manganese-superoxide oxidoreductases (SODs) with molecular mass ~48 kDa and no Fe or S^{-2}. Unlike other SODs, St2 cannot convert O^-_2 into O_2 and H_2O_2. Some diazotrophs are able to utilize the H_2 evolved from N_2 fixation via uptake hydrogenases (Evans et al., 1985). These enzymes are found in N_2-fixing and non-N_2-fixing bacteria and in cyanobacteria. The uptake hydrogenases in *Anabaena* are present only in heterocysts, which are the specialized N_2-fixing cells of cyanobacteria; interestingly, the hydrogenase genes are rearranged during heterocyst differentiation (Carrasco et al., 1995).

Hitherto, ammonium has been accepted as the primary product of N_2 fixation and as a reactant in the biosynthesis of all nitrogen-containing molecules made by N_2-fixing organisms. Because ammonia excretion has been considered a beneficial characteristic enabling N_2 fixers to establish symbioses with other organisms such as plants, it has been generally assumed that the ammonium assimilation enzymes are depressed in symbiotic bacteria. However, *Bradyrhizobium japonicum*, which forms nodules and fixes nitrogen in soybean plants has been shown to excrete alanine preferentially and not ammonium (Waters et al., 1998). Whether this generally occurs in rhizobia is still controversial (Youzhong et al., 2002; Lodwig et al., 2003; Lodwig et al., 2004). The ratio of alanine to ammonia excretion seems to be related to the oxygen concentration and the rate of respiration (Li et al., 1999). For the cyanobacterium *Nostoc*, which can establish symbiosis with many organisms including *Gunnera*, ammonia excretion accounts for only 40% of the nitrogen released (Peters and Meeks, 1989). Different plant endophytes have been found to release (excrete) riboflavin during N_2 fixation (Phillips et al., 1999b). Lumichrome, a compound obtained from riboflavin, has been reported to stimulate root respiration and promote alfalfa seedling growth (Phillips et al., 1999a). Production of riboflavin-lumichrome by plant-associated bacteria is favored by a high N-to-C ratio in the media, and possibly N_2 fixation also promotes the synthesis of nitrogen-containing compounds (other than ammonia), such as lumichrome, that can benefit plants.

Nitrogenase Structure The classical nitrogenase is a complex, two-component metalloprotein composed of an iron (Fe) protein and a molybdenum-iron (MoFe) protein. The properties of nitrogenase have been reviewed (Howard and Rees, 1994; Burgess and Lowe, 1996; Eady, 1996; Seefeldt and Dean, 1997). The iron-molybdenum cofactor (Fe-Moco), the prototype of a small family of cofactors, is a unique prosthetic group that contains Mo, Fe, S, and homocitrate in a ratio of 1 : 7 : 9 : 1, and it is the active site of substrate reduction (Hoover et al., 1989; Kim and Rees, 1992b). All substrate reduction reactions catalyzed by nitrogenase require the sequential association and dissociation of the two nitrogenase components.

A great deal of effort to define the structure of nitrogenases has been expended. *Azotobacter vinelandii* has been suitable for these studies because it produces large amounts of the enzyme, it is amenable to genetic manipulation, and it has *nif* and *nif*-associated genes of known sequence (Brigle et al., 1985; Jacobson et al., 1989; Bishop and Premakumar, 1992). A major achievement in the biochemistry of nitrogenases has been the establishment of the structure of the Fe (Georgiadis et al., 1992) and the MoFe proteins (Kim and Rees, 1992b; Bolin et al., 1993; Schindelin et al., 1997) involving high resolution X-ray crystallographic analysis (Peters et al., 1997; Schlessman et al., 1998). A ~2.2 Å resolution has been reported for the *Azotobacter vinelandii* MoFe-protein (Peters et al., 1997), the *A. vinelandii* Fe-protein (Av2), and the *Clostridium pasteurianum* Fe-protein (Schlessman et al., 1998). The knowledge of the Fe protein structure has contributed to understanding how MgATP functions in nitrogenase catalysis. The Fe-protein is a homodimer with two ATP-binding sites, and the nucleotide binding causes conformational changes in the protein. ATP hydrolysis occurs in the transient complex formed between the component proteins. Molecular interactions were proposed from mutagenesis studies of the nitrogenases (Kent et al., 1989; Dean et al., 1990; Scott et al., 1990). Site-specific mutagenesis studies based on the FeMo protein crystal structure (Kim and Rees, 1992a) have been aimed at amino acids related to the FeMo-cofactor (espe-

cially at the residues proposed to be involved in the entry and exit path for substrates, inhibitors and products) and also at those residues involved in FeMo-cofactor insertion during biosynthesis. The spectroscopic and kinetic properties of the resulting mutant proteins are studied (Dilworth et al., 1998).

The use of biophysical, biochemical and genetic approaches have facilitated the analysis of the assembly and catalytic mechanisms of nitrogenases. The synthesis of the prosthetic groups of nitrogenases has been a challenge for chemists. The different substrates utilized by the nitrogenases seem to bind to different areas of the FeMo-cofactor (Shen et al., 1997). Nitrogenase structural changes that occur after the formation of the active complex are thought to produce transient cavities within the FeMo protein, which when opened allows the active site to become accessible (Fisher et al., 1998). The FeMo-cofactor also is found associated with the alternative nitrogenase, *anf*-encoded proteins (AnfDGK; Gollan et al., 1993; Pau et al., 1993).

The *nifDK* genes of *Azotobacter vinelandii* were fused and then translated into a single large nitrogenase protein that interestingly has nitrogen fixation activity (Suh et al., 2003). This shows that the MoFe protein is flexible. However a substitution of tungsten for Mo abolished nitrogenase activity (Siemann et al., 2003).

Nitrogen Fixation Genes The complete nucleotide sequence of the *Klebsiella pneumoniae* 24-kb region required for N_2 fixation was reported in 1988 (Arnold et al., 1988). Genes for transcriptional regulators were found to cluster contiguously with the structural genes for the nitrogenase components and genes for their assembly. The N_2 fixation (*nif*) genes are organized in seven or eight operons containing the following *nif* genes: *J*, *H*, *D*, *K*, *T*, *Y*, *E*, *N*, *X*, *U*, *S*, *V*, *W*, *Z*, *M*, *F*, *L*, *A*, *B* and *Q* (Fig. 2). The products of at least six N_2 fixation (*nif*) genes are required for the synthesis of the iron-molybdenum cofactor (FeMo-co): *nifH*, *nifB*, *nifE*, *nifN*, *nifQ*, and *nifV*. NifU and NifS might have complementary functions mobilizing the Fe and S respectively needed for nitrogenase metallocluster assembly in *A. vinelandii*. Notably, some of the gene products required for forma-

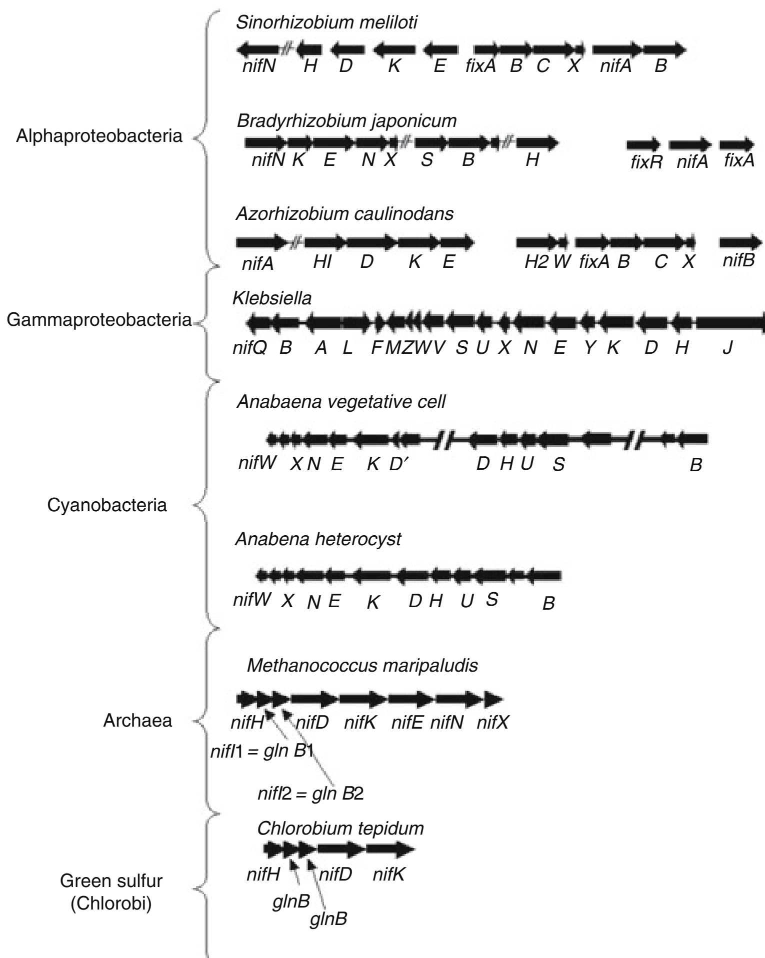

Fig. 2. Arrangements of *nif* genes in dinitrogen-fixing prokaryotes. The *nif* gene organization in *Methanococcus maripaludis* is from Kessler et al. (2001).

tion of the Mo-dependent enzyme are also required for maturation of alternative nitrogenases (Kennedy and Dean, 1992). The *nifJ* gene of *Klebsiella* is required for N_2 fixation, but in the cyanobacterium *Anabaena*, NifJ is required for N_2 fixation only when Fe is limiting (Bauer et al., 1993), whereas in *R. rubrum*, a NifJ protein does not seem to be required for N_2 fixation (Lindblad et al., 1993). The organization of *nif* genes in *Anabaena* is unique and different from that of other N_2 fixers because *nifD* is split between two DNA fragments separated by 11 kb. Recombination events are required to rearrange a contiguous *nifD* gene in N_2-fixing cells (Haselkorn and Buikema, 1992; Fig. 2).

A detailed analysis of the gene products of *nifDK* and *nifEN* (Brigle et al., 1987) revealed a possible evolutionary history involving two successive duplication events. A duplication of an ancestral gene that encoded a primitive enzyme with a low substrate specificity might have occurred before the last common ancestor of all living organisms emerged (Fani et al., 1999).

Nitrogenase structural genes are located on plasmids in some bacteria (such as *Rahnella aquatilis* [Berge et al., 1991], *Enterobacter*, and *Rhizobium* spp. [Martńez et al., 1990]) but are chromosomally encoded in the majority of prokaryotes including bradyrhizobia and most mesorhizobia.

The repeated sequences clustered around the *nif* region of the *Bradyrhizobium japonicum* genome may be involved in recombination thereby facilitating the formation of deletions (Kaluza et al., 1985). In *R. etli* bv. *phaseoli*, multiple copies of the *nif* operon promote major rearrangements in the symbiotic plasmid at high frequency (Romero and Palacios, 1997). Differences in the promoter sequences of the *nifH* regions in *R. etli* are correlated with the different levels of *nif* gene expression (Valderrama et al., 1996). The symbiotic plasmid of *R. etli* bv. *mimosae* is closely related to that of bv. *phaseoli* but its *nif* gene has a different restriction fragment length polymorphism (RFLP) pattern as revealed by *nifH* gene hybridization (Wang et al., 1999a).

A conserved short nucleotide sequence upstream of genes regulated by oxygen (i.e., an anaerobox) has been detected upstream of *Azorhizobium caulinodans nifA* (Nees et al., 1988), *Bradyrhizobium japonicum hemA*, *S. meliloti fixL*, *fixN*, *fixG*, in front of an open reading frame located downstream of *S. meliloti fixS*, within the coding region of *R. leguminosarum* bv. *viciae fixC*, i.e., upstream of the *nifA* gene and upstream of the *fnr* gene (*fixK*-like).

Alternative nitrogenase genes, *anfH*, *anfD* and *anfG* (Mo-independent) are found in the termite gut diazotrophs. The sequences of these genes are similar to those found in bacteria even though the gene organization with contiguous GlnB-like proteins resembles that found in the Archaea (Noda et al., 1999).

The existence of structural genes for three different nitrogenases was revealed when the complete genome sequence of the photosynthetic bacterium *Rhodopseudomonas palustris* was determined (Larimer et al., 2004). Previously, only *Azotobacter* sp. was known to possess three nitrogenases. The expression of *nif* genes of *Azotobacter vinelandii* was determined directly in soil by PCR amplification of reverse transcribed *nifH* gene fragments using *nifH* primers specific for *A. vinelandii* (Bürgmann et al., 2003).

Regulation of Nitrogen Fixation Genes Since nitrogen fixation is an energy expensive process, it is finely tuned, with transcriptional as well as posttranslational regulation. *nif* genes are normally not expressed and require transcriptional activation when N is limiting and conditions are appropriate for nitrogenase functioning. If little is known about the extant diazotrophs, less is known about N_2 fixation gene regulation from a global phylogenetic perspective. Most studies have been directed to Proteobacteria. For actinobacteria and firmicutes there is almost no information. Cyanobacteria and more recently Archaea were studied and showed very different regulation mechanisms from the ones observed in Proteobacteria. In Archaea, a repressor of *nif* genes has been identified (Lie and Leigh, 2003) and no *nifA* has been found in cyanobacteria (Herrero et al., 2001).

Novel regulatory elements, their fine interaction, and a huge complexity of regulatory networks are being revealed as the regulation of nitrogen fixation is studied in depth in model bacterial species. The results are revealing a very complicated sequence of regulatory cascades (Dixon, 1998; Nordlund, 2000; Forchhammer, 2003; Zhang et al., 2003). Regulatory elements such as P_{II} (also known as *glnB*), DRAT (that transfers a ribosyl to nitrogenase and interferes with its activity), and DRAG (that removes the ribosyl) have been found in many diverse nitrogen fixing or non-nitrogen fixing Proteobacteria, Actinobacteria and Archaea (Ludden, 1994; Zhang et al., 2003). Very diverse modes of regulation of *nif* genes have been described that vary between species or even between strains in a single species (D'hooghe et al., 1995; Girard et al., 2000). Detailed studies have been carried out in *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Azospirillum brasilense*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, etc. The most common nitrogenases studied are inactivated by oxygen, and accordingly, the expression

of *nif* genes is negatively regulated by high oxygen concentrations. Different oxygen protection mechanisms have been described (reviewed by Vance, 1998).

Some of the bacterial diazotrophs share a common mechanism of transcriptional initiation of *nif* genes using a RNA polymerase holoenzyme containing the alternative sigma factor σ^N (σ^{54}) and the transcriptional activator NifA (Kustu et al., 1989). Regulators of NifA vary among different diazotrophs. Factor σ^N is competent to bind DNA, but the formation of the open promoter complex (active for transcriptional initiation) is catalyzed by NifA in a reaction requiring nucleoside triphosphate hydrolysis (Lee et al., 1993; Austin et al., 1994). The dual regulation by σ^{54} and NifA may be required to ensure a stringent regulation of *nif* gene expression, and this may be so because biological N_2 fixation represents a major energy drain for the cell. In addition it seems reasonable that *nif* genes are negatively regulated by ammonia to avoid production of the enzyme in the presence of available fixed nitrogen; accordingly, nitrogenase enzymes are inactivated by ammonia but to a lesser degree in *Gluconacetobacter diazotrophicus* (Perlova et al., 2003).

In vivo DNA protection analysis demonstrated that NifA binds to the upstream activator sequences of *nif* genes (Morett and Buck, 1988). In the Alpha- and Betaproteobacteria, the activity of NifA is modulated negatively by the antiactivator NifL, which is a flavoprotein. The integrated responses to fixed nitrogen, oxygen, and energy status are mediated via NifL. The oxidized form of NifL inhibits NifA activity. A potential candidate Fe-containing electron donor involved in the signal transduction of NifL may be a flavohemoglobin, which may act as a global intracellular oxygen sensor (Poole et al., 1994). The expression of *nifL* and *nifA* in *Klebsiella pneumoniae* are coupled at the translational level (Govantes et al., 1998). Mutant forms of NifA were obtained that are no longer inhibited by NifL in *Azotobacter vinelandii* (Reyes-Ramŕez, 2002).

In other diazotrophic Proteobacteria, the NifA protein itself senses oxygen probably via a cysteine-rich motif between the central domain and the C-terminal DNA-binding domain (Fischer et al., 1988). Oxygen-tolerant variants of the *S. meliloti* NifA proteins have been obtained (Krey et al., 1992). Ammonium-insensitive NifA mutants have been reported with modifications involved in the N-terminus of the molecule in *Herbaspirillum seropedicae*, *Azospirillum brasilense* and *Rhodobacter capsulatus* (Souza et al., 1995; Arsene et al., 1996; Kern et al., 1998).

In *Klebsiella pneumoniae*, the *nif* mRNAs were found to be very stable under conditions favorable to N_2 fixation, but the half lives of the *nifHDKTY* were reduced several fold when adding O_2 or fixed nitrogen. A fragment of the *nifH* sequence is required for the O_2-regulation of mRNA stability, and NifY may be involved in the sensing process (Simon et al., 1999).

Symbiotic nitrogen fixation shares common elements with free-living nitrogen fixation, but there are substantial differences as well. In *Rhizobium*, N_2 fixation only takes place inside the nodule. Still not well understood is how the plant partner influences the N_2-fixing activity of the microsymbiont, and the same is true for termite-diazotroph symbioses as well as for cyanobacteria in plants. In the latter case, the plant seems to stimulate the formation of heterocysts, which are differentiated cells that fix N_2 (Wolk, 1996). Even among symbiotic bacteria of legumes (*Sinorhizobium*, *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*), differences in the fine mechanisms regulating N_2 fixation exist and have been reviewed (Fischer, 1994; Kaminski et al., 1998). In *S. meliloti*, *fixLJ* (David et al., 1988) gene products belong to a two-component regulatory family of proteins that are responsive to oxygen. FixL is a high affinity oxygen sensor hemoprotein that has kinase-phosphate activity and is involved in phosphorylation of FixJ in microoxic or anoxic conditions (Gilles-Gonzalez et al., 1994). Upon phosphorylation, FixJ binds to the *nifA* and *fixK* promoters and allows their transcriptional activation (Waelkens et al., 1992).

Nitrogen fixation takes place in heterocysts in some cyanobacteria. Heterocyst differentiation is regulated by HetR, a protease (Haselkorn et al., 1999), and is inhibited by ammonia (Wolk, 1996). The expression of *nif* genes is also downregulated by ammonium or nitrate (Thiel et al., 1995; Muro-Pastor et al., 1999). NtcA is a regulator required for expression of ammonium-repressible genes; in a *ntcA* mutant, induction of *nifHDK* and *hetR* is abolished or minimal (Frias et al., 1994; Wei et al., 1994). The *ntcA* gene, which is conserved among cyanobacteria, bears a DNA-binding motif close to the C-terminus and is homologous to *E. coli* Crp and to *S. meliloti* FixK. The NtcA protein binds to defined sequence signatures that are located upstream of ammonium-regulated promoters (Luque et al., 1994). However, no such signature has been identified upstream of *nif* or *hetR* genes. The *ntcA* gene is autoregulated and presumed activators or cofactors may render NtcA active (Muro-Pastor et al., 1999).

Biological N_2 fixation requires a minimum of 16 ATP molecules and 8 reducing equivalents per molecule of N_2 reduced. Under physiological conditions, a small electron carrier such as a ferredoxin or a flavodoxin is thought to transfer electrons to nitrogenase. In the photosynthetic

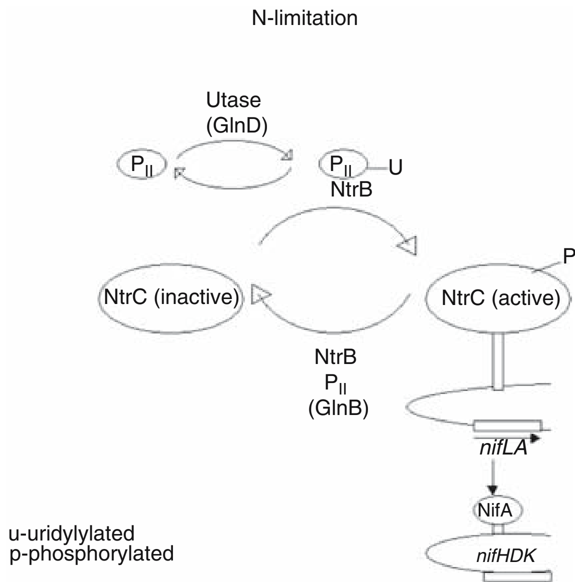

Fig. 3. Cascade regulatory mechanisms in the γ and β *Proteobacteria* under N-limited conditions. Uridylylated P_{II}, as a cofactor of NtrB promotes the phosphorylation of NtrC that then becomes active to bind the upstream regulatory sequences (UAS) of the *nifLA* promoter. NifA in turn binds the UAS of the nitrogenase structural genes in many dinitrogen-fixing prokaryotes studied, allowing their expression and consequentely nitrogen fixation.

bacterium *Rhodobacter capsulatus*, a ferredoxin Fd1 was identified as the major electron donor to nitrogenase (Schatt et al., 1989; Schmehl et al., 1993).

Conclusions Dinitrogen fixation is an important biological process carried out only by prokaryotes. Research on nitrogen fixation has followed a multidisciplinary approach that ranges from studies at the molecular level to practical agricultural applications. Support for research in this area has been driven by economic and environmental imperatives on the problems associated with the use of chemically synthesized nitrogen fertilizer in agriculture (Brewin and Legocki, 1996; Vance, 1998). However, the contributions of researchers in N_2 fixation to gene regulation, biochemistry, physiology, microbial ecology, protein assembly, and structure, and more recently to genomics are highly meritorious achievements in themselves.

Dinitrogen fixation research is a fast evolving field with specific model systems studied in great depth and an extensive knowledge of a larger diversity of N_2-fixing prokaryotes more slowly developing. The advent of molecular biology has certainly enriched our knowledge of the reservoir of N_2-fixing microorganisms and their ecology, but still the estimates of the amounts of nitrogen fixed in nature are uncertain. Human activities are liberating huge amounts of fixed nitrogen to the environment (Socolow, 1999; Karl et al., 2002; McIsaac et al., 2002; Van Breemen et al., 2002), and as a consequence, nitrogen could become less limiting in nature and this may counterselect N_2-fixing prokaryotes. Will some of them disappear without ever been known? After more than a century of research on N_2 fixation, there are still ambitious goals to achieve.

Acknowledgements My thanks to Julio Martínez Romero for technical help, and to Otto Geiger and Michael Dunn for reviewing the manuscript.

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Prokaryotes (2006) 2:818–841
DOI: 10.1007/0-387-30742-7_25

CHAPTER 1.25

Root and Stem Nodule Bacteria of Legumes

MICHAEL J. SADOWSKY AND P. H. GRAHAM

Introduction

The root and stem nodule bacteria of legumes (collectively called the rhizobia) comprise a genetically diverse group of organisms characterized by the ability to produce swellings or nodules on the stems or roots of most, but not all, leguminous plants (peas, beans, clover, etc.). Not all legumes form nodules with rhizobia.

Within the nodules, rhizobia convert atmospheric dinitrogen (N_2) gas into ammonia. This fixed nitrogen (N) is subsequently assimilated by the host, and improves plant growth and productivity. Approximately 300 million hectares (Mha) of legumes are grown worldwide, and they collectively fix about 60 tere grams (Tg) (6×10^7 metric tons) of N each year (Kinzig and Socolow, 1994). Overall, N_2 fixation supplies about 50% of the N used in agriculture, and because the fixed N is used directly by the host plant without initial passage through the soil, the process is generally considered environmentally friendly (Vance, 1998). Fixation rates vary with plant species, length of the growing season, presence of a suitable microsymbiont, and environmental conditions, but rates commonly are in the range of 100–200 kg of N_2 fixed ha^{-1} yr^{-1} (Sparrow et al., 1995; Unkovich et al., 1997). Because of the practical benefits of nodulation and N_2 fixation, the rhizobia have been extensively studied, particularly the genetic basis for their symbiotic interactions. However, the rhizobia are also good *saprophytes*, with soil populations of 10^3 to 10^4 rhizobia g^{-1} being common in soils previously used for legume growth. Thus, the ecological attributes of these organisms also have been studied extensively.

Phylogeny

The taxonomy of the organisms producing root and stem nodules on legumes is in a state of flux. Though this ever-changing taxonomy affects what the organisms are called and how they are distinguished, it has little impact on their phylogenetic relationships. Small subunit rRNA sequence analysis (SSU rRNA) supports division of these organisms into three major groups (*Rhizobium* [including *Agrobacterium*, *Allorhizobium*, *Sinorhizobium*, and *Mesorhizobium*], *Bradyrhizobium* and *Azorhizobium*) within the ∀ subclass of the Proteobacteria (Martinez-Romero and Caballero-Mellado, 1996; Young and Haukka, 1996b).

Wang et al., 1998 show that *Bradyrhizobium* and *Azorhizobium* are only distantly related to fast-growing *Rhizobium* and their relatives. Figure 1 also highlights divisions within *Rhizobium* that in the late 1980s through 1990s, led to subdivision of this genus as indicated above (Chen et al., 1988; DeLajudie et al., 1994; de Lajudie et al., 1998a; Jarvis et al., 1997). These changes, however, are currently under challenge (Kuykendall et al., 2000). Also notable in this figure is the overlap between species of *Rhizobium* and *Agrobacterium*. Amalgamation of *Rhizobium* and *Agrobacterium* has been proposed on a number of occasions (Graham, 1964; Heberlein et al., 1967; De Ley, 1968; Sawada et al., 1993; Parker, 1957), suggesting that the rhizobia may have evolved from plant pathogenic bacteria. Nonpathogenic *Agrobacterium* are well known as nodule contaminants (Hofer, 1941; Graham, 1976; de Lajudie et al., 1999), and often are confused with the nodule-forming rhizobia. Relative to the large number of species of *Rhizobium* that have been described, only a limited number of *Bradyrhizobium* and *Azorhizobium* species have been distinguished. This is likely to change as additional tropical legume species are studied. Additional groups of bradyrhizobia have already been identified, but not detailed phylogenetically (So et al., 1994; Graham et al., 1995). Moreover, links between *Rhizobium* and related root nodule bacteria (*Phyllobacterium*, *Brucella*, and Bartonella) and between *Bradyrhizobium* and *Rhodopseudmonas*, *Blastobacter*, and *Afipia* have been described, but need additional study.

The ability to form nodules is restricted to a clade of plants including both legume and actinorhizal species. Not all legumes bear nodules, the percentage of plant species with nodules increasing from only 23% in the more primitive

Fig. 1. Initiation of nodule formation on the roots of *Phaseolus vulgaris* (L.) by *Rhizobium etli* 8 days after inoculation. Photo by permission of M. H. Chaverra.

Caesalpinioideae to 97% in the *Papilionoideae*. Because these groups of legumes differ in the frequency of nodulation (and because *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* are so different), it has been suggested that the ability to nodulate and fix N_2 could have arisen on more than one occasion (Parker, 1968; Doyle, 1994; Doyle and Doyle, 1997). Doyle, 1994 suggested that nodulation has arisen on at least three previous occasions, including in the genus *Chamaecrista*. Species within *Chamaecrista* can be distinguished from the non-nodulated, but closely related, Cassia on the basis of randomly amplified polymorphic DNA (RAPD) analysis (Whitty et al., 1994). Within Chamaecrista, species differ in the retention and release of rhizobia from infection threads during differentiation (Naisbitt et al., 1992). It seems unlikely that legumes as different as *Phaseolus* and *Acacia* could nodulate with both *Rhizobium* and *Bradyrhizobium* (Lange, 1961; Michiels et al., 1998).

The rhizobia associated with a particular legume host can show significant diversity (Pinero et al., 1988; Souza et al., 1994). However, some caution in interpreting results from biodiversity studies is advisable because a number of studies predate recent phylogenetic advances and changes in rhizobial taxonomy and could have included more than one species of rhizobia. As new legumes are commercialized and exploited, studies to examine the extent of legume/microsymbiont biodiversity near the legume's center of origin, and to explore the consequences of founder effects are warranted.

Taxonomy

Rhizobia have traditionally been a difficult group to classify. Early researchers considered all rhizobia part of a single species that could nodulate any legume. Subsequently each rhizobial strain was shown to only nodulate certain specific legumes. This led to the concept of cross inoculation groups, with rhizobia being distinguished according to the legumes each could nodulate. Thus, rhizobia from alfalfa would generally nodulate medic species and vice versa, but neither would nodulate clover. Using this approach, more than 20 different cross-inoculation groups were identified, and a number of these were raised to species status within the *Rhizobium* (Fred et al., 1932). Fred et al. (1932) stated, "It seems true that the ability of an organism to infect certain plants and not others is as fixed and definite as any phase of the physiology of the organism . . . we feel justified in regarding it as the prime character in species differentiation." Host specificity is still important in the identification of rhizobia but is often at odds with results from numerical and phylogenetic studies (Graham, 1964; DeLey and Rassell, 1965; Heberlein et al., 1967; Moffett and Colwell, 1968). The demonstration that the nodulation genes in *Rhizobium* may be plasmid borne (Nuti et al., 1979; Brewin et al., 1980) or located on chromosomal symbiotic islands and move between organisms has further weakened infection-based taxonomic analyses.

The 1984 edition of *Bergey's Manual of Systematic Bacteriology* divides the *Rhizobiaceae* into four groups, including three genera of nodule- or gall-forming bacteria, *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* (Jordan, 1984). The reduced emphasis on host range and the availability of several new phenotypic and phylogenetic techniques has resulted in the proliferation of new species and genera of nodule bacteria. Currently, 6 genera and 28 species of rhizobia are recognized (Table 1). Kuykendall et al. (2000) question the need for separation of *Rhizobium*, *Agrobacterium*, *Allorhizobium* and *Sinorhizobium*, and instead suggest the consolidation of these organisms into a single genus *Rhizobium*, having three subgenera. In the classification they propose, undicola, galegae and huautlense are included in the subgenus

Fig. 2. A *Bradyrhizobium japonicum* cell showing significant polyhydroxybutyrate accumulation. Photo by T. McDermott, used with permission.

Table 1. Listing of validly published species of root and stem nodule bacteria.

Species	Reference
Rhizobium	Frank, 1989
1. *R. leguminosarum* bvs. *trifolii, viciae* and *phaseoli*	Frank, 1989
2. *R. galegae*	Lindstrom, 1989
3. *R. tropici*	Martinez et al., 1991
4. *R. etli*	Segovia et al., 1993
5. *R. gallicum*	Amarger et al., 1997
6. *R. giardini*	Amarger et al., 1997
7. *R. huautlense*	Wang et al., 1998
8. *R. mongolense*	van Berkum et al., 1998
Sinorhizobium	de Lajudie et al., 1994
1. *S. meliloti*	Dangeard, 1926
	de Lajudie et al., 1994
2. *S. fredii*	Scholla and Elkan, 1984
	de Lajudie et al., 1994
3. *S. saheli*	de Lajudie et al., 1994
4. *S. teranga*	de Lajudie et al., 1994
5. *S. medicae*	Rome et al., 1996
Mesorhizobium	Jarvis et al., 1997
1. *M. loti*	Jarvis et al., 1982, 1997
2. *M. huakuii*	Chen et al., 1991
	Jarvis et al., 1997
3. *M. ciceri*	Nour et al., 1994
	Jarvis et al., 1997
4. *M. tianshanense*	Chen et al., 1995
	Jarvis et al., 1997
5. *M. mediterraneum*	Nour et al., 1995
	Jarvis et al., 1997
6. *M. plurifarium*	de Lajudie et al., 1998
7. *M. amorphae*	Wang et al., 1999
Allorhizobium	de Lajudie et al., 1998
1. *Al. undicola*	de Lajudie et al., 1998
Brudyrhizobium	Jordan, 1982
1. *B. elkanii*	Kuykendall et al., 1992
2. *B. joponicum*	Kirchner, 1895
	Jordan, 1982
3. *B. liaoningense*	Xu et al., 1995
Azorhizobium	Dreyfus et al., 1998
1. *Az. caulinodans*	Dreyfus et al., 1988

Agrobacterium, with three species of plant pathogenic agrobacteria.

With new species of root-nodule bacteria now justified using a polyphasic approach that includes both phenotypic and phylogenetic traits (Graham et al., 1991), the further description of new species of rhizobia based solely on simple characteristics has become increasingly problematic. In the second edition of *The Prokaryotes*, (Elkan and Bunn, 1994) listed phenotypic traits useful in the distinction of *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*. To do so again is a daunting and perhaps not a useful task because older species descriptions may include more than one organism, and because differences in the tests applied and methods used can impact results and their interpretation.

Table 2 lists carbon source utilization differences for many of the species of root and stem nodule bacteria. It was compiled from a number of different studies (Jarvis et al., 1997; de Lajudie et al., 1998b; Wang et al., 1999) and will likely change as new species are identified. Additional distinctive phenotypic differences are urgently needed.

Analysis of rhizobial fatty acid methyl esters (FAME), using gas chromatography (Jarvis and Tighe, 1994; Jarvis et al., 1996), has been recommended as a relatively simple and inexpensive method for the identification of fast-growing rhizobia. Rhizobial FAME profiles correctly identified nearly 95% of almost 200 strains evaluated by (Jarvis and Tighe, 1994 and Jarvis et al., 1996). These studies only erred in identifying some fredii as meliloti and etli as leguminosarum and vice versa (Graham et al., 1999). Ballen and Graham (unpublished observations) have also shown that etli, gallicum, and strains from Dalea and Onobrychisoverlap. Similarly, FAME profiles have been used to distinguish slow-growing japonicum and elkanii (Kuykendall et al., 1992; So et al., 1994; Graham et al., 1995), though in each case additional isolates were identified that did not group with these species.

Table 2. Differences among genera of root nodule bacteria in the carbon compounds used for growth[1].

	Genus of nodule bacteria					
Carbon source	*Rhizobium*	*Sinorhizobium*	*Mesorhizobium*	*Allorhizobium*	*Bradyrhizobium*	*Azorhizobium*
Adonitol	+	+	+	−		−
D-Arabinose	+	+	+	−	+	−
L-Arabinose			(+)	+	+	−
D-Cellobiose	+	+		+	−	−
L-Fucose	+	+/−	+/−	−	−	−
Inositol	+	+	+/−	+	−	−
Gluconate	+	(+)		−	+	+
Lactose	+	+	(+)	+	−	−
L-Lysine		+/−	(−)	−	−	−
DL-Malate	(+)	(+)	+/−	+	(+)	+
D-Maltose	+	+	+	+	−	−
D-Mannose	+	+	(+)	+	+	−
Mannitol	+	(+)	+	+	(+)	−
D-Mellibiose	+	+	(−)	−		−
D-Raffinose	+	+	+/−	−	−	−
Ribose	+	+		+	+	−
L-Rhamnose	+	+	+	+	(+)	−
Sucrose	+	+	+	(−)	+	−
Trehalose	+	+	+	(−)	(+)	−
D-Xylose	+	+	+	−	+	−

Symbols: +, positive reaction; –, negative reaction; +/–, discriminatory within the genus; (+), mainly positive reaction; (–), mainly negative reaction.

[1]Includes data from Elkan and Brunn, 1992; de Lajudie et al., 1994, 1998; Rome et al., 1996; Jarvis et al., 1997 and Wang et al., 1999.

Habitat

Rhizobia through their ability to fix N_2 in symbiosis with legumes play a central role in the N supply of most natural ecosystems. The American tall grass prairie is but one ecosystem in which plant diversity and productivity is controlled in large measure by N availability (Collins et al., 1998). Rhizobia, although thought to be solely soil saprophytes, can also be found in aquatic systems associated with water-growing leguminous plants. Owing to cultural and agricultural practices, the migration of birds and animals, and atmospheric deposition of soil particles, there are relatively few soils in the world that do not contain some rhizobia. Rhizobia have been shown to exist in soils for a relatively long time in the absence of a host plant (Bottomley, 1992; Brunel et al., 1988; Kucey and Hynes, 1989; Sanginga et al., 1994; Slattery and Coventry, 1993; Weaver et al., 1972).

Rhizobia have been recognized as being important for the functioning of soil ecosystems for centuries (Fred et al., 1932). Shortly, after legume root nodules were shown conclusively to assimilate atmospheric N_2 (Hellriegel and Wilfarth, 1888), Nodbe and Hiltner applied for, and were granted, a patent for the use of these microorganisms as legume inoculants (Elkan and Bunn, 1994). This and subsequent farming and cultural practices have led to the dissemination of rhizobia on a global basis.

Rhizobia in soils may be introduced by application of commercial inoculants or, as in many cases, be the normal flora present as microsymbionts of an indigenous legume. Inoculants applied to seed, as recommended by their manufacturer, achieve inoculation rates of 10^3–10^6 rhizobia seed^{-1} (Somasegaran and Hoben, 1994). This corresponds to application rates of up to 8 $\times$ 10^{10} rhizobia ha^{-1} (Brockwell and Bottomley, 1995). At these rates, inoculant strains often dominate in nodulation in the first year of a newly introduced crop (Brockwell et al., 1982; Gibson et al., 1976; Singleton and Tavares, 1986). Moreover, inoculant strains contribute to the rapid buildup of rhizobia in the soil once nodulessenesce and release large numbers of viable rhizobia into the soil system (McDermott et al., 1987; Sutton, 1983). Several studies have documented that inoculant strains dominate in nodules 5–15 years after initial inoculation (Brunel et al., 1988; Diatloff, 1977; Lindstrom et al., 1990). It should be noted, however, that not all introduced legumes receive inoculation, and in such situations, seed, soil or aerial contamination will usually lead to some initial nodule formation, and over a period of 4–5 years, a buildup of soil rhizobial populations (Sadowsky and Graham, 1998a). Moreover, diverse rhizobial populations can develop in association with species that are not initially indigenous to a particular region (Leung et al., 1994). Although it is thought that rhizobia in soil have a clonal

population structure, genetic recombination between groups of soil rhizobia may be contributing to diversity in soils (Demezas et al., 1995; Sullivan et al., 1995). It has been demonstrated that soil rhizobia can transfer plasmids (Jarvis et al., 1989; Kinkle and Schmidt, 1991; Thomas et al., 1994; Young and Wexler, 1988) and chromosomal symbiotic genes (Sullivan et al., 1995).

The rhizobia obtained from any given soil habitat are drastically influenced by the common method of isolation. This usually involves the use of serial dilutions of soil and inoculation on a trap host, followed by recovery from nodules (Somasegaran and Hoben, 1994). This procedure often underestimates the numbers of rhizobia in the soil and biases diversity determinations (Dye et al., 1995). Numerous studies have documented the influence of a trap host on the recovery of particular groups of rhizobia from soils (Bottomley et al., 1994; Bromfield et al., 1995; Brunel et al., 1996; Keatinge et al., 1995; Kumar Rao et al., 1982; van Berkum et al., 1995). Selective culture media, when available, will most likely prove useful in determining the identity of natural populations of rhizobia in soil (Gault and Schwinghamer, 1993; Tong and Sadowsky, 1993).

Lastly, the legume host itself has been shown to strongly influence the prevalence and type of rhizobia in soils (Bottomley, 1992). How this occurs is not known, but it is thought to be due to nonspecific, root-exudate enhanced growth of rhizobia in the rhizosphere, multiplication and release of rhizobia from the nodule, and selection by the trap host of particular groups of rhizobia from mixed soil populations (Sadowsky and Graham, 1998a).

Isolation

Date (1982); Date and Halliday (1987) and Somasegaran and Hoben (1994) have detailed methods for the collection, sampling, isolation, authentication and maintenance of rhizobia. Extensive collection and conservation is necessary because many isolates will prove to be ineffective in symbiosis, or host/strain interactions will be significant. In the case of Stylosanthusscabia, more than 1,000 isolates were evaluated before a strain suitable for use in commercial legume inoculation was identified (Date, 1997).

Collection

The collection of rhizobia is most commonly undertaken as part of a plant introduction program, to supply suitable host germplasm with the rhizobia they need for symbiosis (see the National Plant Germplasm Collection System http://www.ars-grin.gov). Ideally, the collection of nodules should coincide with early season growth and well watered conditions. However, where collection involves remote geographic regions, sample acquisition may be delayed until plant maturity when most nodules may have senesced. Nodule collection may also be limited where the plant species in question is endangered, and no plant harvest is permitted. In both of these cases, soil may be used as a source of rhizobia, using surface-sterilized seed of an appropriate host to "trap" nodule bacteria. Collection of rhizobia also may be undertaken to study the biodiversity of indigenous organisms, or to study success in nodulation, or the soil establishment of bioengineered organisms. In some cases, the culture of rhizobia from nodules may be unnecessary because enough cell material may be present in soils or plant tissue to directly characterize nodule occupants using serological or phylogenetic methods (Sadowsky and Graham, 1998). Somasegaran and Hoben (1994) list several culture collections of rhizobia throughout the world. In addition, the USDA-ARS (National Rhizobium Resource Collectionl) (bldg6.arsusda.gov) provides a searchable database of rhizobia grouped by legume host.

Sampling

Sampling of plants and nodules should be done from undisturbed locations and, where possible, from healthy plants. Accurate site description and record keeping are essential. The number of nodules needed can vary with the reason for collection. Where the aim is to identify inoculant-quality rhizobia, 15–20 nodules per plant, taken from the crown region of the host root system, are usually sufficient. Where the goal is to evaluate strain biodiversity in soil, a large number of nodules should be collected from as much of the root system as possible. Ease of collection may vary; stoloniferous species may have nodules on adventitious roots within 1–2 cm of the surface (Date, 1982), while nodules on tree species may be at a great depth in the soil at some distance from the trunk of the tree. Collected nodules should be protected in *vacutainers* or in vials containing a dessicant (e.g., silica gel) overlain by cotton wool (Somasegaran and Hoben, 1994).

Isolation

Successful isolation of rhizobia from nodules depends on the quality of nodules recovered. When nodules have been stored dry over silica gel or $CaCl_2$, they must first be allowed to imbibe (sterile) water fully before being surface sterilized. Rhizobia also can be frequently recovered from nodules obtained from intact root system frozen at –20°C. Sodium hypochlorite (3%),

hydrogen peroxide (3%) and acidified mercuric chloride (0.1%) are all effective surface sterilants. The former is usually preferred due to its low cost, ready availability and ease of disposal. Surface sterilization procedures are described in detail by Vincent (1970) and Somasegaran and Hoben (1994).

Yeast extract mannitol (YEM) medium is commonly used in the routine isolation and subculture of of rhizobia. Many different formulations for this medium exist (Vincent, 1970; Somasegaran and Hoben, 1994). That used in our laboratory contains:

Mannitol	10.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
K_2HPO_4	0.5 g
$CaCl_2 \cdot 2H_2O$	0.2 g
$FeCl_3 \cdot 6H_2O$	0.01 g
Yeast extract	1.0 g
Agar	20.0 g
Distilled water	1 liter
pH	6.7–7.0

Sterilize by autoclaving 20 min at 103 H 10_3 pascal (15 lb/in_2) pressure.

The medium may be amended with cycloheximide (20 microgram/ml) to reduce fungal contamination and bromthymol blue (BTB; 25 microgram/ml) or Congo red (25 microgram/ml) to facilitate identification of rhizobia. These should be filter-sterilized separately and added to autoclaved, molten YEM medium before plates are poured (Vincent, 1970). Tong and Sadowsky (1993) described a selective medium specific for *Bradyrhizobium*, based primarily on the heavy metal tolerance of these organisms. Media selective for fast-growing rhizobia have been described (Barber, 1979; Louvrier et al., 1995), but they have not proven generally effective.

Rhizobium, *Mesorhizobium*, *Sinorhizobium*, and *Allorhizobium* strains will generally produce moist, gummy colonies on YEM medium that are 4–6 mm in diameter after 7 days incubation. On medium containing BTB, the colonies and surrounding medium are yellow due to acid production by the microorganisms. Slower growing bradyrhizobia produce smaller colonies, usually only 1–2 mm diameter after 7–10 days incubation, which are raised and mucoid. The colonies and surrounding medum are blue in color on YEM containing BTB. Most nodule isolates will produce white or cream colored colonies, though some isolates produce melanin (Cubo et al., 1988), or in the case of bradyrhizobia, a rust red pigmentation in older colonies.

Authentication

Authentication of rhizobia usually involves completion of Koch's postulates with the host from which strains were originally isolated. Somasegaran and Hoben (1994) provide details of this methodology. Inoculated seedlings produced from surface-sterilized seed of a suitable legume host are typically grown in sterile low N medium or on seedling agar in large test tubes, growth pouches or Leonard jars. Plants are examined for nodulation after 25–30 days of incubation under lights. The presence of nodules on uninoculated control plants invalidates the experiment.

Identification

The identity of rhizobia or bradyrhizobia often requires a multiphasic approach using many of the techniques employed in naming new genera, species and strains of rhizobia (Graham et al., 1991). Members of the International Subcommittee on *Rhizobium* and *Agrobacterium*, a subcommittee of the International Committee on Systematic Bacteriology of the International Union of Microbiological Societies, have recommended a minimal set of criteria for naming new species and genera of nodule bacteria (Graham et al., 1995). These criteria are also useful for identifying the genus and species status of unknown rhizobia isolated from nodules. In addition to biochemical, cultural, and symbiotic data, 16S rRNA (rDNA) sequencing (Young, 1996a; Young and Haukka, 1996b), DNA-DNA hybridization (Scholla et al., 1984), FAME (Graham et al., 1995), and multilocus enzyme electrophoresis, (MLEE) (Strain et al., 1995) data are of primary importance for identifying rhizobia isolated from newly surveyed legumes.

Following strain authentication, it is often useful to mark these isolates to facilitate identification in subsequent ecological, genetic or plant studies. This can be done using a variety of techniques. These include intrinsic resistance to a series of different antibiotics (Josey et al., 1979) or the selection of mutants resistant to high levels of antibiotics. In the latter case, selected mutants must be evaluated to show that the acquisition of antibiotic resistance has not influenced nodulation, N_2 fixation or competitive abilities.

Strains can also be identified using strain or group-specific antibodies (Sadowsky et al., 1987a; Schmidt et al., 1968). Antibodies, which are typically produced in rabbits to somatic whole-cell antigens, are useful in strain identification because they do not require genetic modification of strains. Agglutination, immunodiffusion, immunoflourescence, and ELISA techniques all have found wide acceptance in serological identification of rhizobia (Dudman, 1977; Humphrey and Vincent, 1965; Kishinevsky and Jones, 1987; Schmidt et al., 1968). The fluo-

rescent antibody technique is especially useful because it allows for the direct *in situ* examination of rhizobia in soil and nodules (Bohlool and Schmidt, 1973), using direct or sandwich labeling procedures.

Strains also can be genetically modified with β∃-glucuronidase reporter (GUS) (Wilson et al., 1995; Wilson et al., 1999) and lux (Chabot et al., 1996) genes, and these strains have proven especially useful in ecological studies. Again, however, it is essential that such genetically marked strains be plant tested before use in ecological, symbiotic, or field studies.

DNA fingerprinting techniques have been used to identify and study biodiversity of rhizobial strains (Sadowsky, 1994; Versalovic et al., 1998; Sadowsky and Hur, 1998b; Demezas, 1998). Initially, DNA fingerprints of strains were generated following restriction enzyme digestion of total genomic DNA (Glynn et al., 1985; Demezas, 1998). More recently, however, restriction fragment polymorphism (RFLP) analysis techniques, DNA hybridization probes, and DNA primers corresponding to repetitive elements, coupled to the polymerase chain reaction (PCR) technique, have been used in strain identification, competition, and biodiversity studies (de Bruijn, 1992; Judd et al., 1993; Sadowsky, 1994; Sadowsky et al., 1990; Wheatcroft and Watson, 1988).

Cultivation

Rhizobia are relatively robust, ubiquitous, aerobic bacteria with the ability to utilize many different substrates (carbon [C] and nitrogen [N] sources) for growth (Parke and Ornston, 1984). Consequently, rhizobia can be cultivated on a large variety of complex and defined culture media. Only a limited number of rhizobia grow on highly enriched media, such as nutrient broth or LB medium. Medium used in the cultivation of rhizobia depends on the species of nodule bacteria, growth characteristics desired, and the method of cultivation. Most rhizobia are mesophiles and can grow in shake cultures at 25–30°C. However, rhizobia isolated from legumes grown in the Canadian High Arctic grow well at 5°C (Prevost et al., 1987), and high temperature tolerant strains have been isolated in Africa and Brazil.

As stated earlier, most rhizobia grow well in YEM medium (Vincent, 1970), though most produce copious quantities of capsular- and exo-polysaccharides in this medium, limiting its use in biochemical and genetic studies. The bradyrhizobia grow fairly slowly in this medium, with generation times greater than 6 hours. Rhizobia and bradyrhizbia shift the pH of this medium, the rhizobia produce acid and the bradyrhizobia, alkaline byproducts, from growth. Polysaccharide production can be drastically reduced in fast-growing rhizobia by cultivation in TY medium (Beringer, 1978), containing (g/liter): Tryptone (5.0), Yeast extract (3.0) and $CaCl_2 2H_2O$ (0.87), pH 6.9. In this medium turbid cultures, up to 10^9 cells ml^{-1}, can be obtained after overnight incubation at 28°C. The slow-growing bradyrhizobia do not grow in TY medium. A growth medium useful for polysaccharide-free growth by bradyrhizobia is AG medium (Sadowsky et al., 1987b). This medium, which promotes rapid growth of japonicum and elkanii strains, contains (g/liter): HEPES (0.13), MES (0.11), $FeCl_3 6H_2O$ (0.0067), $MgSO_4 7H_2O$ (0.18), $CaCl_2 2H_2O$ (0.013), Na_2SO_4 (0.25), NH_4Cl (0.32), Na_2HPO_4 (0.125), arabinose (1.0), Na-gluconate (1.0) and yeast extract (1.0), pH 6.9. Several defined, minimal, media are also used for the growth of rhizobia (Vincent, 1970; Somasegaran and Hoben, 1994), especially for biochemical and molecular biological studies. We also use AG medium without arabinose, gluconate, and yeast extract, as a minimal medium for the cultivation of prototrophic rhizobia and in genetic mating studies.

Rhizobia for legume inoculants can be grown in shake flasks or fermentors (Somasegaran and Hoben, 1994). Lorda and Balatti (1996) described a glycerol-based culture medium capable of producing approximately 10^{10} cells/ml, even in shake flask culture. In contrast, Stephens and Rask (2000) suggest that carbon-limited media be used to produce legume inoculants, to condition rhizobia to the less favorable conditions found in soil.

Preservation

Rhizobium strains in frequent use are usually maintained on YMA slants in screw capped test tubes stored at 6–10°C. Longer-term storage is achieved by lyophilization with 10% glycerol or 10% sucrose and 5% peptone as cryoprotectants, or by storage at –70°C in 15% glycerol. Gherna (1994) details methodologies for lyophilization and storage at –70°C. Change in *Rhizobium* characteristics with repeated growth on laboratory media has been reported (Herridge and Roughley, 1975) and must be of concern. Some inoculant companies maintain large numbers of ampoules of each *Rhizobium* strain in the freeze-dried state *Rhizobium* and routinely replace all working cultures at 3-month to 1-year intervals.

The Nodulation Process

The nodulation process requires molecular communication between both symbiotic part-

ners and involves the induction and repression of a large number of bacterial and plant genes. Free-living rhizobia infect and form N_2-fixing symbioses with legumes in a series of discrete stages or steps. Stages in the process include: proliferation of rhizobia in the rhizosphere, recognition of host by rhizobia, attachment of rhizobia to susceptible root hair cells, root-hair curling and infection-thread formation, initiation of nodule primordium, and transformation of free-living rhizobia into N_2-fixing bacteroids.

Rhizobia infect their respective host plants and induce root or stem nodules using several different mechanisms. Infection through root hairs is commonly seen with most legumes (Hadri et al., 1998). Rhizobia can also invade the host plant by entry through wounds, cracks, or lesions caused by emergence of secondary roots (Boogerd and van Rossum, 1997), as occurs in peanut and Stylosanthes. In these cases, rhizobia spread intercellularly. There are instances where the same rhizobia infect one legume through root hairs and another via cracks or wounds (Sen and Weaver, 1988). Lastly, rhizobia may initiate infection of the host via cavities surrounding adventitious root primordia on the stems of Sesbania, Aeschynomene, Neptunia, and Discolobium (Boivin et al., 1997). As above, one bacterium may produce both stem and root nodules on different legume plants.

Nodule shape in legumes is determined by the host plant and is regulated by the pattern of cortical cell divisions. There are two basic types of nodules that are formed on legumes: determinant and indeterminant (Franssen et al., 1992). Indeterminant nodules are most commonly formed in symbioses between the fast-growing nodule bacteria and temperate legumes (pea, clover, and alfalfa). Determinate nodules, which are normally induced by bradyrhizobia, are more common on tropical legumes, such as soybean and bean. Morphologically, indeterminate nodules have defined, persistent apical meristems and are elongated and sometimes lobed, whereas determinant nodules do not have persistent meristems and are usually round (Hadri et al., 1998).

In root hair infection, rhizobia attach to susceptible root hairs within minutes of inoculation or contact with the host plant. Rhizobial cells often attach perpendicular to the root hair cell. It has been suggested that adhesion is initially mediated by the calcium (Ca)-binding protein rhicadhesin, or by plant lectins, and subsequent bonding via production of cellulose fibrils (Kijne, 1992). It is hypothesized that rhizobia produce localized hydrolysis of the root hair cell wall. Subsequent penetration of rhizobia through the cell wall leads to root-hair curling, which may be visible 6–18 hours after inoculation. The proportion of root hairs infected is low, the percentage of these giving rise to nodules is low and highly variable, and aborted root hairs can frequently be found.

Within the root hair, rhizobia are enclosed within a plant-derived infection thread, and move down the root hair in the direction of the root cortex. Cell division in the root cortex, in advance of the approaching infection thread, leads to the production of nodule primordia (Kijne, 1992). Spread of the infection thread among cells of the nodule primordium follows, with the release of rhizobia into host cortex by an endocytotic process. Rhizobia are never free in the cytoplasm, but rather are surrounded by a host-derived peribacteroid membrane, which serves to compartmentalize the rhizobia into a symbiosome. One to several rhizobia can be confined to a single symbiosome. Nodulation is usually visible 6–18 days after inoculation, but this varies considerably with the selection of bacterial strain and host cultivar, the inoculant density and placement, and the temperature. Initially nodulation is heaviest in the crown of the root, with secondary nodules appearing on lateral roots as the first-formed nodules senesce. The number of nodules produced on each legume host is tightly controlled by the host and rhizobial genotype, the efficiency of the symbiotic interaction, by environmental factors such as soil N level and the presence of existing nodules (Caetano-Annoles, 1997; Sagan and Gresshoff, 1996; Singleton and Stockinger, 1983).

Genetics

The genetics of the rhizobia has, in most cases, centered on the genetics of nodulation and symbiotic N_2-fixation, key characters that set the rhizobia apart from other soil bacteria. Recent advances in molecular biology and genetics have elucidated a large number of genes with symbiotic functions. Though many of these genes are clustered together (on the chromosome in some organisms and on symbiotic plasmids in others), additional genes may be dispersed or located on different replicons. Consequently, all symbiotically related genes will most likely not be found until total sequencing and functional genomic efforts are completed. Because the scope of this chapter is broad, more detailed information on the genetics of nodulation and N_2 fixation can be found in several recent reviews (Boivin et al., 1997; van der Drift et al., 1998; Schultze and Kondorosi, 1998; Niner and Hirsch, 1998; Denarie et al., 1996; Pueppke, 1996; Spaink, 1995).

Nodulation Genes

In the last several years, a large number of bacterial genes have been identified which are involved in the formation of nodules on leguminous plants. Collectively, more than 65 nodulation genes have been identified in rhizobia, although each strain may only have a subset of these. Niner and Hirsch (1998); Pueppke (1996); and Bladergroen and Spaink (1998) provide a more complete description of the function of a majority of these genes.

Several studies have shown that relatively few genes are required for nodulation of legumes (Göttfert, 1993; Long et al., 1985; Long, 1989; van Rhijn and Vanderleyden, 1995). In the case of the fast-growing rhizobia, a majority of nodulation genes are located on large, indigenous, symbiotic (Sym), and often self-transmissible, plasmids (Broughton et al., 1984; Hombrecher et al., 1981; Kondorosi et al., 1989). The complete genomic sequence of the symbiotic plasmid from *Rhizobium* sp. strain NGR-234, a *Rhizobium* strain with broad nodulation ability (Pueppke and Broughton, 1999), is currently available. In meliloti, the symbiont of alfalfa, nodulation genes (located on an 8.5 kb fragment of the Sym plasmid) contain sequences necessary for the nodulation of a wide variety of legume hosts (Kondorosi et al., 1989; Truchet et al., 1991). These genes, referred to as "common nodulation" genes and designated nodA, nodB and nodC, have homologues in other fast- and slow-growing species. In leguminosarum bvs. trifolii and viceae and meliloti, the common nodulation genes are organized in a similar cluster (Downie et al., 1985; Egelhoff and Long, 1985; Fisher et al., 1985; Nieuwkoop et al., 1987; Putnoky and Kondorosi, 1986; Rolfe et al., 1985; Russell et al., 1985; Schofield and Watson, 1986; van Rhijn and Vanderleyden, 1995). A fourth gene, nodD, is regulatory and together with plant flavonoid signals (see below) activates transcription of other inducible nod genes (Long, 1989; Martinez et. al, 1990; van Brussel et al., 1990). Leguminosarm bvs. viceae and trifolii have single copies of *nodD*. The symbionts meliloti and japonicum have multiple copies of nodD (Göttfert et al., 1986; Göttfert et al., 1990; Honma and Ausubel, 1987). In some instances, nodD also appears to impart host-specificity functions (Spaink et al., 1987). Another nodulation gene cluster, originally designated hsn (for host-specific nodulation), is closely linked to the common nodulation region in meliloti and controls nodulation of specific legume genera (Bachem et al., 1986; Horvath et al., 1986). Mutations in the *hsn* genes (designated *nodFEGH*) cannot be complemented with Sym plasmids from other species of *Rhizobium*. Analogous *hsn* genes also have been isolated from leguminosarum bv. *trifolii* (Djordjevic et al., 1985; Rolfe et al., 1985), leguminosarum bv. *viceae* (Wijffelman et al., 1985), and from *Rhizobium* strain MPIK3030 (Bachem et al., 1986; Bassam et al., 1986; Broughton et al., 1984; Lewin et al., 1987). An *hsn* gene linked to the common nodulation region in japonicum strain USDA 110 has also been reported (Nieuwkoop et al., 1987). This sequence, subsequently called nodZ (Dockendorff et al.,1994), was shown to be involved in the host-specific nodulation of siratro, but not soybean. Hahn and Hennecke (1988) and Göttfert et al. (1990) have identified another *hsn* locus in japonicum strain 110, nodVW, which is essential for the nodulation of siratro, mungbean and cowpea, but not soybean. In japonicum strain USDA 110, the essential nodulation genes are located on the chromosome in several transcriptional units in the order: nolZ, nolA, nodD2, nodD1, nodYABCSUIJmolMNO (Dockendorff et al., 1994). Unlike other rhizobial nodD genes, the japonicum nodD1 is induced by the flavonoids genistein and daidzein (Banfalvi et al., 1988; Kosslak et al., 1987) and by xanthones (Zaat et al., 1987).

Genotype-Specific Nodulation Genes

Although many hsn genes have been identified in *Rhizobium* and *Bradyrhizobium*, there are only limited reports on the identification of genotype-specific nodulation (GSN) genes in the rhizobia (Sadowsky et al., 1991). The GSN genes specifically refer to those bacterial genes that allow nodulation of specific plant genotypes within a given legume species. For example, strain TOM nodulates the pea genotype Pisum sativum cv. Afghanistan (Lie, 1978a; Lie et al., 1978b), but European leguminosarum bv. *viceae* strains fail to nodulate this host. Some GSN-like genes have been found on plasmid pRL5JI of strain TOM (Gotz et al., 1985; Hombrecher et al., 1984). Davis et al. (1988) have identified a single gene on this plasmid, nodX, mediating the O-acetylation of Nod factors (Firmin et al., 1993), which is necessary for the nodulation of "Afghanistan" peas. In fredii strain USDA 257, two other GSN-like loci, nolC (Krishnan and Pueppke, 1991) and nolBTUVW (Meinhardt et al., 1993), allow this strain to nodulate primitive lines of soybean, but not improved soybean varieties, such as "McCall" (Heron et al., 1989). In each case, Tn5 insertions in the gene regions allow fredii to nodulate commercial soybean cultivars. Phenotypically, these regions are similar to that reported by Djordjevic et al. (1985) and Innes et al. (1985) for clover rhizobia. More recently, however, Lewis-Henderson and Djord-

jevic (1991a) reported that nodM in leguminosarum bv. trifolii is a GSN which prevents effective nodulation of subterraneum cv. Woogenellup (Lewis-Henderson and Djordjevic, 1991b). Analysis of leguminosarum bv. trifolii strain TA1 demonstrated that this strain also lacks nodT, and that introduction of nodT from leguminosarum bv. viceae strain ANU843 into TA1 allows effective nodulation of "Woogenellup" (Lewis-Henderson Djordjevic, 1991a). The GSN genes can act in either a positive or negative manner (Djordjevic et al., 1987a; Sadowsky et al., 1990), insertions in a negatively acting nodulation gene extending host-range and insertions in a positively acting GSN gene limiting host range. *Bradyrhizobium* japonicum serogroup 123 strains are restricted for nodulation by PI 377578 (Cregan and Keyser, 1986). The japonicum nolA gene, identified in strain USDA 110, is a positively acting gene that allows serogroup 123 strains to nodulate PI 377578 (Sadowsky et al., 1991). We recently identified a mutant of japonicum strain USDA 110 that has the ability to overcome nodulation restriction conditioned by soybean PI 417566 (Lohrke et al., 1995).

Signal Exchange and Induction of Nod Genes

Although the regulation of nodulation genes in rhizobia is still not fully understood, we know a lot about communication between rhizobia and susceptible legume hosts. Flavonoid signal molecules present in root and seed exudates are necessary for *nod* gene expression (Banfalvi et al., 1988; Boundy-Mills et al., 1994; Djordjevic et al., 1987a; Fellay et al., 1995; Göttfert et al., 1988; Innes et al., 1985; Kosslak et al., 1987; Long, 1989; Mulligan and Long, 1985; Olson et al., 1985; Peters et al., 1986; Price et al., 1992; Sadowsky et al., 1988; van Brussel et al., 1990; Zaat et al., 1987). Other Sym plasmid-borne genes are also induced by root exudates in fredii and *Rhizobium* sp. strain NGR234 (Boundy-Mills et al., 1994; Fellay et al., 1995; Olson et al., 1985; Sadowsky et al., 1988). Flavones, isoflavones, flavanols, flavanones, and closely related compounds have been identified as *nod* gene inducers, and each is specific for a particular legume-*Rhizobium* interaction (Schlaman et al., 1998). Flavanoid compounds are only one of several determinants of host specificity. Spaink et al. (1991) reported differential induction of nodD in various fast-growing rhizobia by a range of flavonoids and exudates. Induction of nodulation genes requires the regulatory *nodD* gene product (Long, 1989; Mulligan and Long, 1985; Shearman et al., 1986). The inducer apparently binds NodD, causing a change in conformation (Kondorosi et al., 1988; Fisher and Long, 1989). Activated NodD then binds to a regulatory, promoter-like sequence, found upstream of rhizobial nod genes, the nod box (Hong et al., 1987; Horvath et al., 1986; Kondorosi et al., 1988; Rostas et al., 1986; Shearman et al., 1986). Repressor proteins have also been suggested to play a role in nod gene regulation (Kondorosi et al., 1988), a repressor encoded by the nolR gene has been identified in S. meliloti strain 41 (Kondorosi et al., 1989; Kondorosi et al., 1991).

Extracellular Nodulation Factors

One of the primary functions of nod genes is the production of extracellular lipochitinoligosaccharide (LCO) molecules, also known as Nod factors (Carlson et al., 1993; Carlson et al., 1994). These molecules, acting at 10^{-8} to 10^{-9} M, can: 1) stimulate the plant to produce more *nod* gene inducers (van Brussel et al., 1990); 2) deform root hairs on homologous hosts (Banfalvi et al., 1989; Faucher et al., 1989); and 3) initiate cell division in the root cortex (Lerouge et al., 1990; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Relic et al., 1993; Spaink et al., 1991). In meliloti these signal molecules are acetylated and sulfated glucosamine oligosaccharides (Lerouge et al., 1990). Similar molecules have been identified in other legume symbiotic systems (Pueppke, 1996 and Downie, 1998 for a review). Numerous observations support the theory that hsn genes control host specificity by decorating Nod factors with various substituents. For example, the meliloti genes nodP, nodQ and nodH are involved in the sulfation of the Nod factor reducing sugar (Faucher et al., 1989; Roche et al., 1991). Disruption of any of these genes affects host specificity. *Rhizobium* spp. NGR234, which can nodulate over 125 different legume species (Pueppke and Broughton, 1999), produces diverse (more than 18) Nod factors, which vary in the substituents attached to a similar backbone structure (Price et al., 1992).

Purified Nod factors, which are structurally similar to those produced by the appropriate rhizobial symbiont, can induce nodules on the specific host plant in the absence of a bacterium (Downie, 1998; Mergaert et al., 1993; Relic et al., 1993, Schultze et al., 1992; Truchet et al., 1991). Nod factors from several strains of japonicum have been characterized (Carlson et al., 1993; Sanjuan et al., 1992). The functions of nod genes and the basic structure of Nod factors for japonicum and several species of the genus *Rhizobium* can be found in (Downie, 1998).

Nitrogen Fixation Genes

Two major types of N_2 fixation genes have been described, *nif* genes and *fix* genes. The *nif* refer

to genes involved in the N_2 fixation process and have structurally and functionally related genes in the free-living diazotrophic microorganism, Klebsiella pneumoniae. pneumoniae was the first N_2-fixing microorganism studied in detail (Kennedy, 1989). As with the nodulation genes, a majority of the *nif* genes are plasmid borne and contiguous in the rhizobia, but chromosomally located in the bradyrhizobia. The N_2 fixation process is catalyzed by the enzyme complex nitrogenase, encoded by the nifDK and nifH genes. The *fix* genes are also involved in the N_2 fixation process, but have no similar structural or functional homologues in pneumoniae. The organization of *nif* genes varies in the rhizobia (Kaminski et al., 1998).

Nitrogenase consists of two protein subunits, a molybdenum-iron (MoFe) protein and an iron-containing (Fe) protein. These structural components of the nitrogenase enzyme complex are often referred to as subunits I and II, respectively. The *nifK* and nifD genes encode the MoFe protein subunits. A FeMo cofactor (FeMo-Co) is required for activation of the MoFe protein and is assembled from the nifB, V, N, and E genes. The nifH gene encodes the Fe subunit protein. In pneumoniae there are at least 20 *nif*-specific genes that are localized in about 8 operons (Dean and Jacobson, 1992). Though the organization of *nif* genes in other organisms varies tremendously (Downie, 1998), nifHD and nifK are conserved in disparate N_2-fixing organisms and rhizobia (Ruvkin and Ausubel, 1980). The gene products NifA and NifL control the regulation of all other nif genes. Whereas NifA is positive activator of transcription of *nif* operons, NifL is involved in negative control. In K. pneumoniae and several other free-living diazotrophic microbes, *nif* gene expression is regulated by oxygen and nitrogen levels (Merrick, 1992). Ammonia (NH_3) causes NifL to act as a negative control and prevents the activator function of NifA. This has been referred to as the N control system, and has been shown to regulate several enzymes that are capable of producing NH_3. Merrick (1992) and Dean and Jacobson (1992) give excellent in-depth reviews of the structure and regulation of N_2 fixation in free-living and symbiotic bacteria.

Other Genes Involved in Symbiotic Nitrogen Fixation

Other plasmid and chromosomally borne bacterial genes also have been found to function indirectly in nodulation and symbiotic N_2-fixation in rhozobia (Table 3). Recent review articles on the structure and function of these and other symbiosis-related genes are provided by Pueppke et al. (1996) and Spaink (1995) and Long (1999).

Ecology

Rhizobia are relatively unique among the majority of soil microorganisms in that they have an extensive soil phase as free-living, saprophytic, heterotrophic microorganisms, yet in conjunction with leguminous plants, they have the ability to form species-specific, N_2-fixing symbiotic associations. The ability to form N_2-fixing nodules affords unique opportunities for the rhizobia. When a legume crop is grown in soil for the first time, few rhizobia are likely to be present and, in most instances, inoculation will most likely be needed for adequate nodulation and subsequent N_2 fixation (Date, 1991; Diatloff, 1977). In contrast, soils surrounding legumes that have been planted for several years usually contain relatively large numbers of rhizobia and do not require added rhizobia. Numerous studies have documented that legume inoculants added to soils containing relatively small populations of rhizobia usually give rise to only a small percentage of the nodules formed (Thies et al., 1991; Date, 1991; Ellis et al., 1984; Ham, 1978). Despite intensive investigations over the last 30 years, however, some of the factors that influence the survival and the persistence of rhizobia in the soil, their ecology and competitiveness for nodulation sites on the host, are only now beginning to be understood. It is beyond the scope of this

Table 3. Some other bacterial genes involved in symbiotic nitrogen fixation.

Gene designation	Phenotype or function	Reference
Exo	Exopolysaccharide	Bechei and Pηhler, 1988 Glazebrook and Walker, 1989
Hup	Hydrogen uptake	Maier, 1986
Gln	Glutamine synthase	Carlson et al., 1987
Dct	Dicarboxylate transport	Finan et al., 1983 Jiang et al., 1989
Nfe	Nodulation efficiency	Sanjuan and Olivares, 1989
Ndv	∃-1,2 Glucans	Breedveld and Miller, 1998
LPS	Lipopolysaccharide	Carlson et al., 1987

chapter to present all that is known about the ecology and soil biology of the rhizobia. The reader is directed to more extensive reviews by Bottomley (1992) and Sadowsky and Graham (1998a) on this material.

The establishment of the symbiotic state results in the production of a nodule populations of more than 10^{10} rhizobia g^{-1} nodule tissue (McDermott et al., 1987). When these nodules senesce at the end of the growing season, large numbers of rhizobia are released into the soil. Nodule bacteroids are subject to changes in surface chemistry (Roest et al., 1995) and are susceptible to osmotic and other soil stresses (Sutton, 1983). However, many of the released organisms manage to persist as free-living, heterotrophic, saprophytes in the soil until a susceptible legume is again planted. As a consequence of this, most soils contain at least some rhizobia, and a dramatic buildup in their numbers occurs when a leguminous host is included in a crop rotation, pasture or natural setting.

Ellis et al. (1984) reported soil populations of bradyrhizobia approaching 10^6 cells g^{-1} in soils of the American Midwest following cultivation of soybean, and rhizosphere populations can reach 10^8 cells g^{-1} (Bottomley, 1992). The distribution of rhizobia in soil is not uniform. Postma et al. (1990) reported that the greatest number of rhizobia are associated with soil aggregates of larger than 50 µm, and Mendes and Bottomley (1998) noted that the percentage of *Rhizobium* recovered from aggregates of different sizes varied over the course of a growing season.

Rhizobia are excellent soil saprophytes and can persist for many years in the absence of their host (Brunel et al., 1988; Kucey and Hynes, 1989; Bottomley, 1992). Chatel et al. (1968) used the term saprophytic competence to describe this ability, but the factors involved have yet to be determined. Even though Bushby, 1990 noted surface electrophoretic charge in bradyrhizobia correlated to the pH of soils from which they came, Rynne et al., 1994 found no correlation between catabolic ability and strain persistence. Inoculant strains used at the time a particular host was introduced may still occupy a large percentage of the nodules formed on that host 10–15 years after their introduction (Diatloff, 1977; Brunel et al., 1988; Lindstrom et al., 1990). However, many studies have shown that inoculant strains may also decline in nodule representation over time, or quite quickly disappear from soil.

The growth of rhizobia in the rhizosphere may also be stimulated by specific root exudates (Van Egaraat, 1975). Rhizobia, in turn, also stimulate growth and respiration of leguminous plants (Phillips et al., 1999). Several research studies have sought to create biased rhizospheres, in which plants transformed to synthesize opines, favor the growth of rhizosphere bacteria utilizing this substrate (Rossbach et al., 1994; Oger et al., 1997; Savka and Farrand, 1997). The inability of strains to compete for nodulation sites on the host legume does not necessarily mean their displacement from the soil population. Bromfield et al. (1995) compared populations of meliloti recovered from soil and nodules and found significant differences in the frequency with which particular genotypes were recovered. Similarly, Segovia et al. (1991) found the population of noninfective bean rhizobia in soil numerically superior to those capable of inducing nodule formation.

Environmental factors, particularly soil pH, temperature and water availability, often affect rhizobial survival in soil, and the balance between particular genotypes. In soils of pH >7.0, Brockwell et al., 1991 found an average of 89,000 meliloti g^{-1} soil, whereas in soils of pH <6.0, the number was only 37 g^{-1}. Even more striking is the replacement of the normal bean microsymbiont etli, by the acid-tolerant tropici to which beans were introduced (Anyango et al., 1995; Hungria et al., 1997). This change occurred in the relatively short time since Spanish and Portuguese colonization of Latin America, and despite the fact that tropici is less competitive than etli in nodule formation with beans (Martinez-Romero and Rosenblueth, 1990; Chaverra and Graham, 1992). In contrast, Richardson and Simpson (1989) found that many rhizobia from acid soils are sensitive to acidity, suggesting that microniches in soils protect these microorganisms from extremes of soil pH.

Temperature also has a marked influence on survival and persistence of rhizobial strains. (Eaglesham et al., 1981) found cowpea rhizobia from the hot, dry Sahelian savannah of West Africa to be temperature tolerant, with good growth at 37°C. More than 90% of the strains isolated from this region grew well to 40°C, whereas rhizobia from the more humid Onne region of West Africa generally failed to grow at this temperature. Soil temperature might contribute to the number of noninfective rhizobia found in some soils (Segovia et al., 1991). Temperature effects appear to be both strain and soil dependent. Marshall (1964) studied a clover nodulation problem in which autumn-sown plants nodulated well, but frequently failed to do so in subsequent regrowth. He found *Bradyrhizobium* sp. (Lupinus) less susceptible than leguminosarum bv. *trifolii* to high soil temperatures, but also noted amelioration of this problem in sandy soils amended with montmorillonite and illite.

Although rhizobia comprise only 0.1 to 8.0% of the total bacterial population in soil, and 0.01 to 0.14% of its biomass (Bottomley, 1992; Schor-

temeyer et al., 1997), their biodiversity in soil, and the factors which can affect it, have been extensively studied.

The development of improved techniques for strain fingerprinting, particularly restriction fragment length polymorphism (RFLP) and PCR analyses (de Bruijn, 1992; Judd et al., 1993; Dye et al., 1995; Madrzak et al., 1995; Richardson et al., 1995; Brunel et al., 1996; Labes et al., 1996; Paffetti et al., 1996; Rome et al., 1996; Sadowsky and Hur, 1998b), multilocus enzyme electrophoresis (MEE) (Pinero et al., 1988; Eardly et al., 1990; Demezas et al., 1991; Demezas et al., 1995; Bottomley et al., 1994; Dupuy et al., 1994; Souza et al., 1994; Strain et al., 1994; Strain et al., 1995) and SDS-PAGE analysis of total cell proteins (Roberts et al., 1980; Dupuy et al., 1994) have been used extensively to study strain biodiversity.

Population biodiversity among the rhizobia for a particular legume species tends to be greatest near the center of origin/domestication of that legume (Lie et al., 1987). Pinero et al. (1988) recorded a mean genetic distance per enzyme locus of 0.691 for 51 isolates of etli from the Mesoamerican center of origin for Phaseolus vulgaris (L.), while Souza et al. (1994) grouped 372 bean rhizobia into 7 clusters comprising 95 electrophoretic types. In the later study, rhizobia isolated from wild bean populations grouped by location and Phaseolus species, whereas those from cultivated beans were very heterogeneous. An emerging consideration in data such as this is the promiscuity of *Phaseolus vulgaris*. This host is nodulated by at least five different species of rhizobia (Michiels et al., 1998), necessitating care in distinguishing between intra- and extraspecific diversity. At the other extreme, the movement of rhizobia as seed-borne contaminants (Perez-Ramirez et al., 1998) can give the impression of limited biodiversity, more properly identified as founder effects (Hagen and Hamrick, 1996).

Environmental stresses noted above also can have profound effects on the biodiversity of rhizobia in soil. In an extreme case (Hirsch et al., 1996), application of manure containing heavy metals reduced the biodiversity of leguminosarum bv. trifolii in soil to a single biotype. Caballero-Mellado and Martinez-Romero (1999) also reported fertilizer effects on strain biodiversity in soil.

Applications

It is unlikely that soils contain appropriate rhizobia when a legume species is planted in a new area for the first time. In these cases, inoculation is usually required for adequate nodulation and N_2 fixation. Yield increases following inoculation with appropriate inoculant-quality rhizobia can exceed 50%, with clear differences between inoculated and uninoculated plants as shown in the figure.

Fig. 3. Response of soybean to inoculation in newly cultivated areas of Puerto Rico. The yellow-green plants in the center of the picture were not inoculated with *Rhizobium*. Courtesy of R. Stewart Smith.

Where plants are not inoculated with rhizobia in the first year of introduction into a new area, nodulation will most likely be limited to that coming from seed-borne or aerial contaminants. It is common that these rhizobia are less than fully effective in symbiosis with their host (Guar and Lowther, 1980). Over several years of cultivation these rhizobia will increase in numbers in the soil, limiting subsequent inoculation response. It is important, therefore, that inoculation with an inoculant-quality organism lead to early establishment and persistence of effective rhizobia in the soil, ensuring long-term benefits. Where this is done, the original inoculant strain(s) may still dominate in nodulation 10–15 years later (Brunel et al., 1988; Kucey and Hines, 1989; Bottomley, 1992). In Thailand, an interesting result of the need for inoculation in the establishment year is illustrated by farmer's willingness to adopt inoculant technologies: 80% of farmers in new areas of soybean production but only 30% of farmers in older established areas are willing to inoculate, and many of the latter apply fertilizer N (Hall and Clark, 1995).

Inoculation of legumes with suitable rhizobia has been practiced for more than 100 years. Initially this involved the collection of soil from areas where particular legumes had established successfully, and the mixing of soil and seed prior to planting. Inoculation with pure cultures of rhizobia followed, and today the provision of rhizobial inoculants to farmers, home gardeners and others is a multimillion dollar industry with approximately 4 tons peat culture sold annually in the United States alone (Burton, 1980). More than 80% of this is for two crops, soybean and

Fig. 4. PulseR.™ A presterilized-peat based inoculant for soybean. Photograph: H. Mc Ives, Agribiotics.

alfalfa. In Brazil where there was an early commitment to N_2 fixation as the principal source of plant N, Dobereiner et al. (1995) estimate the benefits of symbiotic and associative symbioses at more than \$1.8 billion each year. Most inoculant preparations are peat based (Smith, 1992; Brockwell and Bottomley, 1995), but frozen, granular, liquid, and other preparations also are used. Applied as recommended, these preparations will supply between 10^9 and 10^{13} rhizobia ha^{-1}, equivalent to 10^3 to 10^6 rhizobia $seed^{-1}$ (Lupwayi et al., 1999).

Many factors have to be considered if inoculation is to function properly. This website (www.Rhizobium.umn.edu) also provides linkages to a range of inoculant manufacturers and inoculant-related information. Most important in legume inoculation is use of the correct rhizobia. Specificity in nodule formation between host and rhizobia has been referred to already; specificities between host and *Rhizobium* in terms of N_2 fixation also exist (Burton, 1967) and impact inoculant strain selection. Liphatec (Milwaukee, WI) provides more than 100 different strain preparations for legume inoculation (Smith, 1988).

Consistent quality in the inoculant material is also essential, but can be surprisingly variable. A number of countries regulate inoculant quality. Thus, for example, inoculants sold in Canada, Australia, Uruguay and France must contain in excess of 108 or 109 rhizobia g^{-1} peat carrier, and be essentially contaminant free (Lupwayi et al., 1999). Inoculant quality in these countries is often controlled by a government testing agency and, subject to law. Best results have traditionally been with inoculant formulations that are sterilized before introduction of the rhizobia (D. J. Hume and J. A. Omelian, personal communication). These maintain higher numbers of rhizobia and have a longer shelf life than unsterile peat or granular inoculants. Whereas the large-scale production of inoculants is a relatively simple process, not all inoculants meet the quality standards mentioned above. For example, the 18 Argentine inoculants examined by Gomez et al. (1997) ranged in cell count from 0 to 10^9 rhizobia g^{-1}, and 14 contained more contaminants than rhizobia.

Environmental conditions also can affect inoculation success. The effect of soil acidity on rhizobia has already been mentioned, and has necessitated both a search for more acid-tolerant rhizobia (Graham et al., 1982; Graham, 1994; Howieson et al., 1988; Howieson and Ewing, 1989) or the use of pelleted, inoculated seed that provides a neutral environment prior to infection (Somasegaran and Hoben, 1994). Higher inoculation rates may be needed where high temperatures at seeding limit rhizobial survival (Smith et al., 1981; Smith and del Rio Escurra, 1982). A recent trend is for mixed inoculants, for example containing *Rhizobium* plus biocontrol, phosphate-solubilizing or growth hormone-producing organisms (Rice et al., 1995; Burdmann et al., 1996; Xi et al., 1996; Zhang et al., 1997). Results of coinoculation experiments with Azospirillum are promising; more detailed field experimentation is needed to establish the value of the other combined formulations. A concern in all such preparations must be the compatability and survival of the various inoculant organisms used.

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Prokaryotes (2006) 2:842–862
DOI: 10.1007/0-387-30742-7_26

CHAPTER 1.26

Magnetotactic Bacteria

STEFAN SPRING AND DENNIS A. BAZYLINSKI

Introduction

Magnetotactic bacteria are Gram-negative, motile prokaryotes that synthesize intracellular crystals of magnetic iron oxide or iron sulfide minerals. These apparently membrane-bounded crystals are called magnetosomes (Balkwill et al., 1980) and cause the bacteria to orient and migrate along geomagnetic field lines. Magnetotactic bacteria are indigenous in sediments or stratified water columns where they occur predominantly at the oxic-anoxic transition zone (OATZ) and the anoxic regions of the habitat or both. They represent a diverse group of microorganisms with respect to morphology, physiology and phylogeny. Despite the efforts of a number of different research groups, only a few representatives of this group of bacteria have been isolated in axenic culture since their discovery by (Richard P. Blakemore, 1975), and even fewer have been adequately described in the literature. Therefore, little is known about their metabolic plasticity, whereas their diverse morphology and phylogeny has been analyzed to some extent by culture-independent methods. To date, the only validly described species of magnetotactic bacteria are members of the genus *Magnetospirillum*. Representatives of this genus have been isolated reproducibly from various aquatic environments and can be grown relatively easily in mass culture. Therefore, most of the knowledge about the metabolism and biochemistry of magnetotactic bacteria relies on results obtained with strains of this genus.

Ecology

Magnetotactic bacteria are ubiquitous and common in sediments of freshwater or marine habitats, but also in stratified water columns (Bazylinski et al., 1995) and wet soils (Fassbinder et al., 1990). The occurrence of magnetotactic bacteria appears to be dependent on the presence of opposing gradients of reduced and oxidized compounds, usually represented by reduced sulfur species and oxygen, in the sediments or water columns. It has been shown in some cases that the distribution and abundance of these bacteria in the environment might also be dependent on the availability of soluble iron (Stolz et al., 1986). The highest numbers of magnetotactic bacteria are observed at the OATZ of sediments or stratified water columns. Magnetotactic bacteria can therefore be considered as typical examples of gradient organisms. In one study (Fig. 1), the number of a morphologically-distinct magnetotactic bacterium, "*Magnetobacterium bavaricum*," at the OATZ in a freshwater sediment was determined to be up to 7×10^5 live cells per cm^3 (Spring et al., 1993).

"*Magnetobacterium bavaricum*" is very large (average volume ca. 25.8 ± 4.1 μm^3) and could account for approximately 30% of the microbial biovolume in this layer of the sediment. In some environments, magnetotactic bacteria, particularly those that produce iron sulfide minerals, can also be detected in the anoxic region of the habitat, but are only rarely found at sites or regions of water columns or sediments exposed to high levels of oxygen.

The detection of magnetotactic bacteria in environmental samples is relatively easy due to their permanent magnetic dipole moment. One simple method is to put a drop of water or sediment on a microscope slide and place a bar magnet on the microscope stage in such a way that all the magnetotactic bacteria are guided in one direction until they reach and accumulate at the edge of a drop of water and/or sediment where they can be visualized. Alternatively, it is possible to magnetically enrich for higher numbers of cells by placing the south pole (in the Northern Hemisphere; the north pole of the magnet is used in the Southern Hemisphere) of a bar magnet adjacent to the outer wall of a jar filled with sediment and water. If magnetotactic bacteria are abundant in the sample, a brownish or sometimes grayish to white (if the cells contain elemental sulfur globules) spot consisting mainly of magnetotactic bacteria will form next to the inside of the glass wall closest to the south pole of the bar magnet. This material can easily be removed from the jar with a Pasteur pipette and

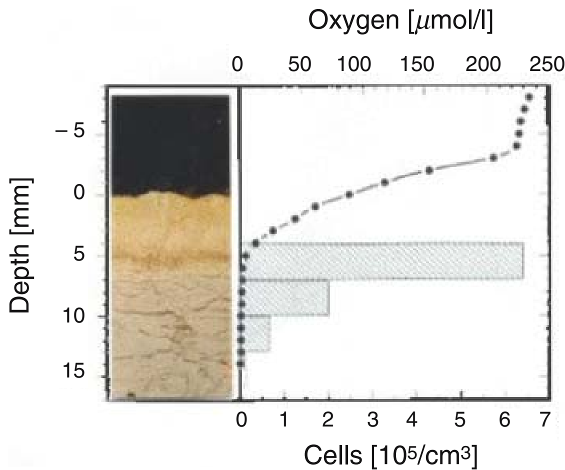

Fig. 1. Vertical distribution of "*Magnetobacterium bavaricum*" in the stratified sediment of a freshwater lake in Bavaria, Germany (Lake Chiemsee). The analyzed sediment sample was stored in an aquarium for several weeks before the measurements. The crosshatched bars indicate counts for successive depth fractions (3 mm intervals). Oxygen was measured every 1 mm (solid circles). A color photograph of the corresponding section of the aquarium is shown on the left. The upper brownish gray layer followed by a thin reddish brown and then a gray layer is characteristic of Lake Chiemsee sediments. The water-sediment interface corresponds to 0 mm.

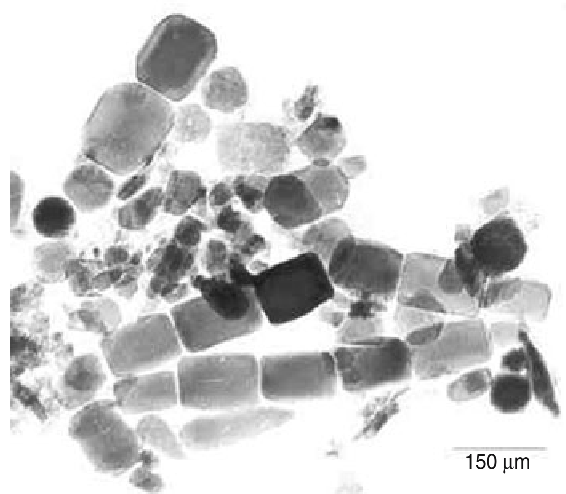

Fig. 2. Brightfield TEM image of a magnetic separate from surface sediments collected from the Irish Sea. Note the presence of parallelepipedal, cubo-octahedral, and tooth-shaped crystals of magnetite, presumed "magnetofossils" left from magnetotactic bacteria. Courtesy of Z. Gibbs.

examined as described above. Magnetotactic bacteria commonly enrich (i.e., increase in numbers) in sediment samples in jars or aquaria stored in dim light at room temperature. The process may take several weeks to months, however. In several cases, successions of different magnetotactic bacterial morphotypes have been observed during the enrichment process. Astonishingly, magnetotactic bacteria sometimes remain active for several years in the aquaria without addition of any nutrients.

When magnetotactic bacteria die and lyse, magnetosome crystals sometimes remain stable in sediments at sites where these bacteria were abundant, resulting in a change of the remanent magnetization of those sediments. Fossil magnetosome crystals consisting of magnetite ("magnetofossils") have been retrieved from many sites including deep sea sediments up to 50 million years old (Petersen et al., 1986) and ancient consolidated sediments up to 2 billion years old (Chang and Kirschvink, 1989a; Chang et al., 1989b). The bacterial origin of particles like those shown in Fig. 2 is assumed based on the unique morphology and size distribution of these magnetite crystals (see Magnetite-type Magnetosomes in this Chapter). Magnetite particles of similar sizes and shapes to those of magnetotactic bacteria have also been discovered within the Martian meteorite ALH84001 where they are thought to be the result of biological processes and therefore have been used as evidence for the presence of life on ancient Mars (McKay et al., 1996).

Nanometer-sized magnetite particles have also been found in soil samples. There the occurrence of magnetite seems to be strongly dependent on environmental conditions favoring the growth of magnetotactic bacteria, a fact which is also of some archaeological interest. It has been speculated that changes in the magnetic susceptibility of top soils, which frequently indicate locations of buried remains of archaeological objects, are caused by magnetotactic bacteria (Fassbinder and Stanjek, 1993). It is thought that the decay of wooden posts or palisades in wet soil leads to localized sites rich in organic material where the growth of magnetotactic bacteria is stimulated, thereby leading to the localized production and accumulation of magnetite. However, it is also possible that the magnetite is produced by iron-reducing bacteria through a biologically-induced mineralization process. In this case, Fe^{2+}, resulting from the bacterial enzymatic reduction of Fe^{3+}, reacts chemically with ferrihydrite to form extracellular magnetite.

Magnetotaxis, Chemotaxis and Aerotaxis

Soon after the discovery of magnetotactic bacteria, a model was proposed to explain the function of the bacterial magnetosome and the biological advantage of magnetotaxis. The original theory,

proposed by Blakemore (1975), was based on the assumption that all magnetotactic bacteria are microaerophilic and indigenous in sediments. Richard B. Frankel and co-workers clearly showed that these bacteria passively align and actively swim along the inclined geomagnetic field lines as a result of their magnetic dipole moment. Blakemore called this behavior magnetotaxis and proposed that magnetotaxis helps to guide the cells down to less oxygenated regions of aquatic habitats at the surface of sediments. Once cells have reached their preferred microhabitat they would presumably stop swimming and adhere to sediment particles until conditions changed, as for example, when additional oxygen was introduced. By this mechanism, a conventional aerotactic response which is a three dimensional search problem could be reduced to an one dimensional search problem in which cells only swim downward, thereby increasing the efficiency of the organism in finding an optimal oxygen concentration in the sediments. This theory is supported by the predominant occurrence of magnetotactic bacteria that are North-seeking (i.e., swim in the direction indicated by the North-seeking pole of a magnetic compass needle) under oxic conditions in the Northern hemisphere whereas bacteria are predominately South-seeking in the Southern hemisphere. Due to the negative and positive sign of the geomagnetic field inclination in the Northern and Southern hemispheres, respectively, magnetotactic bacteria in both hemispheres therefore swim downward toward the sediments (Blakemore, 1982).

Recent findings, including the discovery of large populations of magnetotactic bacteria at the OATZ of chemically stratified aquatic habitats and the isolation of obligately microaerophilic, coccoid magnetotactic bacterial strains, make it necessary to rethink this view of magnetotaxis. The traditional model does not completely explain how bacteria in the anoxic zone of a water column benefit from magnetotaxis, nor does it explain how the magnetotactic cocci form microaerophilic bands of cells in semi-solid oxygen gradient medium. Spormann and Wolfe (1984) showed earlier that magnetotaxis is somehow controlled by aerotaxis in some magnetotactic bacteria, but this alone does not help to explain all observed effects of magnetotaxis. More recently, it was demonstrated (with pure cultures of magnetite-producing magnetotactic bacteria) that magnetotaxis and aerotaxis work together in these bacteria (Frankel et al., 1997). The behavior observed in these strains has been referred to as "magneto-aerotaxis," which is a more accurate description than magnetotaxis because these cells do not try to reach a distinct magnetic pole or field as the term magnetotaxis implies. Thus, the term magnetotaxis is a misnomer.

The traditional model also fails to explain the various types of magnetotactic behavior which had been observed by several authors but without recognizing the fundamental differences between these behaviors (Moench and Konetzka, 1978; Blakemore et al., 1980; Spormann and Wolfe, 1984). Only when distinct morphotypes of magnetotactic bacteria were isolated and grown in pure culture for detailed studies using thin, flattened capillaries (Frankel et al., 1997), it became clear that two types of mechanisms have been observed, which apparently occur in different bacteria, termed polar and axial.

The distinction can be seen by examination of cells in wet mounts under oxic conditions using a microscope and a magnet of a few gauss parallel to the plane of the slide (Fig. 3). Polar magnetotactic bacteria, particularly the magnetotactic cocci, swim persistently along the magnetic field lines without reversing their direction or turning. If the magnetic field is reversed, the bacteria reverse their swimming direction and continue swimming persistently in the same direction relative to the magnetic field. Bacteria from Northern hemisphere habitats predominately swim parallel to the magnetic field, corresponding to northward migration in the geomagnetic field. Bacteria from the Southern hemisphere swim antiparallel to the magnetic field. It was this consistent swimming behavior that led to the discovery of magnetotactic bacteria by Blakemore (1975). On the other hand, axial magnetotactic bacteria, especially the freshwater spirilla, orient and swim in both directions along the magnetic field lines with frequent reversals of swimming direction and some accumulating in approximately equal numbers on both sides of the water drop (Fig. 3a).

The distinction between polar and axial magneto-aerotaxis can also be seen in flattened capillary tubes containing suspensions of cells in reduced medium with one or both ends of the capillary tube open. In the first situation, where one end of the capillary is open (the right end of the capillaries in Fig. 3b) and the other sealed, a single oxygen gradient forms beginning at the open end of the capillary. Cells of strain MC-1 in these capillaries rotate 180° after a reversal of B, the magnetic field, and the band separates into groups of cells swimming in opposite directions along B, away from the position of the band before the reversal. A second reversal results in the reformation of a single band. Cells of *M. magnetotacticum* also rotate 180° in these capillaries but the band of cells does not separate and remains intact (Fig. 3). In the second situation (not shown), where both ends of the capillary

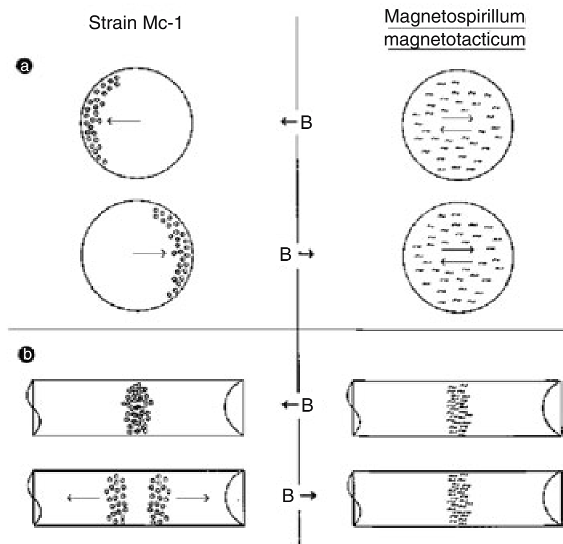

Fig. 3. Two types of magnetotaxis. (a) Depictions of the polar magnetotactic behavior of strain MC-1 and axial magnetotactic behavior of *Magnetospirillum magnetotacticum* in water drops under oxic conditions on a microscope slide (B, magnetic field; arrow points northward). Cells of strain MC-1 swim persistently parallel to B (North-seeking motility) and accumulate at the edge of the drop. When B is reversed, cells continue to swim parallel to B (North-seeking motility) and accumulate at the other side of the drop. Cells of *M. magnetotacticum* swim in either direction relative to B and continue to do so when the field is reversed. (b) Illustrations of aerotactic bands of strain MC-1 and *M. magnetotacticum* in flat capillaries. The right ends of the capillaries are open to air and the left ends are sealed. After reversal of B, cells of strain MC-1 rotate 180° and the band separates into groups of cells swimming in opposite directions along B, away from the position of the band before the reversal. A second reversal results in the reformation of a single band. Cells of *M. magnetotacticum* also rotate 180° but the band of cells remains intact. Figure adapted from Frankel et al. (1997).

tubes are open, diffusion of oxygen into the ends of the tubes creates an oxygen gradient at each end of the tube, oriented in opposite directions. Polar magnetotactic bacteria incubated in a magnetic field oriented along the long axis of the tube form an aerotactic band at only one end of the tube, whereas axial magnetotactic bacteria form bands at both ends of the tube. Thus for polar magnetotactic bacteria the magnetic field provides an axis and direction for motility, whereas for axial magnetotactic bacteria the magnetic field only provides an axis of motility, pointing to different magneto-aerotactic mechanisms occurring in two types of bacteria.

Axial Magneto-Aerotaxis

Almost all magnetotactic spirilla available in axenic culture and grown in liquid media, exhibit axial magneto-aerotaxis (Figs. 3 and 4). Other bacteria that show axial magnetotaxis are microaerophilic or anaerobic chemoheterotrophs, or facultative chemolithoautotrophic bacteria that are either monopolarly or bipolarly flagellated. In most habitats, axial magnetotactic bacteria appear to represent only a very small fraction of the total count of magnetotactic bacteria, although these organisms are harder to detect in wet mounts using a microscope. Cells representing this type of magnetotaxis were referred to as two-way swimmers because in a homogeneous medium they swim in either direction along the magnetic field, B (Fig. 4). In the presence of an oxygen gradient, cells swim parallel or antiparallel to B with aerotaxis determining the direction of migration. Therefore, an aerotactic band of cells forms at both ends of the tube in capillaries where both ends are open, whereas cells displaying a polar magnetotaxis form only one band at the end of the tube corresponding to their magnetic polarity. The aerotactic, axial magnetotactic spirilla appear to use a temporal sensory mechanism for oxygen detection as do most microaerophilic bacteria studied so far (Frankel et al., 1998). Changes in oxygen concentration measured during swimming determine the sense of flagellar rotation. Cells moving away from the optimal oxygen concentration consequently reverse their swimming direction. In this model, changes in oxygen concentration are measured within short intervals implying that these bacteria must be actively motile in order to quickly measure and respond to concentration gradients in their habitat. The combination of a passive alignment along geomagnetic field lines with an active, temporal, aerotactic response provides the organism with an efficient mechanism to find the microoxic or suboxic zone in its habitat. Therefore the term magneto-aerotaxis is also an appropriate descriptive term for this tactic behavior.

Fig. 4. Sequence showing magnetotactic spirilla displaying axial magnetotaxis. For the video, see the online version of *The Prokaryotes*.

Polar Magneto-Aerotaxis

The large majority of naturally-occurring magnetotactic bacteria display polar magnetotaxis (Figs. 3 and 5). The following mechanism for polar magnetotaxis was proposed based on experimental data obtained with an axenic culture of a marine magnetotactic coccus. It was demonstrated that these cocci can swim in both directions along a static magnetic field, B, without the need of turning around by reversing the sense of flagellar rotation. It seems that a two-state sensory mechanism determines the sense of flagella rotation leading to parallel or antiparallel swimming along the geomagnetic field lines.

Under higher than optimal oxygen tensions, the cell is presumably in an "oxidized state" and swims persistently parallel to B (Fig. 5), i.e., downward in the Northern hemisphere. Under reducing conditions or suboptimal oxygen concentrations, the cell switches to a second state, the "reduced state", which leads to a reversal of the flagellar rotation and to a swimming antiparallel to B (upward in the Northern hemisphere). This two-state sensing mechanism results in an efficient aerotactic response, provided that the oxygen-gradient is oriented correctly relative to B, so that the cell is guided in the right direction to find either reducing or oxidizing conditions. This is especially important because adaptation, which would lead to a spontaneous reversal of the swimming direction, was never observed in controlled experiments with the cocci. The redox sensor, which controls this two-state response, might be similar to the FNR (fumarate and nitrate reduction) transcription factor found in *Escherichia coli* and other bacteria. The FNR factor is sensitive to oxygen and activates gene expression in the reduced state thereby promoting the switch between aerobiosis and anaerobiosis in *E. coli* (De Graef et al., 1999). The sensory mechanism in the examined magnetotactic cocci is not only affected by oxygen. Cells exposed to light of short wavelengths (=500 nm) also showed a response similar to a switch to the "oxidized state" (Frankel et al., 1997).

Fig. 5. Sequence showing magnetotactic cocci displaying polar magnetotaxis. For the video, see the online version of *The Prokaryotes*.

Revised Model of Magnetotaxis

Based on these observations, we would like to extend the current model of a magnetically-guided aerotaxis (magneto-aerotaxis) to a more complex redoxtaxis. In this case, the unidirectional movement of magnetotactic bacteria in a drop of water would be only one aspect of a sophisticated redox-controlled response. One hint for the possible function of polar magnetotaxis could be that most of the representative microorganisms are characterized by possessing either large sulfur inclusions or magnetosomes consisting of iron-sulfides. Therefore, it may be speculated that the metabolism of these bacteria, being either chemolithoautotrophic or mixotrophic, is strongly dependent on the uptake of reduced sulfur compounds which occurs in many habitats only in deeper regions at or below the OATZ due to the rapid chemical oxidation of these reduced chemical species by oxygen or other oxidants in the upper layers. To overcome the problem of separated pools of electron donor and acceptor, several strategies have been developed by sulfide-oxidizing bacteria. Microorganisms belonging to the genus *Thioploca*, for example, use nitrate, which is stored intracellularly (most of the internal space of the cell is vacuolar) to oxidize sulfide and have developed vertical sheaths in which bundles of motile filaments are located. It is assumed that *Thioploca* uses these sheaths to efficiently move in a vertical direction in the sediment, thereby accumulating sulfide in deeper layers and nitrate in upper layers (Huettel et al., 1996). For some magnetotactic bacteria, it might also be necessary to perform excursions to anoxic zones of their habitat in order to accumulate reduced sulfur compounds. In our model, shown in Fig. 6, we propose that polar magnetotaxis helps to guide bacteria, depending on their internal redox-state, either downward to accumulate reduced sulfur species or upward to oxidize stored sulfur with oxygen. Thus, we hypothesize that magnetotactic bacteria displaying polar magnetotaxis alternate between two internal redox states. The "oxidized state" would result from the almost complete consumption of stored sulfur, the assumed electron donor. In this state, cells seek deeper anoxic layers where they could replenish the depleted stock of electron donor using nitrate or other compounds as alternative electron acceptor. Finally, they would reach a "reduced state." According to our model, cells in this redox state would have accumulated a large amount of sulfur which cannot be efficiently oxidized under anaerobic conditions leading to a surplus of reduction equivalents. Therefore, cells must return to the microoxic zone where oxygen is available to them as an electron acceptor. In addition, oxygen may be necessary for the synthesis of magnetosomes in some bacteria (Blakemore et al., 1985). The advantage of polar magnetotaxis is that an oxygen gradient is not necessary for efficient orientation in the anoxic zone, thereby enabling a rapid return of the cell along large distances to the preferred microoxic conditions. A further benefit would be that cells avoid the waste of energy by constant movement along gradients, but instead can attach to particles in preferred microniches until they reach an unfavorable internal redox state that triggers a magnetotactic response either parallel or antiparallel to the geomagnetic field lines. In any case, greater than optimal concentrations of oxygen would switch cells immediately to an "oxidized state" provoking the typical down-seeking response of magnetotactic bacteria visible under the microscope. The observation of significant numbers of microaerophilic, magnetotactic bacteria in the anoxic zone of some sediments and the attachment of "*Magnetobacterium bavaricum*" to sediment particles in

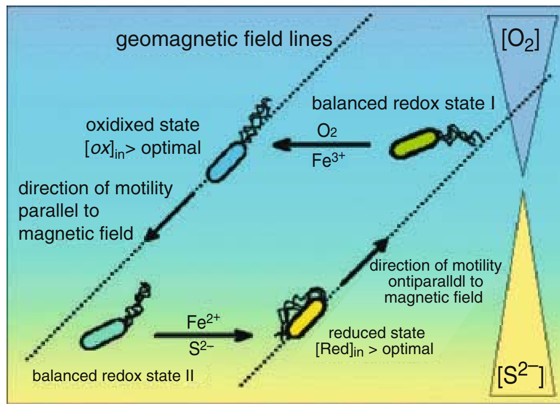

Fig. 6. Hypothetical model of the function of polar magnetotaxis in bacteria (Northern hemisphere). Cells are guided along the geomagnetic field lines depending on their "redox-state" either downward to the sulfide-rich zone or upward to the microoxic zone, thereby enabling a shuttling between different redox layers.

microcolony-like aggregates, fits well with this model, which is summarized in the following Fig. 6.

Morphologic and Phylogenetic Diversity

Morphotypes

The diversity of magnetotactic bacteria is reflected by the high number of different morphotypes found in environmental samples of water or sediment. Commonly observed morphotypes include coccoid to ovoid cells, rods, vibrios and spirilla of various dimensions. One of the more unique morphotypes is an apparently multicellular bacterium referred to as the MMP many-celled magnetotactic prokaryote.

Regardless of their morphology, all magnetotactic bacteria studied so far are motile by means of flagella and have a cell wall structure characteristic of Gram-negative bacteria. The arrangement of flagella differs and can be either polar, bipolar, or in tufts. Another trait which shows considerable diversity is the arrangement of magnetosomes inside the bacterial cell. In the majority of magnetotactic bacteria, the magnetosomes are aligned in chains of various lengths and numbers along the cell's long axis of the cell, which is magnetically the most efficient orientation. However, dispersed aggregates or clusters of magnetosomes occur in some magnetotactic bacteria usually at one side of the cell, which often corresponds to the site of flagellar insertion. Besides magnetosomes, large inclusion bodies containing elemental sulfur, polyphosphate, or poly-β-hydroxybutyrate are common in magnetotactic bacteria collected from the natural environment and in pure culture.

The most abundant type of magnetotactic bacteria occurring in environmental samples, especially sediments, are coccoid cells possessing two flagellar bundles on one somewhat flattened side. This bilophotrichous type of flagellation gave rise to the tentative genus "*Bilophococcus*" for these bacteria (Moench, 1988). One representative strain of this morphotype is in axenic culture (see Other Magnetotactic Strains in Pure Culture in this Chapter). In contrast, two of the morphologically more conspicuous magnetotactic bacteria, regularly observed in natural samples but never isolated in pure culture, are the MMP and a large rod containing large numbers of hook-shaped magnetosomes ("*Candidatus Magnetobacterium bavaricum*").

The MMP, a Many-Celled Magnetotactic Prokaryote A magnetotactic aggregation of cells that swims as an entire unit and not as separate cells was first reported and described by Farina et al. (1983). Similar morphotypes were later found also in sulfide-rich marine and brackish waters and in sediments along the coasts of North America and Europe (Mann et al., 1990a). The MMP (for many-celled magnetotactic prokaryote) consists of about 10 to 30 coccoid to ovoid Gram-negative cells, roughly arranged in a sphere with a diameter ranging from approximately 3 to 12 µm (Fig. 7).

Cells are asymmetrically multiflagellated on their outer surfaces exposed to the external surroundings. Magnetosomes consist of the magnetic iron-sulfide greigite, Fe_3S_4, and several nonmagnetic precursors to greigite (see "Iron-Sulfide Type Magnetosomes"). The magnetosome crystals are generally pleomorphic although cubo-octahedral, rectangular prismatic, and tooth-shaped particles have also been observed in cells. They are usually loosely arranged in short chains or clusters in individual cells. The total magnetic moment of the MMP was determined and ranges from 5×10^{-16} to 1×10^{-15} Am^2, which is sufficient for an effective magnetotactic response. The type of magnetotaxis displayed by the MMP appears to be polar, but aggregates have been observed to reverse direction. Under oxic conditions in a uniform magnetic field, the swimming speed in the preferred direction averages 105 µm/s. After reaching the edge of a water drop, aggregates sometimes spontaneously reverse their swimming direction and show short excursions of 100 to 500 µm with twice the speed of the forward motion in the opposite direction of their polarity (Rodgers et al., 1990) as shown in Fig. 8. This so

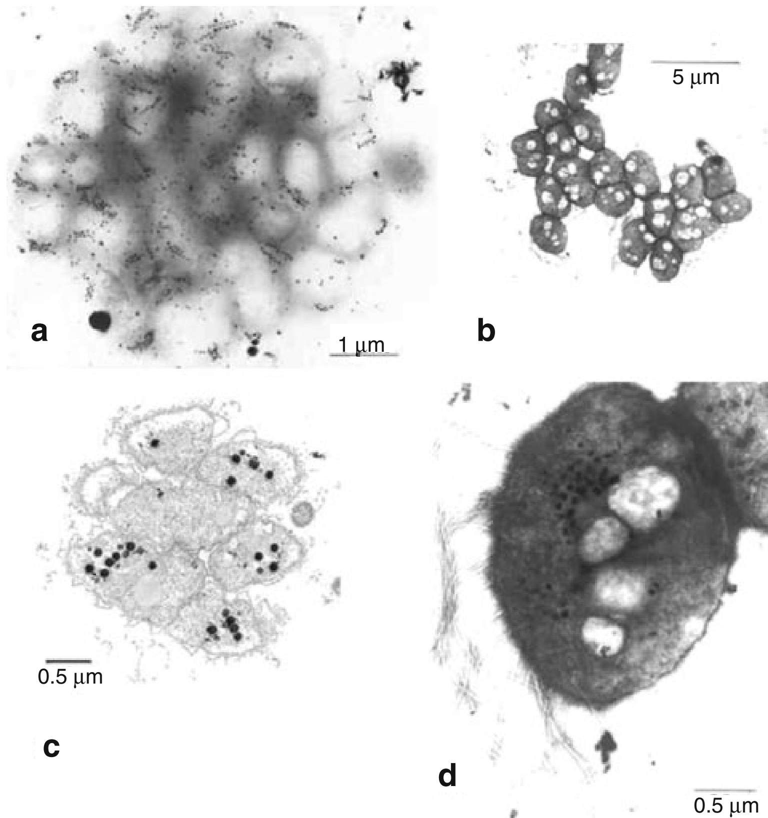

Fig. 7. Brightfield TEM micrographs of the many-celled magnetotactic prokaryote (MMP). (a) An unstained, single MMP revealing the numerous greigite-containing magnetosomes within the organism mostly arranged in short chains. (b) Negatively-stained preparation (2.5% ammonium molybdate, pH 7.0) of a single MMP that is disrupted to reveal separated individual cells. (c) Thin-section of an MMP again showing its many-celled nature. (d) Negatively-stained individual cell of the MMP. Note the asymmetric distribution of flagella which cover the cell on one side, the pleomorphism of the greigite-containing magnetosomes, and the electron-lucent vacuoles resembling poly-β-hydroxybutyrate (PHB) granules.

called "ping pong" motion seems to be a peculiarity of this organism.

Fig. 8. Sequence showing the typical "ping-pong" motility of the MMP. For the video, see the online version of *The Prokaryotes*.

In one study, it was reported that individual cells within the aggregate are connected by intercellular membrane junctions (Rodgers et al., 1990a; 1990b). However, the cohesive force among individual cells seems to be relatively weak because a lowering of the osmotic pressure leads to an immediate disruption of the aggregate into single nonmotile cells.

"*Candidatus Magnetobacterium bavaricum*" The first phenotypic description of this morphotype by Vali et al. (1987) was based on cells collected from material retrieved from the littoral sediments of a large freshwater lake in Southern Germany (Lake Chiemsee). Later, similar bacteria were also found in sediments of other freshwater habitats in Germany and Brazil. After the determination of its phylogenetic relationship (Spring et al., 1993), this organism was given the candidatus status due to its unusual phenotypic traits which distinguish it from all other magnetotactic bacteria. "*Magnetobacterium bavaricum*" displays polar magnetotaxis and is preferentially found in the microoxic zone of sediments, although significant numbers are also found in anoxic regions of their habitat (Fig. 1). In situ hybridizations using a specific fluorescently-labeled oligonucleotide probe targeting the 16S rRNA of this organism enabled the detection of microcolonies of this bacterium on microscope slides immersed into sediment for several weeks (Fig. 9). Thus, there appears to be a tendency for "*Magnetobacterium bavaricum*" to adhere to particles located in microsites with preferred environmental conditions.

Cells of "*M. bavaricum*" are large rods having dimensions of 1–1.5 x 6–9 μm and are motile by a polar tuft of flagella. The most impressive trait of this bacterium is the extremely high number of magnetosomes per cell. A single cell may contain up to a thousand hook-shaped magnetosomes usually arranged in 3–5 rope-shaped bundles oriented parallel to the long axis of the cell (Fig. 10).

The magnetosomes consist of magnetite (Fe_3O_4) and have a length of 110–150 nm. The average total magnetic moment per cell was experimentally determined to be approximately 3×10^{-14} Am^2, which is about an order of magnitude higher than that of most other magnetotactic bacteria. The presence of large sulfur inclusions is typical for this bacterium and seems

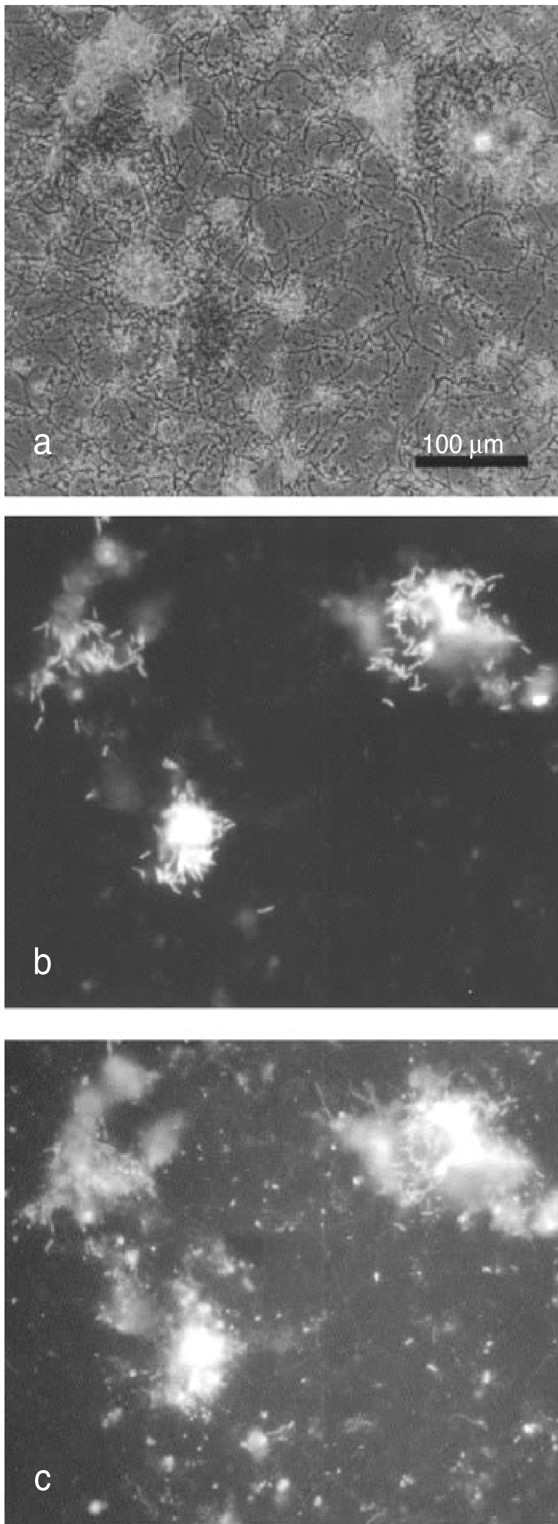

Fig. 9. In situ hybridization of a microscope slide grown over with sediment bacteria using fluorescently labeled oligonucleotide probes. (a) Phase contrast micrograph. (b+c) Same field viewed with epifluorescence microscopy enabling the detection of a specific probe binding to a signature region of the 16S rRNA of "*M. bavaricum*" (b), and of a probe with broad specifity hybridizing with the 16S rRNA of most known bacteria (c).

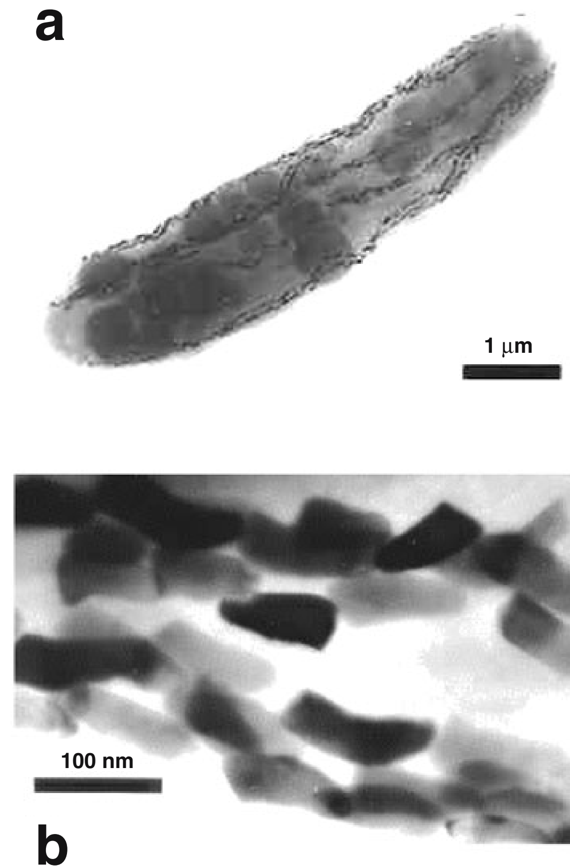

Fig. 10. Brightfield transmission electron microscope (TEM) micrographs of "*M. bavaricum*." (a) Whole cell displaying bundles of magnetosome chains and sulfur globules. (b) Hook-shaped magnetite-type magnetosomes. Courtesy of M. Hanzlik.

to be dependent on environmental conditions. In an unidirectional magnetic field, cells swim forward (i.e., northward in the Northern Hemisphere) with an average speed of 40 μm/s with the flagella wound around the rotating cell. Gradients of some chemical substances lead to a reversal of the sense of flagellar rotation resulting in a swimming in the opposite direction for a short time.

Composition and Structure of Magnetosome Crystals

The magnetosome mineral phase in magnetotactic bacteria are tens-of-nanometer-sized crystals of an iron oxide and/or an iron sulfide. The mineral composition of the magnetosome is specific enough for it to be likely under genetic control, in that cells of several cultured magnetite-producing magnetotactic bacteria still synthesize an iron oxide and not an iron sulfide, even when hydrogen sulfide is present in the growth medium

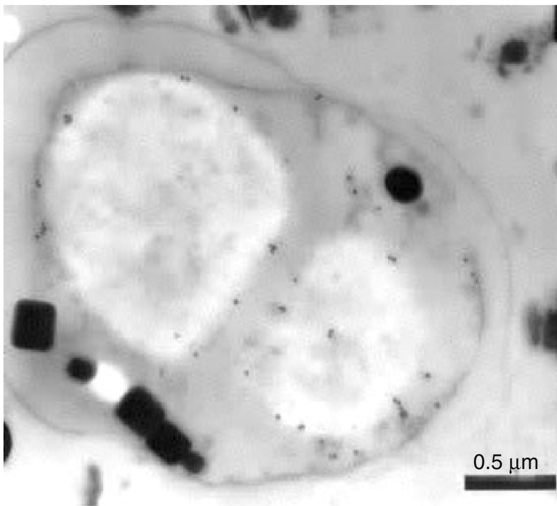

Fig. 11. Unusually large magnetite crystals identified in coccoid magnetotactic bacteria retrieved from a lagoon near Rio de Janeiro, Brazil. Small black dots represent gold-labeled antibodies detecting a specifically bound polynucleotide probe complementary to a highly variable region of the 16S rRNA of these cells.

(Meldrum et al., 1993a; Meldrum et al., 1993b). The size of the magnetosome mineral crystals also appears to be under control of the organism because the large majority of magnetotactic bacteria contain crystals displaying only a very narrow size range, from about 35 to 120 nm (Frankel et al., 1998). Magnetite and greigite particles in this range are stable single magnetic domains (Butler and Banerjee, 1975; Diaz-Rizzi and Kirschvink, 1992). Smaller particles would be superparamagnetic at ambient temperature and would not have stable, remanent magnetization. Larger particles would tend to form multiple domains, reducing the remanent magnetization. However, in some uncultured bacteria from the Southern Hemisphere exceptionally large magnetite-magnetosomes have been observed in some uncultured bacteria from the Southern hemisphere (Fig. 11), having dimensions well above the theoretically determined size limits of single domain magnetite (Spring et al., 1998). It remains unclear if the crystals in these bacteria are still of single-domain size or are multi-domain particles and why such unusually large crystals are formed by certain bacteria, but, interestingly, it seems that the crystal-size corresponds with the size and/or the growth phase of these bacteria, i.e., large cells possess larger crystals than smaller cells of the same type.

In contrast to chemically synthesized magnetite and greigite crystals, biologically produced magnetosome mineral particles display a range of well-defined morphologies which can be classified as distinct idealized types (Fig. 12).

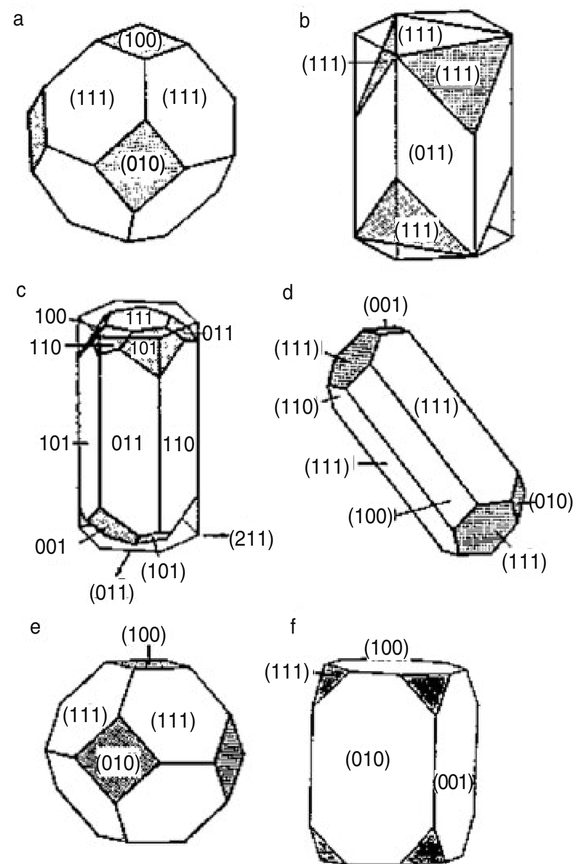

Fig. 12. Idealized magnetite (a–d) and greigite (e–f) crystal morphologies derived from high resolution TEM studies of magnetosome crystals from magnetotactic bacteria: (a) and (e) cubo-octahedrons; (b), (c), and (f) variations of pseudo-hexagonal prisms; (d) elongated cubo-octahedron. Numbers within parentheses refer to the faces of the crystal lattice planes on the surface of the crystal. Figure adapted from Heywood et al. (1991) and Mann and Frankel (1989).

The consistent narrow size range (Devouard et al., 1998) and morphologies of the intracellular magnetosome particles represent typical features of a biologically controlled mineralization and are clear indications that the magnetotactic bacteria exert a high degree of control over the biomineralization processes involved in magnetosome synthesis.

Magnetite-Type Magnetosomes The iron oxide-type magnetosomes consist solely of magnetite, Fe_3O_4. The particle morphology of the magnetite crystals in magnetotactic bacteria varies but is extraordinarily consistent within cells of a single bacterial species or strain (Bazylinski et al., 1994). Three general morphologies of magnetite particles have been observed in magnetotactic bacteria using transmission electron microscopy (TEM; Blakemore et al., 1989; Mann et al.,

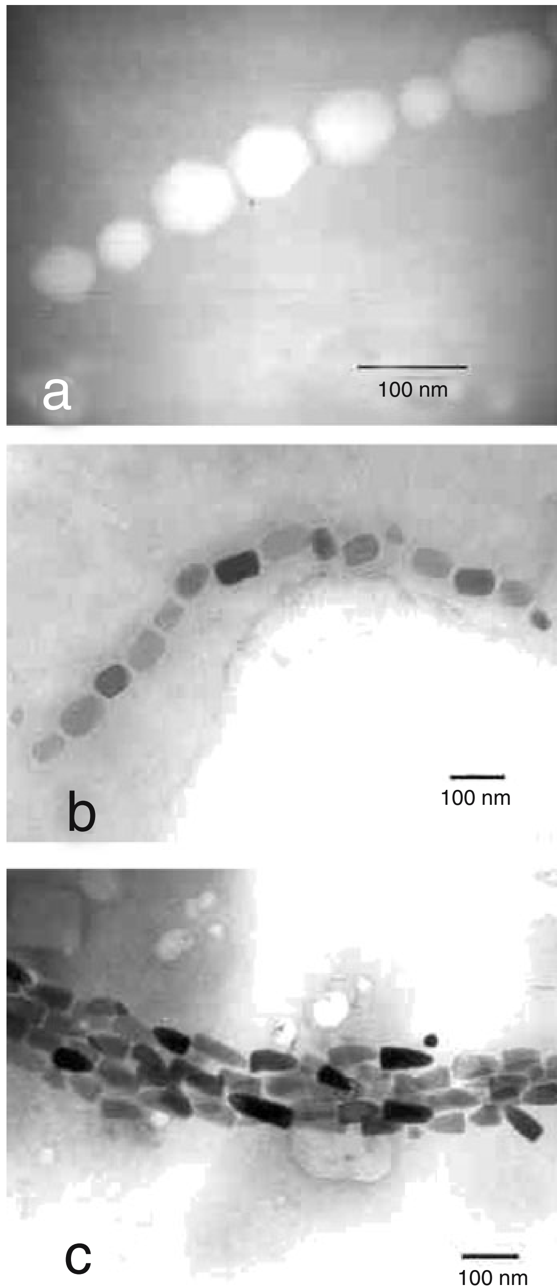

Fig. 13. Morphologies of intracellular magnetite (Fe_3O_4) particles produced by magnetotactic bacteria. (a) Darkfield scanning-transmission electron microscope (STEM) image of a chain of cubo-octahedra in cells of an unidentified rod-shaped bacterium collected from the Pettaquamscutt Estuary, Rhode Island, USA, viewed along a [111] zone axis for which the particle projections appear hexagonal. (b) Brightfield TEM image of a chain of prismatic crystals within a cell of strain MV-2, a marine vibrio, with parallelepipedal projections. (c) Brightfield TEM image of tooth-shaped (anisotropic) magnetosomes from an unidentified rod-shaped bacterium collected from the Pettaquamscutt Estuary.

1990a; Stolz, 1993; Bazylinski et al., 1994). They include: 1) roughly cuboidal (Balkwill et al., 1980; Mann et al., 1984); 2) parallelepipedal (rectangular in the horizontal plane of projection; Moench and Konetzka, 1978; Towe and Moench, 1981; Moench, 1988; Bazylinski et al., 1988); and 3) tooth-, bullet-, or arrowhead-shaped (anisotropic; Mann et al., 1987a; Mann et al., 1987b; Thornhill et al., 1994).

High resolution TEM and selected area electron diffraction studies have revealed that the magnetite particles within magnetotactic bacteria are of relatively high structural perfection and have been used to determine their idealized morphologies (Matsuda et al., 1983; Mann et al., 1984a; 1984b; 1987a; 1987b; Meldrum et al., 1993a; Meldrum et al., 1993b). These morphologies are all derived from combinations of {111}, {110} and {100} forms (a form refers to the equivalent symmetry related lattice planes of the crystal structure) with suitable distortions (Devouard et al., 1998). The roughly cuboidal particles are cubo-octahedra ([100] + [111]), and the parallelepipedal particles are either pseudohexahedral or pseudooctahedral prisms. Examples are shown in Fig. 12a–d. The cubo-octahedral crystal morphology preserves the symmetry of the face-centered cubic spinel structure, i.e., all equivalent crystal faces develop equally. The pseudo-hexahedral and pseudo-octahedral prismatic particles represent anisotropic growth in which equivalent faces develop unequally (Mann and Frankel, 1989; Devouard et al., 1998). The synthesis of the tooth-, bullet- and arrowhead-shaped magnetite particles (Figs. 10b, 13c) appears to be more complex than that of the other forms. They have been examined by high resolution TEM in one uncultured organism (Mann et al., 1987a; Mann et al., 1987b) and their idealized morphology suggests that growth of these particles occurs in two stages. The nascent crystals are cubo-octahedra which subsequently elongate along the [111] axis parallel to the chain direction.

Whereas the cubo-octahedral form of magnetite can occur in inorganically-formed magnetites (Palache, 1944), the prevalence of elongated pseudohexahedral or pseudo-octahedral habits in magnetosome crystals imply anisotropic growth conditions, e.g., a temperature gradient, a chemical concentration gradient, or an anisotropic ion flux (Mann and Frankel, 1989). This aspect of magnetosome particle morphology has been used to distinguish magnetosome magnetite from detrital or magnetite produced by biologically induced mineralization (by the anaerobic iron-reducing bacteria), using electron microscopy of magnetic extracts from sediments (e.g., Petersen et al., 1986; Chang and Kirschvink, 1989a; Chang et al., 1989b; Stolz et al., 1986; Stolz et al., 1990; Stolz, 1993).

Iron-Sulfide Type Magnetosomes Virtually all freshwater magnetotactic bacteria have been found to synthesize magnetite as the mineral phase of their magnetosomes. In contrast, many marine, estuarine, and salt marsh species produce iron sulfide-type magnetosomes consisting primarily of the magnetic iron sulfide, greigite, Fe_3S_4 (Heywood et al., 1990; Heywood et al., 1991; Mann et al., 1990b; Pósfai et al., 1998a; 1998b). Reports of non-magnetic iron pyrite (FeS_2; Mann et al., 1990b) and magnetic pyrrhotite (Fe_7S_8; Farina et al., 1990) have not been confirmed and may represent misidentifications of additional iron sulfide species occasionally observed with greigite in cells (Pósfai et al., 1998a; Pósfai et al., 1998b). Currently recognized greigite-producing magnetotactic bacteria includes the MMP (Farina et al., 1983; Rodgers et al., 1990a; 1990b; DeLong et al., 1993) and a variety of relatively large, rod-shaped bacteria (Bazylinski et al., 1990; Bazylinski et al., 1993a; Heywood et al., 1990; Heywood et al., 1991; Bazylinski and Frankel, 1992).

The iron sulfide-type magnetosomes contain either particles of greigite (Heywood et al., 1990; Heywood et al., 1991) or a mixture of greigite and transient non-magnetic iron sulfide phases that appear to represent mineral precursors to greigite (Pósfai et al., 1998a; Pósfai et al., 1998b). These phases include mackinawite (tetragonal FeS) and possibly a sphalerite-type cubic FeS (Pósfai et al., 1998a; Pósfai et al., 1998b). Based on TEM observations, electron diffraction, and known iron sulfide chemistry (Berner, 1967; Berner, 1970; Berner, 1974), the reaction scheme for greigite formation in the magnetotactic bacteria appears to be: cubic FeS $\rightarrow$ mackinawite (tetragonal FeS) $\rightarrow$ greigite (Fe_3S_4; Pósfai et al., 1998a; Pósfai et al., 1998b).

The de novo synthesis of non-magnetic crystalline iron sulfide precursors to greigite aligned along the magnetosome chain indicates that chain formation within the cell does not involve magnetic interactions. Interestingly, under the strongly reducing, sulfidic conditions at neutral pH in which the greigite-producing magnetotactic bacteria are found (Bazylinski et al., 1990; Bazylinski and Frankel, 1992), greigite particles would be expected to transform into pyrite (Berner, 1967; Berner, 1970) which has not been unequivocally identified in magnetotactic bacteria. It is not known if and how cells prevent this transformation.

As with magnetite, three particle morphologies of greigite have been observed in magnetotactic bacteria (Fig. 14): 1) cubo-octahedral (the equilibrium form of face-centered cubic greigite) (Heywood et al., 1990; Heywood et al., 1991); 2) pseudo-rectangular prismatic as shown in Fig. 14 and 12e–f (Heywood et al., 1990; Heywood et al.,

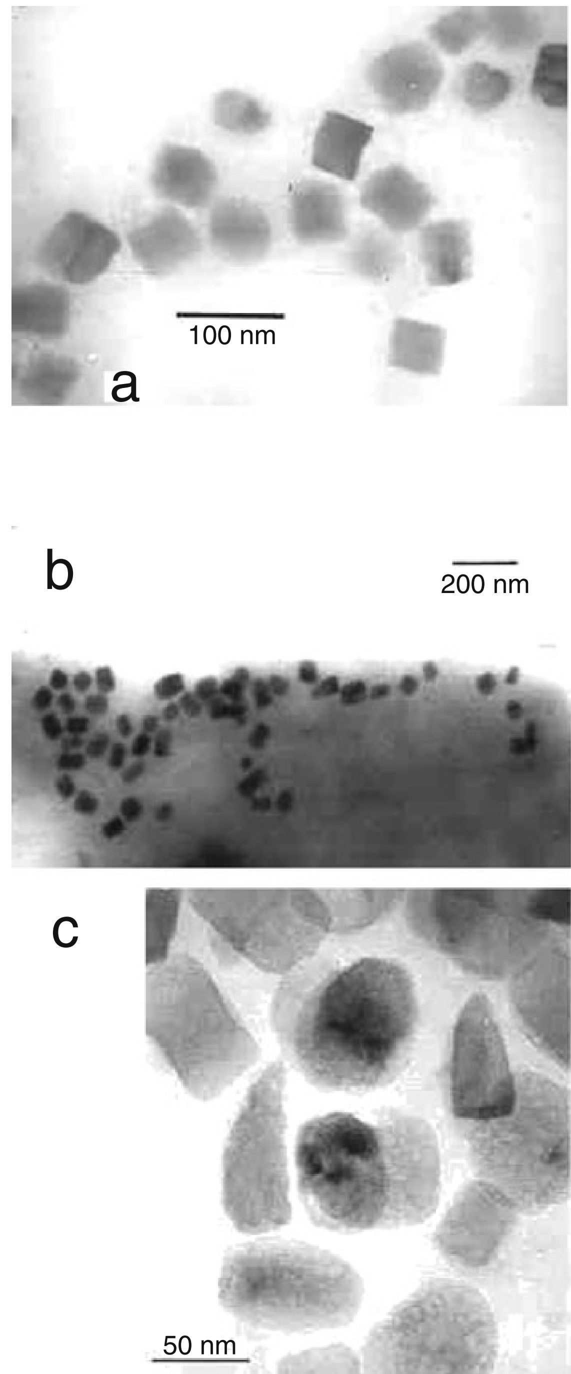

Fig. 14. Morphologies of intracellular greigite (Fe_3S_4) particles produced by magnetotactic bacteria. (a) Brightfield STEM image of cubo-octahedra in an unidentified rod-shaped bacterium collected from the Neponset River estuary, Massachusetts, USA. (b) Brightfield STEM image of rectangular prismatic particles in an unidentified rod-shaped bacterium collected from the Neponset River estuary, Massachusetts, USA. (c) Brightfield TEM image of tooth-shaped and rectangular prismatic particles from the many-celled magnetotactic prokaryote (MMP), courtesy of M. Pósfai and P. R. Buseck.

1991); and 3) tooth-shaped (Pósfai et al., 1998a; Pósfai et al., 1998b).

Like that of their magnetite counterparts, the morphology of the greigite particles also appears to be species- and/or strain-specific, although confirmation of this observation will require controlled studies of pure cultures of greigite-producing magnetotactic bacteria, none of which is currently available. One clear exception to this rule is the MMP (Farina et al., 1983; Bazylinski et al., 1990; Bazylinski et al., 1993a; Mann et al., 1990b; Rodgers et al., 1990a; 1990b; Bazylinski and Frankel, 1992). This unusual microorganism, found in salt marsh pools all over the world and some deep sea sediments, has been found to contain pleomorphic, pseudorectangular prismatic, tooth-shaped, and cubo-octahedral greigite particles. Some of these particle morphologies are shown in Fig. 7 and 14c. Therefore the biomineralization process(es) appear(s) to be more complicated in this organism than in the rods with greigite-containing magnetosomes or in magnetite-producing, magnetotactic bacteria.

Magnetite and Greigite Crystals in a Single Bacterium One slow-swimming, rod-shaped bacterium, collected from the OATZ from the Pettaquamscutt Estuary, was found to contain arrowhead-shaped crystals of magnetite and rectangular prismatic crystals of greigite co-organized within the same chains of magnetosomes (this organism usually contains two parallel chains of magnetosomes) (Bazylinski et al., 1993b; Bazylinski et al., 1995). In cells of this uncultured organism, the magnetite and greigite crystals occur with different, mineral-specific morphologies and sizes and are positioned with their long axes oriented along the chain direction. Both particle morphologies have been found in organisms with single mineral component chains (Mann et al., 1987a; Mann et al., 1987b; Heywood et al., 1990; Heywood et al., 1991), which suggests that the magnetosome membranes surrounding the magnetite and greigite particles contain different nucleation templates and that there are differences in magnetosome vesicle biosynthesis. Thus, it seems likely that two separate sets of genes control the biomineralization of magnetite and greigite in this organism.

Phylogeny

The phylogeny of many morphotypes of magnetotactic bacteria, including both those in pure culture and those collected from natural environments, has been determined by sequencing their 16S rRNA genes. To date, representatives of the magnetotactic prokaryotes are phylogenetically associated with three major lineages within the Bacteria. Although most are located within the *Proteobacteria*, "*Magnetobacterium bavaricum*" is affiliated with another phylum, the newly designated *Nitrospira* group. Those within the *Proteobacteria* are distributed among the delta- and alpha-subclasses. The uncultured greigite-producing, MMP and the magnetite-producing, sulfate-reducing magnetotactic bacterium RS-1, which is available in pure culture, are located in the delta-subclass, whereas members of the genus *Magnetospirillum* and various vibrios and coccoid magnetotactic bacteria, all of which produce magnetite, belong to the alpha-subclass (Fig. 15). Although these results suggest that the trait of magnetotaxis in bacteria has multiple evolutionary origins (DeLong et al., 1993), it is also possible that the ability of magnetosome

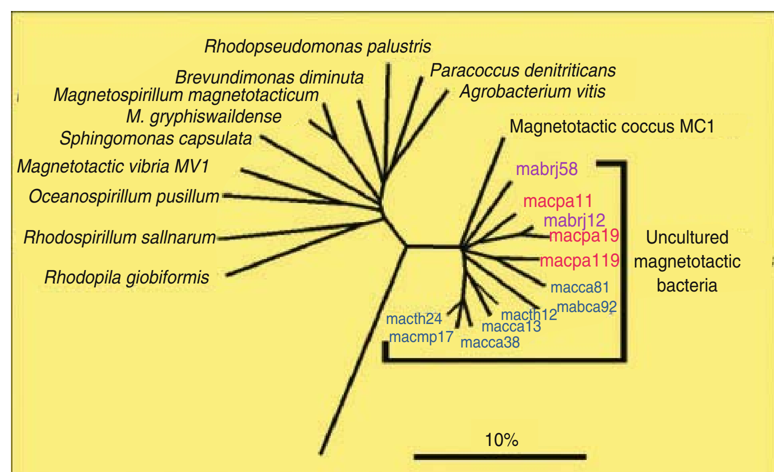

Fig. 15. Phylogenetic tree based on 16S rRNA sequences showing the positions of cultured and uncultured magnetotactic bacteria within the alpha-subclass of *Proteobacteria*. Sequences of uncultured magnetotactic bacteria retrieved from freshwater habitats are in blue, from marine habitats in red, and from a lagoon in pink.

formation was spread among various phylogenetic groups of bacteria and even eukaryotes by lateral gene transfer.

To date, most of the 16S rRNA sequences of magnetotactic bacteria retrieved from environmental samples form a deep-branching group within the alpha-subclass (Fig. 15). This phylogenetic assemblage consists (up to now) exclusively of bacteria displaying magnetotaxis. Similarity values of 16S rRNA sequences within this monophyletic group of magnetotactic bacteria range from 88.0 to 99.3%. Using in situ hybridization with fluorescently-labeled oligonucleotide probes, it was demonstrated that members of this coherent phylogenetic cluster represent the dominant fraction of magnetotactic bacteria in many environments like lagoons, marine and freshwater sediments (Spring et al., 1992; Spring et al., 1994; Spring et al., 1998). Magnetotactic bacterial morphotypes in this group, as evidenced by in situ hybridization, are mainly represented by coccoid to ovoid bacteria, but also include one rod- to vibrio-shaped bacterium (mabcs92; Fig. 15). Despite continuous effort in several laboratories, most members of this group have resisted attempts (in several laboratories) at isolation to axenic culture. One major reason may be their adaptation to and requirement for gradient systems not easily replicated in synthetic growth media. The only exception is the marine magnetotactic coccus strain MC-1, which can be cultivated in a synthetic oxygen gradient medium.

Cultivation and Physiology

The Genus *Magnetospirillum*

TAXONOMY Magnetospirilla are found in freshwater habitats where they usually occur in low numbers as, for example, compared with the magnetotactic cocci. These clockwise spirilla have dimensions of 0.2–0.7 by 1–20 μm and display an axial magneto-aerotaxis, at least when grown in liquid culture. The genus *Magnetospirillum* currently comprises the two validly described species, *M. magnetotacticum* and *M. gryphiswaldense*, and several partially characterized strains. *M. magnetotacticum* was the first magnetotactic bacterium isolated and grown in pure culture and was originally assigned to the genus *Aquaspirillum* based on a number of phenotypic characteristics (Maratea and Blakemore, 1981). At that time, this genus contained a large number of phylogenetically diverse, nonphototrophic, freshwater spirilla with the type species *A. serpens* phylogenetically located among the beta-subclass of the *Proteobacteria*. Phylogenetic analyses of *Magnetospirillum* strains later revealed that they all belong to a phylogenetic branch within the alpha-subclass of the *Proteobacteria* and are closely related to phototrophic spirilla of the genus Phaeospirillum (Fig. 15; Burgess et al., 1993; Schüler et al., 1999). Therefore, it was justified to propose the new genus *Magnetospirillum* for these strains (Schleifer et al., 1991). Members of this genus can be distinguished from other freshwater spirilla by their ability to produce membrane-enveloped cubo-octahedral magnetite crystals, averaging about 42 nm in diameter (Balkwill et al., 1980; Schleifer et al., 1991), arranged in a single chain within the cell. Other characteristic traits of members of this genus include bipolar monotrichous flagellation and a preference for microoxic growth conditions. Several strains of magnetospirilla can grow also anaerobically with nitrate as terminal electron acceptor or aerobically with atmospheric concentrations of oxygen. Magnetite synthesis appears to only occur under microaerobic conditions in most species while *Magnetospirillum* strain AMB-1 appears to synthesize magnetite under anaerobic conditions as well (Matsunaga and Tsujimura, 1993). Preferred substrates are intermediates of the tricarboxylic acid cycle and acetate. Carbohydrates are not utilized. Catalase and oxidase may be present or not. The guanine-plus-cytosine content of DNA ranges from 64 to 71 mol% (Burgess et al., 1993).

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF MAGNETOSOME FORMATION There has been much interest in the elucidation of magnetosome formation because the crystals synthesized by magnetotactic bacteria are of great structural perfection, have consistent particle morphologies and narrow size distributions, possible indications that the particles may have novel magnetic, physical and/or electrical properties. Understanding the factors controlling the biomineralization of iron in magnetosome synthesis within bacteria could also be helpful for the elucidation of similar processes in animals and man or for the artificial synthesis of biominerals. Despite the dedicated and elaborate efforts in studying magnetosome synthesis in bacteria, published results are rather sparse. This is partly due to the lack of a significant number of magnetotactic bacteria strains and the difficulty in culturing them reproducibly in the laboratory, which would be a prerequisite for the establishing of biochemical or genetic model systems. Nevertheless, some interesting results have been obtained using the few available, but fastidious *Magnetospirillum* strains.

In general, the bacterial magnetite synthesis can be divided into three steps. Initially, extracellular iron has to be transported across the cell wall to the inside of the cell. Once within the cell, iron must accumulate in specialized compart-

ments, the magnetosome vesicles. There, the iron presumably precipitates and transforms or grows into a single-magnetic-domain magnetite crystal with a specific morphology. It is assumed that the membrane vesicle is synthesized prior to the precipitation of iron but since there is currently little evidence to support this idea, it is possible that the precipitation of iron and crystal nucleation occurs first and the magnetosome membrane then forms around the growing crystal. The uptake of iron from the surrounding environment by cells of *Magnetospirillum* strains has been analyzed by several groups (Paoletti and Blakemore, 1986; Nakamura et al., 1993b; Schüler and Baeuerlein, 1996; Schüler and Baeuerlein, 1998). Generally, the results suggest that iron is taken up by the cell in the ferric form and transported across the membrane by an energy-dependent reductive process. Iron-binding siderophores were thought to be involved in iron uptake by *M. magnetotacticum* (Paoletti and Blakemore, 1986), which appeared to produce a hydroxamate siderophore under high, but not low, iron conditions. However, this finding was never confirmed by other laboratories. Spent culture fluid stimulates the uptake of ferric iron in *M. gryphiswaldense* although there was no evidence for the production of a siderophore by this species. This stimulation may be due to the production of unknown compounds, produced by cells during growth, which mediate iron uptake by an unrecognized novel mechanism (Schüler and Baeuerlein, 1996). In this respect, it is noteworthy that most magnetotactic bacteria are adapted to microenvironments, like the oxic-anoxic transition zone of sediments, where soluble iron is available to the cell in sufficient quantities for magnetite synthesis (generally about 10–20 μM iron; Blakemore et al., 1979). Thus, magnetotactic bacteria probably have no need for high-affinity transport systems like many other aerobic bacteria growing under iron deficient conditions. This is consistent with experiments performed with cells of *M. gryphiswaldense*. Under iron deficient conditions, cells of *M. gryphiswaldense* do not or cannot distinguish between the use of incorporated iron either as a cofactor for cellular proteins or for magnetite synthesis and store this essential element as an inorganic mineral, magnetite, at the expense of their own growth (Schüler and Baeuerlein, 1996). The marine magnetotactic vibrio, strain MV-1, behaves similarly.

The fate of iron taken up by cells was studied by Frankel et al. (1983) in *M. magnetotacticum* using Fe^{57} Moessbauer spectroscopy. It was proposed that the ferrous iron taken up by cells is immediately reoxidized to form a low-density hydrous Fe(III) oxide. It is not yet clear if this step takes place in the cytoplasm or in the magnetosome vesicles. How iron is transported from the cell membrane into the magnetosome vesicle is also not known. Iron is precipitated within the magnetosome vesicle presumably through a dehydration step as ferrihydrite (a high-density Fe(III) hydroxide). Finally, magnetite (Fe_3O_4) is produced by the reduction of one-third of the Fe(III) ions in ferrihydrite and further dehydration steps. The crystallization process(es) involved in magnetite formation is apparently linked closely to the magnetosome membrane and may be controlled by specific proteins present in this membrane. The chemical transformation of amorphous Fe(III) precursors to crystalline magnetite is sensitive to environmental conditions like ion concentration, pH and redox potential (Mann et al., 1990c) which have to be therefore precisely regulated by the magnetosome membrane or by conditions within the magnetosome membrane vesicle. Growth of the magnetite crystal, i.e., its orientation, shape and size, must also be under strict control because these characteristics are specific for one strain and/or species of bacteria and to a great extent independent from the growth conditions (Bazylinski et al., 1994).

Because the magnetosome membrane seems to play a key role in the synthesis of magnetite crystals, its structure and composition has been analyzed in several studies. By analyzing the magnetosome membrane in this way, some clues relating to how magnetite biomineralization occurs within the cell may be found. Gorby et al. (1988) showed that the magnetosome membrane in *M. magnetotacticum* has an architecture similar to that of the cytoplasmic membrane and consists of a lipid bilayer and numerous proteins, some of which appear to be unique to the magnetosome membrane. Okuda et al. (1996) found three proteins with molecular weights of 12, 22 and 28 kDa, specifically associated with the magnetosome membrane in *M. magnetotacticum*. They successfully identified and sequenced the gene encoding for the 22 kDa protein, which was found to belong to a family of protein import receptors common in mitochondria and peroxisomes. The role of this protein in magnetosome synthesis remains unclear however. A gene likely involved in magnetite synthesis was identified and characterized by Matsunaga et al. (1992). They used a genetic approach using the microorganism *Magnetospirillum* strain AMB-1, which forms colonies of magnetite-forming cells on agar surfaces, thereby facilitating the screening for nonmagnetic mutants. The gene, designated *magA*, encodes for a membrane protein showing sequence similarities to some cation efflux proteins. Based on experiments with the recombinant protein, it was proposed that the MagA

protein plays a role in the energy-dependent transport of iron across membranes.

ENRICHMENT AND ISOLATION Magnetotactic spirilla have been repeatedly isolated from various freshwater habitats, so that it is possible to give some guidelines for their succesful enrichment and isolation.

Several morphotypes of magnetotactic bacteria can be enriched in the laboratory by putting mud and overlying water from a sampling site into aquaria or jars, which are loosely covered and stored in dim light. After several days to weeks, the number of magnetotactic bacterial cells generally increases significantly. Magnetotactic spirilla, however, are in most cases not among the dominating morphotypes and therefore are only rarely detected using light microscopy. Consequently, the usefulness of this method for the enrichment of representatives of the genus *Magnetospirillum* remains questionable. To date, no selective growth media are known for the cultivation of magnetotactic spirilla, so that a successful isolation procedure will in most cases depend on the purity of the inoculum. Because of the magnetic dipole moment of these bacteria, physical separation from nonmagnetotactic contaminants is possible. A commonly used method for the separation of magnetotactic bacteria from sediment samples was described by Moench and Konetzka (1978). They concentrated magnetotactic bacterial cells using a bar magnet (e.g., stirring bar) fixed to the outer wall of a jar filled with sediment and water. Directed magnetotactic bacteria eventually accumulate at the side of the jar and become concentrated enough to form (opposite to the magnet) a brownish spot from where they can be transferred into a sterile cap using a Pasteur pipette. The sample containing the concentrated magnetotactic cells still cotains non-magnetotactic bacteria, so that a further purification step is advisable before it is used as an inoculum for growth media. The "capillary racetrack" devised by Spormann and Wolfe (Wolfe et al., 1987) has been successfully used for this purpose (Schüler et al., 1999).

All isolated strains of magnetospirilla, with the possible exception of *Magnetospirillum* strain AMB-1, appear to prefer low oxygen tensions for growth and magnetite synthesis. Thus the creation and maintenance of microoxic conditions in growth media is especially important for the isolation of these organisms starting from small inocula. The growth medium should contain 10–20% of sterilized mud or water from the respective habitat and low concentrations of agar to allow the establishment of a semisolid oxygen gradient. Suitable carbon sources are intermediates of the tricarboxylic acid cycle, e.g., malate or succinate. An oxygen-sulfide gradient medium was successfully used by Schüler et al. (1999) for the effective isolation of magnetotactic spirilla from a freshwater pond. Screw-capped culture tubes are filled with 1 ml of solid sulfide agar (4 mM Na_2S, 1.5% agar, pH 7.4) and overlaid with 10 ml of slush-agar. The slush-agar consists of (per 800 ml deionized water): 200 ml of filtered pond water, 1 ml of vitamin elixir, 2 ml of mineral elixir (Wolin et al., 1963), 0.05 g, sodium succinate, 0.05 g, yeast extract, 0.05 g, NH_4Cl, 0.05 g, $MgSO_4$, 0.5 mM potassium phosphate buffer (pH 7.0); 2 mg of resazurin and 2 g of agar. After adjusting the pH to 7.0 and autoclaving, sterile solutions of ferric citrate and neutralized cysteineHCl are added (final concentrations 10 μM and 0.01%, respectively). The culture tubes can be inoculated after the establishment of sulfide and oxygen gradients within the medium, which takes about 24 hours. Several days to weeks of incubation at room temperature in the dark may be required until growth becomes apparent, usually as fluffy pinpoint colonies.

Although the original inoculum always contains various types of magnetotactic bacteria, in most cases only magnetotactic spirilla grow and are eventually isolated in pure culture. Modifications of this medium may eventually prove useful for the isolation of hitherto uncultured types of magnetotactic bacteria.

Following isolation, most strains of magnetospirilla can be cultured in liquid media without added water or mud from the sampling site. However, the gas composition of the headspace of the cultures is crucial for good growth and magnetite synthesis by most species. The maximum concentration of oxygen allowing growth and/or magnetite synthesis differs among the described *Magnetospirillum* strains. *M. magnetotacticum* grows optimally and produces the highest number of magnetosomes at an oxygen tension of 1% in the headspace and tolerates higher initial oxygen concentrations only if a large number of cells is inoculated into the growth medium. In contrast, *Magnetospirillum* strain AMB-1 grows (but does not produce magnetosomes) aerobically under atmospheric concentrations of oxygen (approximately 21% O_2; Matsunaga et al., 1991b). Magnetite synthesis is inhibited by all *Magnetospirillum* strains when cells are cultured under oxygen concentrations above 2 to 6% (Blakemore et al., 1985; Schüler and Baeuerlein, 1998).

Strain MV-1, a Facultatively Anaerobic Magnetotactic Vibrio

A marine magnetotactic vibrioid to helicoid bacterium, strain MV-1, was isolated by Bazylinski et al. (1988). Cells of strain MV-1 are small, rang-

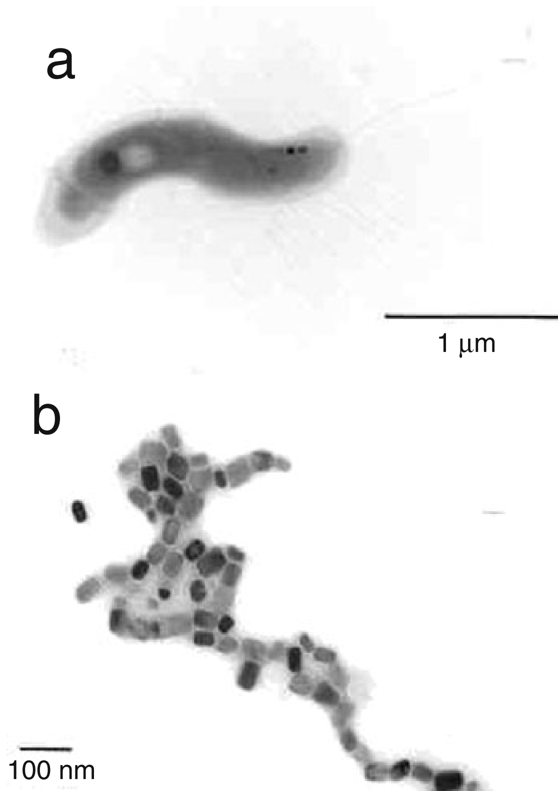

Fig. 16. Brightfield TEM image of negatively stained cell and magnetosomes of strain MV-1. (a) Cell stained with uranyl acetate showing a single polar flagellum and a chain of magnetite-containing magnetosomes. (b) Preparation of purified magnetosomes from strain MV-1 stained with 2% aqueous sodium phosphotungstate, pH 7.0. The "magnetosome membrane" is visualized as an electron-lucent area surrounding each individual crystal and is easily removed with detergents such as sodium deodecyl sulfate.

ing from 1–5 μm by 0.2–0.5 μm, and possess a single, unsheathed, polar flagellum (Fig. 16a). Cells grow and synthesize pseudohexahedral prismatic crystals of magnetite, averaging 53 by 35 nm in size (Fig. 16b; Sparks et al., 1990), in their magnetosomes microaerobically and anaerobically, with nitrous oxide as the terminal electron acceptor. Cells appear to produce more magnetite under anaerobic conditions than under microaerobic conditions (Bazylinski et al., 1988) and, like *M. magnetotacticum*, synthesize a number of magnetosome membrane proteins that are not present in other cellular fractions (Dubbels et al., 1998). A stable, spontaneous nonmagnetotactic mutant strain of MV-1 that does not produce magnetosomes has recently been isolated and partially characterized (Dubbels and Bazylinski, 1998).

Strain MV-1 is nutritionally versatile being able to grow chemoorganoheterotrophically with organic and some amino acids as carbon and energy sources, and chemolithoautotrophically with thiosulfate or sulfide as energy sources oxidizing them to sulfate, and carbon dioxide as the sole carbon source (Kimble and Bazylinski, 1996). Cells produce intracellular sulfur deposits when grown with sulfide (Kimble and Bazylinski, 1996). As do virtually all aerobic chemolithoautotrophic bacteria, strain MV-1 uses the Calvin-Benson cycle for autotrophic carbon dioxide fixation (McFadden and Shively, 1991). Cell-free extracts from thiosulfate-grown cells of strain MV-1 show ribulose bisphosphate carboxylase/oxygenase (rubisCO) activity (Kimble and Bazylinski, 1996), and recently (Dean and Bazylinski, 1999a) the gene for a form II rubisCO enzyme (*cbbM*) was cloned and sequenced from strain MV-1. There was no evidence for a *cbbL* gene (encodes for form I rubisCO enzymes) in DNA hybridization analyses despite using *cbbL* gene probes from several different organisms. Because many uncultured magnetotactic bacteria collected from natural habitats thrive in oxygen-sulfide inverse gradients, as previously mentioned, and contain internal sulfur deposits (Moench, 1988; Spring et al., 1993; Frankel and Bazylinski, 1994; Iida and Akai, 1996; Kimble and Bazylinski, 1996), it seems many species are likely chemolithoautotrophs that obtain energy from the oxidation of sulfide and perhaps other reduced sulfur sompounds. Using pulsed-field gel electrophoresis (PFGE), the genome of strain MV-1 was found to consist of a single, circular chromosome of approximately 3.7 Mb (Dean and Bazylinski, 1999b). There was no evidence of linear chromosomes or extrachromosomal DNA such as plasmids. The guanine-plus-cytosine content of the DNA of this strain is 52.9 mol% as determined by HPLC and 53.5 mol% by T_m.

A virtually identical strain to strain MV-1, designated MV-2, was isolated from the Pettaquamscutt Estuary (DeLong et al., 1993; Meldrum et al., 1993b). Cells of this strain produce the same morphological type of magnetite crystals as strain MV-1 (Meldrum et al., 1993b) and display many of the same phenotypic traits as strain MV-1 (such as anaerobic growth with nitrous oxide as a terminal electron acceptor, heterotrophic growth with organic and amino acids, and chemolithoautotrophic growth on reduced sulfur compounds). However, strain MV-2 shows slightly different restriction fragment patterns in pulsed-field gels than strain MV-1 using the same restriction enzymes (Dean and Bazylinski, 1999b). As with strain MV-1, the genome of strain MV-2 consists of a single, circular chromosome of a similar size, about 3.6 Mb (Dean and Bazylinski, 1999b). The guanine-plus-cytosine content of the DNA of this strain is 56.2 mol% as determined by HPLC and 56.6% by T_m.

Strain RS-1, a Sulfate-Reducing Magnetotactic Bacterium

It was thought for a long time that all magnetotactic bacteria are obligate or facultative microaerophiles (*Magnetospirillum* strain AMB-1 and the marine vibrio, strain MV-1, grow anaerobically with nitrate and nitrous oxide, respectively, as well as with oxygen) adapted to the microoxic zone of their environment. With the isolation of an obligately anaerobic strain from a sulfidic freshwater habitat by Sakaguchi et al. (1993), this assumption is clearly incorrect. Cells of this organism, designated strain RS-1, are 0.9–1.5 by 3–5 µm with a helicoid to rod-shaped morphology and possess a single polar flagellum. They exhibit an axial magnetotaxis coupled with a strong anaerotaxis reflecting their obligate anaerobic metabolism. According to the revised model of magnetotaxis, they may have developed an axial magnetotaxis because they do not have to oscillate between microoxic and anoxic zones of their habitat, which would select for polar magnetotaxis.

Strain RS-1 is a dissimilatory sulfate-reducing, chemoorganoheterotrophic bacterium that utilizes a variety of organic substrates, e.g., pyruvate, lactate, ethanol and fumarate). Cells can use sulfate or fumarate as electron acceptor but not oxygen. They are catalase positive and oxidase negative. The guanine-plus-cytosine content of DNA was determined by HPLC to be 66 mol%.

Sequencing of the 16S rRNA gene of strain RS-1 showed that it is phylogenetically affiliated to the delta-subclass of the *Proteobacteria* (Kawaguchi et al., 1995). The nearest neighbors in a phylogenetic tree are members of the genus *Desulfovibrio*, typical representatives of the obligately anaerobic, dissimilatory sulfate-reducing bacteria. In contrast to Desulfovibrio sp., cells of strain RS-1 are able to produce intracellular bean-shaped crystals of magnetite, responsible for its magnetotactic response. Consequently, the production of magnetosomes consisting of magnetite is found in bacteria belonging to three different phylogenetic groups, viz. the α- and δ-subclasses of *Proteobacteria* and the *Nitrospira* group ("*Magnetobacterium bavaricum*"), indicating multiple evolutionary origins of intracellular magnetite synthesis or lateral gene transfer between different phylogenetic groups.

Other Magnetotactic Strains in Pure Culture

Several other pure cultures of magnetotactic bacteria exist, but they appear to be obligate microaerophiles and grow poorly (D. A. Bazylinski, unpublished results). Hence, very little is known about them. Strain MC-1 (Fig. 17), a marine biliophotrichous coccus, was isolated from water collected from the Pettaquamscutt Estuary, a chemically-stratified semi-anaerobic basin in Rhode Island, USA. Cells of this strain produce pseudohexahedral prisms of magnetite, averaging 72 by 70 nm in size (when grown autotrophically), and grow chemolithoautotrophically with thiosulfate or sulfide as an electron and energy source (Meldrum et al., 1993a; Frankel et al., 1997). Cells may also be able to grow chemoorganoheterotrophically. Like all magnetotactic cocci observed, cells of strain MC-1 show polar magneto-aerotaxis regardless of whether they are grown in liquid or semi-solid oxygen gradient media. This strain has a genome size of approximately 4.5 Mb as determined by pulsed-field gel electrophoresis (Dean and Bazylinski, 1999b). The guanine-plus-cytosine content of the DNA of strain MC-1, as determined by HPLC, is 55.8 mol%. This organism has not been completely characterized and described.

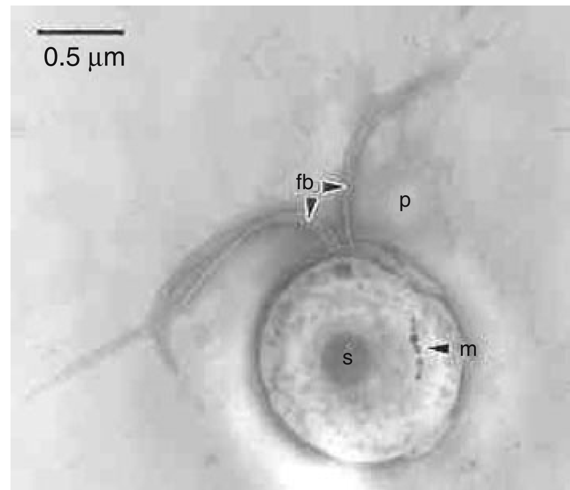

Fig. 17. Brightfield TEM image of a cell of the bilophotrichously flagellated marine coccus strain MC-1 negatively stained with uranyl acetate. Note the two flagellar bundles (fb), the presence of pili (p), sulfur globules(s), and chain of Fe_3O_4-containing magnetosomes (m).

Strain MV-4 (Fig. 18), a small marine spirillum, was isolated from sulfide-rich mud and water collected from School Street Marsh, Woods Hole, Massachusetts, USA. Cells of this strain produce elongated octahedrons of magnetite, averaging 61 by 52 nm in size, and grow chemolithoautotrophically with thiosulfate or chemoorganoheterotrophically with succinate (Meldrum et al., 1993b). Unlike most freshwater magnetotactic spirilla, this strain shows polar magneto-aerotaxis at least when grown in semi-solid oxygen gradient media. Like strain MC-1,

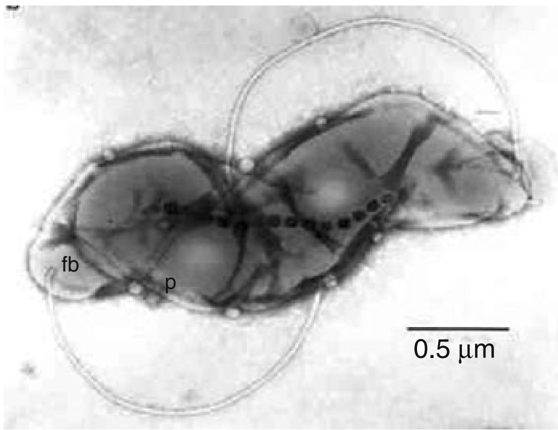

Fig. 18. Brightfield TEM image of a cell of the marine spirillum strain MV-4 negatively stained with uranyl acetate showing bipolar flagellation and a chain of Fe_3O_4-containing magnetosomes.

this strain has not been completely characterized and described.

Biotechnological Applications

It was not long after the discovery of magnetotactic bacteria that publications of physical studies and of commercial and medical applications involving the magnetotactic cells, isolated magnetosomes and/or magnetite crystals began to appear. It is clear that magnetotactic bacterial cells and their magnetic crystals have novel physical, magnetic and possibly electrical properties. In addition, in certain types of applications, bacterial magnetite offers several advantages compared to chemically synthesized magnetite. Bacterial magnetosome particles, unlike those produced chemically, have a consistent shape, a narrow size distribution within the single magnetic domain range, and a membrane coating consisting of lipids and proteins. The magnetosome envelope allows for easy couplings of bioactive substances to its surface, a characteristic important for many applications.

Magnetotactic bacterial cells have been used to determine south magnetic poles in meteorites and rocks containing fine-grained magnetic minerals (Funaki et al., 1989; Funaki et al., 1992) and for the separation of cells after the introduction of magnetotactic bacterial cells into granulocytes and monocytes by phagocytosis (Matsunaga et al., 1989). Magnetotactic bacterial magnetite crystals have been used in studies of magnetic domain analysis (Futschik et al., 1989) and in many commercial applications including: the immobilization of enzymes (Matsunaga and Kamiya, 1987); the formation of magnetic antibodies in various fluoroimmunoassays (Matsunaga et al., 1990) involving the detection of allergens (Nakamura and Matsunaga, 1993a) and squamous cell carcinoma cells (Matsunaga, 1991a), and the quantification of IgG (Nakamura et al., 1991); the detection and removal of *Escherichia coli* cells with a fluorescein isothiocyanate conjugated monoclonal antibody, immobilized on magnetotactic bacterial magnetite particles (Nakamura et al., 1993c); and the introduction of genes into cells, a technology in which magnetosomes are coated with DNA and "shot" using a particle gun into cells that are difficult to transform using more standard methods (Matsunaga, 1991a). Unfortunately, the prerequisite for any large scale commercial application is mass cultivation of magnetotactic bacteria or the introduction and expression of the genes responsible for magnetosome synthesis into a bacterium, e.g., *E. coli*, that can be grown relatively cheaply to extremely large yields. Although some progress has been made, the former has not been achieved with the available pure cultures.

Acknowledgements. We thank F.C. Meldrum, M. Pósfai, P.R. Buseck, and M. Hanzlik for use of figures and L. Cox, D. Schüler and R.B. Frankel for helpful discussions and suggestions concerning this chapter. S.S. is grateful to K.-H. Schleifer and the Deutsche Forschungsgemeinschaft (DFG) for continuous support. Research in the laboratory of D.A.B. is supported by U.S. National Science Foundation grant CHE-9714101 and U.S. National Aeronautics and Space Administration grant NAG 9-1115.

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Prokaryotes (2006) 2:863–892
DOI: 10.1007/0-387-30742-7_27

CHAPTER 1.27

Luminous Bacteria

PAUL V. DUNLAP AND KUMIKO KITA-TSUKAMOTO

Introduction and Historical Perspective

"The smallest lamps in the world, luminous bacteria, are no different from ordinary bacteria except in their ability to luminesce."
—E. N. Harvey, 1940

The luminous bacteria are those bacteria that contain naturally acquired genes for light production, the *lux* genes. Most currently known luminous bacteria express the *lux* genes at high levels in laboratory culture (Fig. 1) or in nature, leading to the emission of easily visible levels of light. Bacterial light production is one of several biochemically distinct types of bioluminescence (Hastings, 1995).

The existence of bacterial luminescence and of many of the luminous bacteria themselves has been known for some time. During the 1700s and 1800s, various animal products (such as meats, fish and eggs), the decaying bodies of marine and terrestrial animals, and even human wounds and corpses, were reported to produce light (Harvey, 1940; Harvey, 1952). Many years before those observations and long before bacteria were known to exist, Boyle (1668) demonstrated that the "uncertain shining of Fish," the light coming from decaying fish, required air. Indeed, encounters with luminous objects and substances extend back to the beginnings of recorded history in Greece and China (Harvey, 1957), and they continue in modern times to be causes of concern and wonder. Many of these encounters can be attributed to the saprophytic or pathogenic growth of luminous bacteria on marine and terrestrial animals. According to Harvey (1940), J. F. Heller in 1854 was the first to give a name, *Sarcina noctiluca*, to the suspected responsible organism. As the science of bacteriology developed during the period from 1860 through 1910, individual types of light-producing bacteria were grown and distiguished from other bacteria, notably through the work of Bernhard Fischer (Fischer, 1887) and Martin Beijerinck (Beijerinck, 1889), among many others (Zobell, 1946; Harvey, 1952; Harvey, 1957). During the first half of the 20th century, luminous bacteria were isolated from various habitats, the chemistry of bacterial light production, and culture requirements for growth and luminescence were characterized, and they were placed in the evolving system of microbial taxonomy (e.g., Zobell and Upham, 1944; Farghaly, 1950; Johnson, 1951). In the latter half of the 20th century, those efforts paralleled the growth of microbiology, incorporating the tools and knowledge developing from advances in biochemistry, physiology and genetics (Baumann and Baumann, 1977; Baumann and Baumann, 1981; Farmer and Hickman-Farmer, 1992; Hastings and Nealson, 1977; Hastings and Nealson, 1981; Hendrie et al., 1970; Nealson and Hastings, 1992; Singleton and Skerman, 1973). Much has been learned during the past 50 years about the enzymes and genes involved in bacterial light production and about the phylogeny and ecology of light-emitting bacteria. However, much remains to be learned about these topics and about the evolutionary origins and cellular functions of bacterial luminescence.

In the past, the luminous bacteria were often considered to be a separate microbial group, distinguished by their distinctive and unifying phenotype, the production of light. They are seen now more properly as representative prokaryotes with much to reveal about the fundamental biology of bacteria. This view develops from (and is supported by) a deepening understanding of phylogenetic relationships and the realization that these bacteria are metabolically similar to other well-established bacteria (Baumann and Baumann, 1981; Baumann and Schubert, 1984a; Baumann et al., 1984b). Indeed, light-emission is a biochemical trait shared by several but not all species and strains of the genera *Vibrio*, *Photobacterium*, *Shewanella* and *Photorhabdus*. As members of these genera, the luminous bacteria are for the most part typical Gram negative bacteria similar in fundamental ways to terrestrial enterobacteria (Baumann and Baumann, 1977). They occur together with closely related nonluminous types in many habitats, responding in the same metabolic and physiological ways as other bacteria, and carrying out with them

Fig. 1. Bacterial luminescence. Colonies of the luminous marine bacterium *V. fischeri* growing on a seawater-based complete medium photographed by the light they produce. From Meighen and Dunlap (1993).

ecologically important activities unrelated to luminescence.

Supporting this view is a growing appreciation that light production in luminous bacteria is tightly integrated with cellular metabolism and global gene regulation (Ulitzur and Dunlap, 1995; Callahan and Dunlap, 2000). Light production is sensitive to the physiological state of the cell, and expression of the *lux* genes, along with many other sets of genes of diverse functions, is coordinately regulated in response to that state. Therefore, despite its phenotypic distinctiveness, luminescence is not an independent biochemical activity of the cell; it is instead an integral feature of the biology of these bacteria. Studies of luminescence therefore are likely to reveal basic processes in Gram-negative prokaryotes.

An example of how bacterial luminescence can lead to insights of fundamental importance in microbiology is quorum sensing. Previously called "autoinduction" and studied as the special cell density-dependent mechanism by which luminous bacteria control light production, quorum sensing has now been identified in many nonluminous Gram-negative bacteria, including several pathogens of animals and plants (Fuqua et al., 1996; Greenberg, 1997; Dunlap, 1997; Swift et al., 1999; Hastings and Greenberg, 1999). Early studies of luminous bacteria would not have led to predictions that autoinduction of luminescence would become a new bacterial regulatory paradigm (Nealson, 1999). However, the multitude of scientific advances and research opportunities opened up by the revelations of quorum-sensing control of luminescence suggest that studying luminous bacteria (as representative Gram-negative prokaryotes) and luminescence (as an integral aspect of their biology) will continue to reveal insights and themes of biological importance.

Biochemistry of Bacterial Light Production

Light emission in bacteria is catalyzed by luciferase, a heterodimeric protein of approximately 80 kD, composed of α (40-kDa) and β (37-kDa) subunits. Bacterial luciferase mediates the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde (RCHO) by molecular oxygen (O_2) to produce blue-green light (Fig. 1) according to the following reaction.

$$FMNH_2 + O_2 + RCHO \xrightarrow{luciferase} FMN + H_2O + RCOOH + h\nu(490\ \text{nm})$$

In the luminescence reaction, binding of $FMNH_2$ by the enzyme is followed by interaction with O_2 to form a luciferase-bound 4a-peroxyflavin. Association of this complex with aldehyde forms a highly stable intermediate, the slow decay of which results in oxidation of the $FMNH_2$ and aldehyde substrates and the emission of light. Quantum yield for the reaction has been estimated at 0.1 to 1.0 photons. The reaction is highly specific for $FMNH_2$, and the aldehyde substrate in vivo is likely to be tetradecanal. Synthesis of the long-chain aldehyde is catalyzed by a fatty-acid reductase complex composed of three polypeptides, an NADPH-dependent acyl protein reductase (called "r," 54 kDa), an acyl transferase ("t," 33 kDa), and an ATP-dependent synthetase ("s," 42 kDa). The complex has a stoichiometry of $r_4s_4t_{2\text{-}4}$, and its activity is essential for the production of light in the absence of exogenously added aldehyde. The genes *luxA* and *luxB* for the α and β luciferase subunits and *luxC*, *luxD* and *luxE* for the r, s and t polypeptides of the fatty-acid reductase, respectively, are contiguous and coordinately expressed in all luminous bacteria examined to date. Furthermore, as described in a later section, luciferases from different species of luminous bacteria exhibit substantial sequence identity, consistent with a common evolutionary origin. For references and detailed information on the biochemistry of bacterial light production, the reader is directed to reviews by Hastings (1995), Hastings et al. (1985), Meighen (Meighen, 1988; Meighen, 1991) and Meighen and Dunlap (1993).

Species and Phylogeny of Luminous Bacteria

The currently known luminous bacteria are members of the genera *Vibrio*, *Photobacterium*, *Shewanella* and *Photorhabdus* (Table 1). These bacteria are Gram-negative γ-Proteobacteria, nonsporulating, chemoorganotrophic heterotrophs, most of which are facultatively aerobic. Two of the marine luminous bacteria, however, *Shewanella hanedai* (Jensen et al., 1980) and *Shewanella woodyi* (Makemson et al., 1997), differ from the other luminous bacteria in using only a respiratory mode of metabolism. Detailed information on the metabolism, physiology and morphology of these bacterial groups and individual species can be found in Baumann and Baumann (1981), Baumann et al. (1984b), Farmer and Hickman-Brenner (1992), Boemare et al. (1993) and Forst et al. (1997). The luminous *Photobacterium* and *Shewanella* species and most of the luminous *Vibrio* species occur in the marine environment, whereas *Photorhabdus* species are terrestrial. *Vibrio cholerae* may be the only species with luminous strains occurring in brackish environments and freshwater.

Certain of the species listed in Table 1 were described in the late 1990s (Makemson et al., 1997; Fischer-Le Saux et al., 1999). Furthermore, luminous strains of species previously described as nonluminous are being found. Examples include *Vibrio salmonicida*, a pathogen of salmonid fish (Fidopastis et al., 1999) and intensely luminous strains of *Photobacterium angustum* isolated from the Sea of Cortez (K. Kita-Tsukamoto et al., manuscript in preparation). Indeed, light production does not define a phylogenetically exclusive or consistent grouping. The genera *Vibrio*, *Photobacterium* and *Shewanella* contain many nonluminous species. Even species characterized as luminous can contain strains that do not produce light and that lack the genes necessary for light production. An example is a strain of *Photorhabdus luminescens* symbiotic with entomopathogenic nematodes (Akhurst and Boemare, 1986; Forst and Nealson, 1996). Adding to this complexity, some species or strains carry the *lux* genes and produce a high level of light under natural conditions but produce little or no light when grown in laboratory culture. Examples include luminous bacteria infecting crustaceans (Giard and Billet, 1889b) and strains of *V. fischeri* symbiotic with the Hawaiian sepiolid squid, *Euprymna scolopes* (Boettcher and Ruby, 1990). Furthermore, many nonluminous strains of *V. cholerae* carry *lux* genes (Palmer and Colwell, 1991; Ramaiah et al., 2000). Relevant to the question of which species and strains of bacteria produce light is the observation that luminescence often is not phenotypically stable. Strains luminous on primary isolation often become dim or dark in laboratory culture (Nealson and Hastings, 1979b; Akhurst, 1980; Silverman et al., 1989; Nealson and Hastings, 1992). Therefore, it is reasonable to assume that luminescence has been overlooked in many species, especially those represented primarily by laboratory strains or those studied under clinical settings at temperatures where luminescence may not be produced. With environmental isolates and previously characterized species, the use of cooler temperatures (10–20°C) for growth and examination, utilization of conditioned media, inducers and luciferase substrates (Fidopiastis et al., 1999), and the application of probes for *luxA* and other *lux* genes (Wimpee et al., 1991) will undoubtedly reveal many more types of bacteria with the ability to produce light.

From the perspective of 16S rRNA sequence-based phylogeny, the luminous bactera are representative members of the γ-Proteobacteria, with luminous species in four genera (*Vibrio*, *Photobacterium*, *Photorhabdus* and *Shewanella*) within three families (Vibrionaceae, Enterobacteriaceae and Alteromonadaceae; Fig. 2). Genera containing species or strains of luminous bacteria are a small fraction of the162 genera in 20 families of the γ-Proteobacteria (see *Bergey's Manual of Systematic Bacteriology*, May 2001). Most of the luminous species are members of the Vibrionaceae (genera *Vibrio* and *Photobacterium*), which also contain many nonluminous species. Diverging from lineages within the Vibrionaceae are several uncultured luminous symbionts of anomalopid (flashlight) and ceratioid (deep-sea) anglerfish (Fig. 2). The anomalopid and ceratioid symbionts form separate monophyletic groups, and these symbionts are sufficiently divergent from known luminous bacteria to suggest they represent new species or genera within the Vibrionaceae (Haygood, 1990; Haygood, 1993a; Haygood and Distel, 1993b).

The intermingling of luminous and nonluminous species in the Vibrionaceae contrasts with the phylogenetic separateness of the luminous species within the Enterobacteriaceae and Alteromonadaceae (Fig. 2). Within these latter two families, the luminous species occur on branches that appear distal to other species. Placement of *Photorhabdus* in the Enterobacteriaceae, though generally accepted, is controversial (Janse and Smits, 1990; Rainey et al., 1995), however. Characteristics of *Photorhabdus* species not typical of members of the Enterobacteriaceae include luminescence, synthesis of yellow and red pigments and the inability to reduce nitrate (Farmer et al., 1989; Forst and Nealson, 1996).

Table 1. Luminous bacteria.

Species[a]	Representative habitats	Bioluminescent symbiosis	Selected references
Marine			
Vibrio			
fischeri	Temperate coastel seawater	Monocentrid fish, certain sepiolid squids	Boettcher and Ruby, 1990 Fitzgerald, 1977 Lee and Ruby, 1992 Reichelt and Baumann, 1973 Ruby and Nealson, 1976 Ruby and Nealson, 1978
harveyi	Temperate to tropical coastal seawater, sediment	—	O'Brien and Sixemore, 1979 Reichelt and Baumann, 1973 Ruby and Nealson, 1978 Yetinson and Shilo, 1979
logei	Coastal cold seawater, sediment, Arctic and Mediterranean	Certain sepiolid squids	Bang et al., 1978 Baross et al., 1978 Fidiopastis et al., 1998
mediterranea[b]	Coastal seawater	—	Ortiz-Conde et al., 1989
orientalis	Seawater, surfaces of shrimp	—	Yang et al., 1983
salmonicida	Tissue lesions of Atlantic salmon	—	Fidiopastis et al., 1999
splendidus	Coastal seawater, Persian Gulf	—	Nealson et al., 1993
vulnificus	Human blood and tissue, United States	—	Oliver et al., 1986
Unnamed	Not yet cultured	Anomalopid fish	Hygood, 1990 Wolfe and Haygood, 1991
Unnamed	Not yet cultured	Ceratioid fish	Haygood and Distel, 1993 Haygood et al., 1992
Photobacterium			
angustum	Seawater and fish intestines, Sea of Cortez	—	Kita-Tsukamoto et al. (in prep.)
leiognathi	Coastal temperate to tropical seawater leiognathid fish	Acropomatid, apogonid, Certain loligiroid squids	Fukasawa and Dunlap, 1986 Fukasawa et al., 1998 Herring and Morin, 1978 Reichelt et al., 1977
phosphoreum	Coastal and pelagic cold to temperate seawater	Opisthoproctid, chlorophthalmid, trachichthyid, morid, macrourid, steindachnerid fish	Herring and Morin, 1978 Haygood, 1993 Ruby and Morin, 1978 Wimpee et al., 1991
Shewanella			
hanedai	Cold seawater and sediment	—	Jensen et al., 1980
woodyi	Seawater and squid ink, Alboran Sea	—	Makemson et al., 1997
Brackish/Estuarine			
Vibrio cholerae	Temperate to tropical estuaries, bays coastal seawater	—	Palmer and Colwell, 1989 Ramaiah et al., 2000
Terrestrial			
Photorhabdus			
luminescens	Insect larvae infected with heterorhabditid nematodes	—[c]	Boemare et al., 1993 Fischer-Le Saux et al., 1999
temperata	Insect larvae infected with heterorhabditid nematodes	—[c]	Fischer-Le Saux et al., 1999
asymbiotica	Human skin lesions United States and Australia	—	Farmer et al., 1989 Fischer-Le Saux et al., 1999 Peel et al., 1999

[a]Luminous strains. For additional information, see Baumann and Baumann (1981); Farmer and Hickman-Brenner (1992); Hastings and Nealson (1981); and Nealson and Hastings (1992).
[b] Ability of this species to luminesce is not well established.
[c]Symbiotic with entomopathogenic nematodes; on anatomical and behavioral grounds not considered here to be equivalent to bioluminescent symbiosis in fishes and squids.

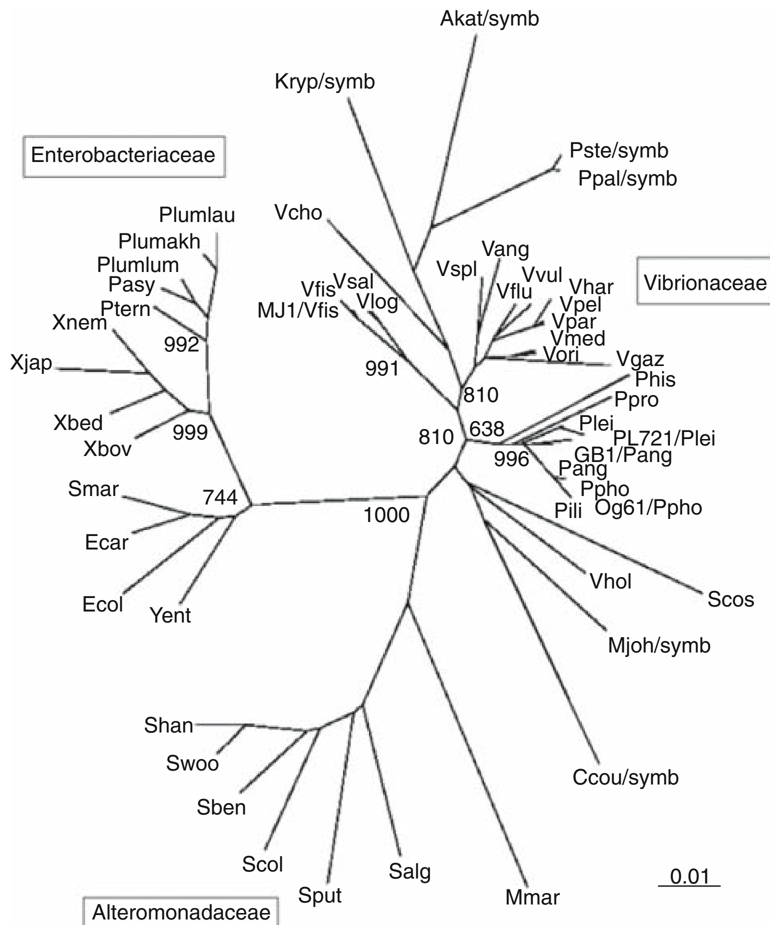


Fig. 2. 16S rDNA-based phylogenetic tree of luminous bacteria. Luminous species and strains are in boldface. Names are abbreviated as the first letter of the genus and the first three letters of species of the bacterium or its symbiotic host, along with the first three letters of the subspecies, where appropriate. See accession numbers as indicated for references. Included in the tree for comparison are sequences of species and strains related to luminous bacteria and sequences of uncultured symbiotic luminous bacteria. The neighbor-joining (NJ; Saitou and Nei, 1987) tree was constructed based on 1,163 unambiguously aligned positions. CLUSTAL W program (ver. 1.60; Thompson et al., 1994) was used for alignment of sequences and realigned manually using MacClade 3.05 (Maddison and Maddison, 1992). The NJ tree was developed from the distance matrix calculated by the algorithm of the Kimura two-parameter model (Kimura, 1980). Bootstrap analysis was done with 1,000 replicates. The scale bar represents 0.01 nucleotide substitutions per position. Alteromonadaceae: *Moritella marina* (T [type strain]), X82142; *Shewanella algae* (T), AF005249; *S. benthica* (T), X82131; *S. colwelliana* (T), AF170794; *S. hanedai* (T), X82132; *S. woodyi* (T), AF003549; and *S. putrefaciens* (T), X81623. Enterobacteriaceae: *Erwinia carotovora* subsp. carotovora (T), M59149; *Escherichia coli*, J01859; *Photorhabdus asymbiotica* (T), Z76755; *P. luminescens* subsp. *akhurstii* (T), AJ007359; *P. luminescens* subsp. *laumondii* (T), AJ007404; *P. luminescens* subsp. *luminescens* (T), X82248; *P. temperata* (T), AJ007405; *Serratia marcescens* (T), M59160; *Xenorhabdus beddingii* (T), X82254; *X. bovienii* (T), X82252; *X. japonicus* (T), Z76739; *X. nematophilus* (T), X82251; and *Yersinia enterocolitica* (T), M59292. Vibrionaceae: *Photobacterium angustum* (T), X74685; *P. angustum* GB-1 (K. Kita-Tsukamoto et al., manuscript in preparation); *P. histaminum*, D25308; *P. iliopiscarium* (T), AB000278; *P. leiognathi* (T), X74686; *P. leiognathi* PL-721, Z21730; *P. phosphoreum* (T), D25310; *P. phosphoreum* Og61, Z19107; *P. profundum*, AB003191; *Salinivibrio costicola* (T), X74699; *Vibrio anguillarum* (T), X16895; *V. cholerae*, X74694; *V. fischeri* (T), X74702; *V. fischeri* MJ-1, Z21729; *V. fluvialis* (T), X74703; *V. gazogenes* (T), X74705; *V. harveyi* (T), X74706; *V. hollisae* (T), X74707; *V. logei*, X74708; *V. mediterranei* (T), X74710; *V. orientalis* (T), X74719; *V. parahaemolyticus* (T), X74720; *V. pelagius* (T), X74722; *V. salmonicida* (T), X70643; *V. splendidus* (T), X74724; and *V. vulnificus* (T), X74726. Uncultured symbiotic luminous bacteria: *Anomalops katoptron* symbiont, Z19081; *Cryptosaras couesi* symbiont, Z19106; *Kryptophanaron alfredi* symbiont, Z19003; *Melanocetus johnsoni* symbiont, Z19105; *Photoblepharon palpebratus* symbiont, Z19085; and *Photoblepharon steinetzi* symbiont, Z19080.

Nonetheless, the separateness of the luminous species in these two families suggests a separate and relatively recent acquisition of *lux* genes by *Photorhabdus* (Forst et al., 1997) and luminous *Shewanella* (discussed below).

Habitats and Ecology of Luminous Bacteria

Marine

Luminous bacteria are globally distributed in the marine environment (Table 1) and can be isolated from seawater, sediment and suspended particulates. They also colonize marine animals as saprophytes, commensal enteric symbionts and parasites (Hastings and Nealson, 1981; Meighen and Dunlap, 1993; Makemson et al., 1997), and certain of them establish bioluminescent symbiosis with marine fish and squids (Dunlap and Greenberg, 1991b; Nealson and Hastings, 1992; Haygood, 1993a; Ruby, 1996). In seawater, numbers of luminous bacteria generally are low (from 0.01 to up to 40 cells per ml of seawater; Nealson and Hastings, 1992). In association with animals, however, luminous bacteria can attain very high numbers (up to 10^{11} cells per ml in symbiotic habitats; Ruby and Nealson, 1976; Dunlap, 1984; Nealson and Hastings, 1992). The very high numbers of luminous bacteria in saprophytic, commensal, parasitic and symbiotic habitats indicates the potential of these habitats to make substantial contributions to the density and distribution of luminous bacteria in seawater, sediments and marine snow (Reichelt et al., 1977; O'Brien and Sizemore, 1979; Ruby and Morin, 1979; Haygood et al., 1984; Nealson et al., 1984; Ramesh et al., 1987; Ruby and Lee, 1998), which in turn serve as environmental sources of these bacteria for re-colonization of animals (Nealson and Hastings, 1992). Except in bioluminescent symbiosis, which is specific to certain luminous bacteria, the luminous species coexist in these habitats with nonluminous bacteria.

Unlike marine animals, marine algae apparently are not commonly colonized by luminous bacteria. Only one luminous bacterium with the ability to digest agar has been reported, a strain of *V. harveyi* (Fukasawa et al., 1987). Although agar digestion is often observed among *Vibrio* spp. and other marine bacteria, searches for other luminous bacteria with this trait, including extensive examination of surfaces of marine algae, have not yet revealed other luminous strains. One can speculate that a rare bacteriophage-mediated transduction between *Vibrio* spp. (Baross et al., 1978a) might have been the mechanism by which the strain of *V. harveyi* acquired genes for agar hydrolysis. Regardless, the surfaces of marine algae are an additional habitat exploited, though apparently rarely, by luminous bacteria and shared with nonluminous forms.

The distributions and numbers of individual species of luminous bacteria correlate with certain environmental factors (Baumann and Baumann, 1981; Hastings and Nealson, 1981). Primary among these factors are temperature and depth (Ruby and Nealson, 1978b; Yetinson and Shilo, 1979; Ruby et al., 1980), salinity (Yetinson and Shilo, 1979; Feldman and Buck, 1984), nutrient limitation and sensitivity to photooxidation (Shilo and Yetinson, 1980). Temperature, along with being an important environmental factor, can influence whether luminous bacteria from environmental samples are detected. For example, *Shewanella hanedai* and *Vibrio logei*, which are psychrotrophic, grow and produce light at low temperature (e.g., 4°C to 15°C, and grow but do not produce light at room temperature (24°C). Therefore, incubation of platings of environmental samples at the lower temperatures may reveal the presence of other psychrotropic luminous species. Temperature relationships would appear to be species-specific, however. For example, *S. woodyi* (found in squid ink and seawater in the Alboran Sea near Gibraltar; Makemson et al., 1997) and *V. fischeri*, species closely related to *S. hanedai* and *V. logei*, respectively, grow and produce light at room temperature.

Studies of the distibution and density of luminous bacteria in the marine environment traditionally have used visual observation of luminescence to identify these bacteria. However, the presence of *lux* genes in bacteria that do not produce light in culture and the physiological crypticity of luminescence in some species (Boettcher and Ruby, 1990; Fidopiastis et al., 1999) reveal that luminous bacteria are more numerous and diverse than identified by the luminescence phenotype. Enzyme assay and antibody methods previously have been used to detect luciferase in several nonluminous *Vibrio* spp. (Nealson and Walton, 1978b; Makemson and Hastings, 1986b; Kou and Makemson, 1988), and *luxA*-based DNA probes from various seawater samples have been used to identify *lux* gene-containing bacteria not producing light in culture (Potrikus et al., 1984; Palmer and Colwell, 1991; Lee and Ruby, 1992; Wimpee et al., 1991; Ramaiah et al., 2000). The efficacy of species- and group-specific *luxA*-based probes for the identification of environmental isolates of luminous bacteria has been demonstrated for two species, *Photobacterium phosphoreum* from the Black Sea and *Vibrio splendidus* from coastal waters of Kuwait (Wimpee et al., 1991; Nealson et al., 1993).

Freshwater

Knowledge of luminous bacteria in freshwater environments is limited to reports that luminous *V. cholerae* exist in freshwater and infect freshwater crustaceans. Luminous strains of *V. cholerae* have been isolated from freshwater and brackish estuarine waters in various locations (Desmarchelier and Reichelt, 1981; West and Lee, 1982; West et al., 1983; Palmer and Colwell, 1991; Ramaiah et al., 2000; Table 1). The first such isolation, in 1893, apparently was by F. Kutscher from the Elbe River in Germany (Harvey, 1952). Then called "*Vibrio albensis*," that strain later was synonymized with *V. cholerae* (Reichelt et al., 1976).

With respect to infecting freshwater animals, Thulis and Bernard in 1786 described the luminescence of a freshwater crustacean (possibly the common amphipod *Gammarus pulex*, which apparently was infected with luminous bacteria) from a river in southern France (Harvey, 1957). Yasaki (1927) reported the isolation of luminous bacteria from intensely luminous specimens of the freshwater shrimp, *Xiphocaridina compressa*, in Lake Suwa, Japan. Initially characterized as *Microspira phosphoreum*, the bacterium was later redescribed as *Vibrio yasakii* (Majima, 1931). More recently, a bacterium responsible for this "light disease of shrimp" was isolated from freshwater shrimp in Lake Biwa, Japan, and identified as non-O1 *V. cholerae* (Shimada et al., 1995). Nonluminous *V. cholerae* also are associated with disease in freshwater crustaceans (Thune et al., 1991).

Terrestrial

Luminous bacteria in the terrestrial environment have been noticed mostly as parasites of insects that cause the infected animal to luminesce. Observations of luminous midges, caterpillars, mole-crickets, mayflies and ants, among other infected insects, have been reported from the 1700s into modern times (Harvey, 1952; Haneda, 1950). As described and summarized by Harvey (Harvey, 1952; Harvey, 1957), other early reports of terrestrial luminescence attributable to luminous bacteria include luminous mutton, veal, eggs of chickens and lizards, human corpses and battlefield wounds. Many, and perhaps all, of the observations of luminous insects result from colonization by members of the genus *Photorhabdus*, of which three species are currently described, *P. luminescens*, *P. temperata* and *P. asymbiotica* (Fischer-Le Saux et al., 1999; Table 1).

Photorhabdus luminescens and *P. temperata* occur as the mutualistic symbionts of entomopathogenic nematodes (commonly found in soil) of the family Heterorhaditidae (Akhurst and Dunphy, 1993; Forst and Nealson, 1996; Forst et al., 1997). They are carried in the intestine of the infective juvenile stage of the nematode and participate in a lethal infection of insect larvae. When the nematode enters the insect, via the digestive tract or other openings, and penetrates the insect's hemocele, the bacteria are released into the hemolymph, where they use its constituents for growth. The bacteria elaborate a variety of extracellular enzymes that presumably break down macromolecules of the hemolymph. Proliferation of the bacteria leads to death of the insect, and its carcass becomes luminous. The bacteria also produce various extracellular and cell surface-associated factors pathogenic for the insect, as well as bacteriocins and hydroxystilbene and anthraquinone antibiotics, which apparently inhibit the growth of other microorganisms in the insect cadaver (Akurst, 1982). Crystalline protein inclusion bodies of unknown function are also produced (Bintrim and Ensign, 1998). The nematodes feed on the bacteria or products of bacterial degradation of the hemolymph enabling them to develop and sexually reproduce (Boemare et al., 1997; Forst et al., 1997). Completion of the nematode life cycle involves reassociation with the bacteria and the emergence from the insect cadaver of the nonfeeding infective juveniles, carrying the bacteria in their intestines. Cells of *P. luminescens* presumably are present in soil, but association with the nematode apparently is important for their survival and dissemination. Luminescence of the infected insect larva might function to attract nocturnally active animals to feed on the glowing carcass, thereby increasing the opportunities for the bacterium and the nematode to be disseminated. However, luminescence is not required for successful symbiosis with the nematode; not all strains of *P. luminescens* produce luminescence (Akhurst and Boemare, 1986; Forst and Nealson, 1996). Furthermore, bacteria in the genus *Xenorhabdus*, which are symbiotic with entomopathogenic nematodes in the family Steinernematidae, are ecologically very similar to *Photorhabdus*, except that they do not produce light (Akhurst and Dunphy, 1993). The similarities between the lifestyles and activities of *Photorhabdus* and *Xenorhabdus* are postulated to be a case of ecological convergence (Forst and Nealson, 1996).

Human clinical infections have yielded *P. asymbiotica*, introduced apparently by spider and insect bites (Farmer et al., 1989; Peel et al., 1999). Luminous battlefield wounds are intriguing because luminescence apparently is a sign that the wound will heal well (Harvey, 1957). Indeed, luminous bacteria will grow and produce light on living mammalian tissue (Johnson, 1988). Perhaps antibiotic-producing *P. lumine-*

scens or *P. temperata* promoted wound healing by preventing the growth of putrefying, pathogenic bacteria. On the other hand, the human pathogenicity of *P. asymbiotica* suggests that this species might have killed rather than healed if introduced into wounds. The recent description of *P. asymbiotica* and *P. temperata*, and the presence of genetically distinct subspecies within *P. luminescens* and *P. temperata* (Fischer-Le Saux et al., 1999; Fig. 2) indicate that additional diversity, possibly at the species level, may exist in this genus.

Along with terrestrial *Photorhabdus* species, marine luminous bacteria might have been responsible for some of the early reports of luminous meats and eggs, especially if brine was used in their preparation or they otherwise were exposed to seawater. Haneda (1950), following the observation by Molisch (1925) of luminous bacteria growing on beef, demonstrated that luminous bacteria could be isolated from certain samples of beef, pork and chicken meat. These meats might have contained enough salt to support the growth of marine forms, and Haneda cultured the bacteria in media containing 0.5% salt. However, whether these bacteria were terrestrial (i. e., *Photorhabdus*), freshwater (i. e., *V. cholerae*), or marine in origin apparently is not known.

Parasitism of Marine Invertebrates

Most of the commonly encountered marine luminous bacteria are not known to be highly invasive or virulent in animals. Many or perhaps all luminous species, however, can act as opportunistic pathogens upon entering an animal's body through lesions resulting from injury or stress. First noted in marine animals apparently by Viviani in 1805 (Harvey, 1957), infections of marine crustaceans by luminous bacteria are common, causing the infected animal to luminesce (Giard, 1889a; Giard and Billet, 1889b; Inman, 1926; Hastings and Nealson, 1981). Luminous bacteria inhabit the gut tract and colonize external surfaces of marine crustaceans (Inman, 1926; Baross et al., 1978b; O'Brien and Sizemore, 1979; Lavilla-Pitogo et al., 1992); many are chitinolytic (Spencer, 1961; Baumann and Schubert, 1984a). The bacteria enter the hemocele of the animal through lesions in the gut or carapace, developing luminescence and killing the animal within a few days. The species of luminous bacteria infecting isopods and amphipods commonly encountered in coastal environments have not been identified in recent times, but they exhibit characters consistent with members of the genera *Vibrio* and *Photobacterium* (Hastings and Nealson, 1981; P. Dunlap, unpublished observation). Nonluminous bacteria undoubtedly cause similar infections that go unnoticed due to the lack of light production.

As opportunistic pathogens of marine crustaceans, luminous bacteria have had a profoundly deleterious effect on commercial prawn mariculture. The development of intensive monoculture of *Penaeus monodon*, the giant tiger prawn, and other penaeids during the 1980s led to a dramatic increase in disease and death of the animals due to luminous bacteria. Shrimp hatchery rearing ponds can become heavily infested with luminous bacteria, with shrimp larvae developing "luminescent vibriosis," a pathogenic state responsible for massive mortalities. The problem continues in grow-out ponds, where the infection localizes to the hepatopancreas in juveniles, limiting the growth of the animals and further increasing losses to mortality (Lavilla-Pitogo and de la Peñ, 1998). Primarily responsible are strains of *V. harveyi*, though other luminous and nonluminous vibrios have been identified (Lavilla-Pitogo et al., 1990; Karunasagar et al., 1994; Lavilla-Pitogo and de la Peñ, 1998; Leano et al., 1998).

Parasitism of Vertebrates

In contrast to the situation with marine invertebrates, luminous bacteria apparently only rarely infect vertebrate animals. The ability of *P. asymbiotica* to infect humans has been mentioned above. *Vibrio harveyi* has been identifed in fish disease, and recently, *V. salmonicida* (a pathogen of salmonids and cod) has been shown to produce light under certain conditions (Fidopiastis et al., 1999).

Clinical strains of *Vibrio vulnificus* and *V. cholerae* typically are nonluminous, but luminous strains of *V. vulnificus* have been isolated from dead humans (Oliver et al., 1986), and luminous strains of *V. cholerae* have been isolated from humans suffering from cholera (Jermoljewa, 1926). Furthermore, Weleminsky (1895) demonstrated that a nonluminous clinical isolate of *V. cholerae* developed luminescence apparently by passage through another animal. *Vibrio cholerae* strains that are luminous or that contain the *luxA* gene are present in relatively high percentages in freshwater and estuarine environments (West and Lee, 1982; West et al., 1983; Palmer and Colwell, 1991; Ramaiah et al., 2000). The light-producing and *luxA* gene-containing strains are the non-O1 type of *V. cholerae* (Palmer and Colwell, 1991; Ramaiah et al., 2000).

Bioluminescent Symbiosis

One of the most remarkable attributes of luminous bacteria is the ability of certain species to

establish luminescence-based symbiotic associations called "bioluminescent symbiosis" with marine animals. These associations have been found in certain teleost fish, some loliginid and sepiolid squids, and possibly in pyrosomes and salps. The treatise by Buchner (1965) and the review by Herring and Morin (1978b) provide comprehensive access to early literature. For pyrosomes and salps, bioluminescent symbiosis with luminous bacteria is controversial (Harvey, 1952; Buchner, 1965). Pyrosome zooids bear a pair of simple photophores containing intracellular bacteroids, but the involvement of bacteria in pyrosome luminescence has been both discounted and supported (Galt, 1978; Herring, 1978a; Mackie and Bone, 1978; Haygood, 1993a). Although the bacteroids have not been cultured, the presence of bacterial luciferase in photophores is consistent with a bacterial origin for pyrosome luminescence (Leisman et al., 1980). In myctophid and stomiiform fishes, a similar proposal that the luminescence of photophores is due to the presence of symbiotic luminous bacteria (Foran, 1991) was shown conclusively to be invalid (Haygood et al., 1994).

In bioluminescent symbiosis of fishes and squids with luminous bacteria (Table 1), the host animal bears one or a pair of specialized gland-like tissues, called "light organs," which house a pure culture of the species-specific symbiotic bacterium. Accessory structures associated with the light organ, i.e. lens, reflector, and light-absorbing shutters and barriers, control, direct and focus the light the bacteria produce. The host animal uses the bacterial light in luminescence displays associated with various behaviors, including predator avoidance by counterillumination and flashing, sex-specific signaling, attracting or locating prey, and orienting in dark and dimly lighted environments (Hastings, 1971; Morin et al., 1975; Nealson and Hastings, 1979b; McFall-Ngai and Dunlap, 1983; McFall-Ngai and Montgomery, 1990; McFall-Ngai and Morin, 1991a). In fishes, the light organs are internal, associated with the gut tract, or external, located below the eye (subocular light organ), in the lower jaw (mandibular light organ) or at the terminus of an elongated dorsal fin ray (escal light organ), whereas in squids, they are found as bilobed organs ventrally within the mantle cavity, associated with the ink sac (Herring, 1977; Hastings and Nealson, 1981; Haygood, 1993a; McFall-Ngai and Ruby, 1991b). Bioluminescent symbiosis, owing to the specificity between host and symbiont, contrasts with other associations of luminous bacteria with animals; the saprophytic, commensal and parasitic associations are nonspecific and often involve assemblages of luminous and nonluminous bacteria (Harvey, 1940; Nealson and Hastings, 1992). Furthermore, a trend toward greater integration between symbiont and host can be envisioned in bioluminescent symbiosis, with certain animals colonized by facultatively symbiotic (i.e., culturable) bacteria and others harboring obligately symbiotic (i.e., not yet cultured) bacteria (Haygood, 1993a). Bioluminescent symbiosis appears to be a unique kind of symbiosis; the bacterial metabolic product needed by the host animal is light, used in bioluminescence displays, rather than a nutrient needed for host development or growth (Claes and Dunlap, 2000).

Four species of luminous bacteria, *V. fischeri*, *V. logei*, *P. leiognathi* and *P. phosphoreum*, have been identified in bioluminescent symbiosis (Table 1). *Vibrio fischeri* and *P. leiognathi* colonize light organs of both fish and squids (Boettcher and Ruby, 1990; Fukasawa et al., 1986; Fukasawa et al., 1988; Reichelt et al., 1977), whereas *P. phosphoreum* so far has been found in association only with fish (Herring and Morin, 1978b; Hastings and Nealson, 1981). *Vibrio logei* was identified recently as the predominant symbiont of the sepiolid squids *Sepiola affinis* and *Sepiola robusta* (Fidopiastis et al., 1998). Two other groups of fishes, the flashlight fish (family Anomalopidae) and deep-sea anglerfish (suborder Ceratioidei) bear light organs with symbiotic luminous bacteria that so far have not been cultured. The anomalopid symbionts, based on analysis of the *luxA* and 16S rRNA genes, are likely to be members of the genus *Vibrio*, and different genera of the fish harbor bacteria that differ at greater than the strain level (Haygood, 1990; Wolfe and Haygood, 1991). The results of 16S rRNA gene sequence analysis of the bacterial symbionts of two ceratioids (representing different families of anglerfish) group these bacteria with other marine enterics phylogenetically similar to *Photobacterium* and *Vibrio* and suggest that these may be new bacterial species in each fish (Haygood and Distel, 1993b).

Symbiont-Host Specificity

Despite the presence of various different species of luminous bacteria in the habitats of animals that form bioluminescent symbiosis, these associations are highly specific. Members of a given family of fishes and of squids consistently harbor the same species of symbiont (Fitzgerald, 1977; Reichelt et al., 1977; Ruby and Morin, 1978a; Ruby and Nealson, 1976; Fukasawa et al., 1986; Hastings and Nealson, 1981; Ruby, 1996). Various selective pressures, alone or in combination might account for this specificity. Physiological conditions of the light organ, which derives from host biology and light organ anatomy, may be important. Light-organ osmolarity, oxygen and iron levels, and types of nutrients presum-

ably interface with the individual physiological capabilities of different symbiotic luminous species and strains, promoting adaptively high levels of luminescence and competitive growth dominance of one type over another (Ruby and Nealson, 1976; Ruby and Nealson, 1977; Nealson, 1979a; Dunlap, 1985a; Haygood and Nealson, 1985a; Haygood, 1993a; Hastings et al., 1987; Graf and Ruby, 1998; Lee and Ruby, 1994a; Visick et al., 2000). Specific recognition and other exclusion mechanisms (Ruby, 1996) also may play a major role. Local abundance of the host also may contribute to specificity (Hastings and Nealson, 1981; Ruby and Lee, 1998).

Along with host-related factors, temperature, as an environmental factor, might play a significant role in host-symbiont specificity. A loose concordance is seen between the temperature of the host's habitat and the temperature sensitivities of the symbiotic bacteria. Fishes dwelling in temperate and tropical shallow waters harbor the more mesophilic species *V. fischeri* or *P. leiognathi*, whereas fishes dwelling in cold, deeper waters tend to harbor the more psychrotrophic species *P. phosphoreum* (Hastings and Nealson, 1981). Indeed, temperature reveals an exception to the pattern of host-symbiont specificity. The closely related species *V. logei* and *V. fischeri* can colonize the same species of sepiolid squid, *Sepiola affinis* and *Sepiola robusta*, forming mixed symbiotic cultures. Whereas lower temperatures favor the more psychrotrophic *V. logei*, warmer temperatures favor the more mesophilic *V. fischeri* (Fidopiastis et al., 1998; Nishiguchi, 2000).

Symbiont Transmission

In the few cases studied, squids and fishes have been found to acquire their symbiotic luminous bacteria by horizontal transfer. Best documented is the sepiolid squid *Euprymna scolopes*, hatchlings of which carry no *V. fischeri* cells or other bacteria in their nascent light organs. Soon after hatching, the animal picks up its symbiotic bacterium from seawater, establishing bioluminescent symbiosis (Wei and Young, 1989; McFall-Ngai and Ruby, 1991b). Symbiont motility is required for this process (Graf et al., 1994). Nascent light organs of juvenile *Siphamia versicolor* (family Apogonidae) at the early larval stage lack bacteria but contain them later in development, consistent with acquisition of the bacteria from the seawater (Haneda, 1965; Leis and Bullock, 1986). Recently, Wada et al. (1999) provided evidence for horizontal transfer of *P. leiognathi* to juvenile *Leiognathus nuchalis* (family Leiognathidae). For anomalopid (flashlight) fish, no evidence was found that the symbiotic bacteria are associated with gonads or eggs, consistent with horizontal transfer in this group as well (Haygood, 1993a).

Symbiont Contributions to Host Survival, Growth, and Development

Bioluminescent symbiosis appears to be a special class of symbiosis, one in which the primary metabolic contribution the symbiotic bacteria make to the host is light. In most bacterial associations with animals and plants, the host is dependent nutritionally on its symbiotic bacteria, via bacterial fixation of carbon or nitrogen, the activity of bacterial extracellular degradative enzymes, such as cellulases, or bacterial provision of vitamins or other essential nutrients (Douglas, 1995). As a consequence, absence of the symbiotic bacteria can have a profound influence on the survival, growth and development of the host. In contrast, the sepiolid squid *E. scolopes* cultured aposymbiotically from hatching to reproductive adulthood survived, grew and developed equally as well as animals colonized by *V. fischeri* (Claes and Dunlap, 2000). These observations indicate that *V. fischeri* apparently makes no major nutritional contribution to the animal. The metabolic dependency of *E. scolopes* on *V. fischeri* therefore seems limited to light production. Selection for the association presumably is ecological, with the squid's ability to counterilluminate using light produced by *V. fischeri* (Singley, 1983; McFall-Ngai and Montgomery, 1990) playing an important role in survival. Whether a similar lack of nutritional dependency of the host on its symbiotic bacteria characterizes other bioluminescent symbioses remains to be determined.

Influence of Symbionts on Light-Organ Morphogenesis

Much progress in understanding host-symbiont relationships in bioluminescent symbiosis has developed from studies of the sepiolid squid *E. scolopes*. The animal maintains a species-specific (and strain-specific) association with *V. fischeri*, harboring the bacteria extracellularly in diverticulated epithelial tubules comprising the core of the bilobed ventral light organ. Associated with the light organ are accessory tissues, specifically the ink sac, and the reflectors and lens, which control and direct the light produced by *V. fischeri*. The tubules lead into a ciliated duct that connects each lobe of the light organ to the mantle cavity (McFall-Ngai and Ruby, 1991b; Ruby, 1996; McFall-Ngai, 1999).

Analysis of the colonization process in hatchling juvenile *E. scolopes* demonstrates a role for the symbiotic bacterium in morphological changes in the light organ. The nascent, rudimentary light organs in hatchlings bear a pair of

lateral ciliated epithelial appendages (CEAs) and contain a pair of three simple sac-like epithelial tubules embedded in the undifferentiated accessory tissues. The proximal portions of these tubules are ciliated and directly connect to the mantle cavity via a lateral pore. Colonization of the epithelial tubules, which is facilitated by ciliary beating of the CEAs, occurs through these lateral pores, which later coalesce, with the formation of a ciliated duct for each light organ lobe. Colonization triggers regression of the CEAs within approximately 4 days. Other morphological changes include alterations in the epithelial cells of the distal portions of the light organ tubules, which develop a dense microvillous brush border (McFall-Ngai and Ruby, 1991b; Montgomery and McFall-Ngai, 1993; Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995; Ruby, 1996; Lamarcq and McFall-Ngai, 1998; McFall-Ngai, 1999).

Presence of the bacteria, however, is not necessary for overall development of the light organ and its accessory tissues, which proceed normally in aposymbiotic animals (Claes and Dunlap, 2000). In possible contrast to light organ development in *E. scolopes*, light organs of the monocentrid fish, *M. japonicus*, had not developed by day 21 in larvae from artificially fertilized eggs (Yamada et al., 1979), suggesting that acquisition of *V. fischeri* may be necessary to initiate the light organ developmental program. Whether development of the light organ in aposymbiotic juvenile leiognathid fish requires colonization by *P. leiognathi* is not yet known (Wada et al., 1999).

Host Contribution to Symbiont Dissemination

Bioluminescent symbiosis is likely to have a significant impact on the density and distribution of the symbiotic bacteria in seawater. Growth of the bacterial population in the light organ leads to the continual or diurnal release of bacterial cells into the environment (Dunlap, 1984; Haygood et al., 1984; Nealson et al., 1984; Lee and Ruby, 1994b; Boettcher et al., 1995). The cells are released either directly into seawater, as in monocentrid fish and sepiolid squids, or indirectly via the gut tract, as in leiognathid fish. Estimates of growth rates for the bacteria indicate the population doubles once to a few times per day (Dunlap, 1984; Haygood et al., 1984; Lee and Ruby, 1994b), so each adult host may release as many as 10^7 to 10^8 symbiont cells per day. This release, which has the potential of dispersing the bacteria into other habitats they colonize (Nealson et al., 1984), may be essential for re-initiation of the association with the next generation of the host animal (Ruby and Lee, 1998).

Physiological Control of Luminescence

Growth conditions can strongly influence the amount of light produced by luminous bacteria in laboratory culture. Oxygen, amino acids, glucose, iron and osmolarity have distinct effects, depending on the species studied (Harvey, 1952; Nealson and Hastings, 1977b; Makemson and Hastings, 1982; Haygood and Nealson, 1985a; Hastings et al., 1987; Dunlap, 1991a). Those factors that stimulate growth rate, such as readily metabolized carbohydrates, tend to decrease light production and luciferase synthesis. They do so presumably by causing oxygen and reducing power ($FMNH_2$) to be directed away from luciferase (McElroy and Seliger, 1962; Coffey, 1967) and by indirectly or directly influencing *lux* gene expression (Dunlap and Greenberg, 1985b; Dunlap, 2000). Conversely, factors that restrict growth rate, such as limitation for iron, tend to stimulate the synthesis and activity of luciferase (Hastings and Nealson, 1977; Haygood and Nealson, 1985a; Hastings et al., 1987; Dunlap, 1991a). The mechanisms by which these factors operate, however, are not well understood (Haygood and Nealson, 1985b; Dunlap, 1992a; 1992b), indicating that much remains to be learned about the interplay between growth physiology of the cell and regulatory elements controlling *lux* gene expression.

Amino Acids, Catabolite Repression, and Control by cAMP

The amino acid arginine and certain structurally and metabolically related compounds can stimulate luminescence in *V. harveyi* growing in minimal medium (Coffey, 1967; Nealson et al., 1970; Hastings and Nealson, 1977). The mechanism for this activity remains unknown. Conversely, yet equally intriguing, mixtures of amino acids can transiently and in a dose-dependent manner block the increase in light production of inducing cultures of *P. leiognathi* (P. Dunlap, unpublished observation). Catabolism of the amino acids might account for this temporary repression of luminescence induction.

Catabolite repression of luminescence generally is attributed to effects on levels of 3′,5′-cyclic AMP (cAMP) and cAMP receptor protein (CRP; Nealson et al., 1972; Meighen and Dunlap, 1993; Dunlap, 1997). Different species of luminous bacteria, however, respond in different ways. In *V. harveyi*, catabolite repression by glucose in batch culture is permanent and is reversed by addition of cAMP (Nealson et al., 1972), whereas glucose repression of luminescence in *V. fischeri* is temporary, is not reversed

by addition of cAMP, and is eliminated by prior growth in the presence of glucose (Ruby and Nealson, 1976). Complicating these differences from studies in batch culture are studies of *V. fischeri* grown in phosphate-limited chemostat culture; glucose repression of luminescence then is permanent and reversible by cAMP (Friedrich and Greenberg, 1983). A further complication for studies of cAMP-control of luminescence in *V. fischeri* is the presence in this species of a novel, exceptionally potent periplasmic cyclic nucleotide phosphodiesterase specific for extracellular 3′, 5′-cyclic nucleotides; activity of the enzyme enables cells to grow on exogenously supplied cAMP as a sole source of carbon and energy, nitrogen and phosphorus (Dunlap et al., 1992d; Dunlap and Callahan, 1993; Callahan et al., 1995). Regardless of the differences in catabolite repression, mutants of *V. harveyi* and *V. fischeri* apparently defective in adenylate cyclase and unable to produce light in the absence of added cAMP have been isolated and characterized (Ulitzur and Yashphe, 1975; Dunlap, 1989a). Furthermore, CRP from *V. harveyi* has been purified and shown to be immunologically and functionally homologous to CRP of *Escherichia coli* (Chen et al., 1985), and the *cya* and *crp* genes of *V. fischeri* have been cloned and found to be highly similar in deduced amino acid residue sequence to *E. coli cya* and *crp* genes (P. Dunlap et al., unpublished observation). Consistent with these observations, the regions upstream of the luminescence operons of *V. harveyi* and *V. fischeri* contain a CRP binding site (Engebrecht and Silverman, 1987; Devine et al., 1988a; Miyamoto et al., 1988b). Studies with *V. fischeri* and with *E. coli* carrying the *V. fischeri* luminescence system indicate that a major effect of cAMP-CRP is to activate the production of LuxR, the luminescence operon transcriptional activator (see below; Dunlap and Greenberg, 1985b; Dunlap and Greenberg, 1988; Dunlap and Kuo, 1992c; Shadel et al., 1990a), although other important *lux* regulatory effects have also been described (Shadel and Baldwin, 1991; Shadel and Baldwin, 1992a; Shadel and Baldwin, 1992b). Regardless, control of the luminescence system by cAMP-CRP in *V. fischeri* demonstrates the integration of luminescence with cellular metabolism and suggests that activity of the luminescence system is part of the cellular response to stresses associated with nutrient limitation and decreasing growth rate. Consistent with that view, a heat-shock protein (GroESL) and a repressor of DNA repair (LexA) have been shown or are suspected of contributing to control of luminescence (Ulitzur and Dunlap, 1995). Those factors that can restrict growth rate while enhancing expression and activity of luciferase, e.g., limiting oxygen, limiting iron and high or low osmolarity (Dunlap, 1991a; Hastings et al., 1987; Meighen and Dunlap, 1993) might operate by influencing the cellular levels of cAMP and CRP or other stress-response elements.

The *Lux* Genes, Luminescence Autoinduction and Quorum Sensing

The *lux* Genes. The bacterial *lux* genes can be grouped in three categories: the core *lux*, accessory *lux* and regulatory genes. The five core *lux* genes, which provide the enzymatic capability for light production, are common to all luminous bacteria examined to date. These genes are *luxA* and *luxB*, encoding the luciferase subunits, and *luxC*, *luxD* and *luxE*, encoding the fatty-acid reductase subunits; they occur contiguously as an operon, *luxCDABE* (Fig. 3). The bacterial*lux*-genes have been used for a variety of applications, primarily as reporters for environmental and regulatory effects in heterologous systems (LaRossa, 1998).

Accessory *lux* genes, which are associated with light production, are found in different species and strains of luminous bacteria. In some cases, these genes are linked to the *lux* operon. *Photobacterium phosphoreum* (Fig. 3) and a strain of *P. leiognathi* bear *luxF*, a gene similar in sequence to *luxB*, between *luxB* and *luxE*, encoding a nonfluorescent flavoprotein. The *lux* operons of the marine luminous bacteria also contain *luxG*, which in *V. fischeri* is followed by a strong transcriptional terminator (Swartzman et al., 1990a). The LuxG protein may be a flavin reductase of the Fre/LuxG family of NAD(P)H-flavin oxidoreductases (Zenno and Saigo, 1994a; Zenno et al., 1994b). The last gene of the *lux* operon in *V. harveyi* is *luxH*. Protein LuxH is homologous to *E. coli* RibB (3,4-dihydroxy-2-butanone 4-phosphate synthase), a key enzyme in riboflavin synthesis (Swartzman et al., 1990b). Recently, the *ribB* gene of *V. fischeri* was identified. In contrast to *luxH* in *V. harveyi*, *ribB* in *V. fischeri* is unlinked to the *lux* operon; nonetheless its expression is controlled coordinately with the *lux* operon (Callahan and Dunlap, 2000). Additional genes involved in riboflavin synthesis have been identified downstream of the *lux* operon in *V. fischeri*, *P. leiognathi* and *P. phosphoreum* (Lee et al., 1994c). Other accessory luminescence genes have been described (O'Kane and Prasher, 1992; Meighen, 1994).

The third category, genes specifying regulatory proteins, has been identified to date only in *V. fischeri* and *V. harveyi* (Fig. 3). In *V. fischeri*, the main *lux* regulatory genes, *luxR*, encoding the acyl-homoserine lactone receptor/*lux* operon transcriptional activator, and *luxI*, encoding acyl-homoserine lactone synthase (Engebrecht et al., 1983; Engebrecht and Silverman, 1984; Schaefer

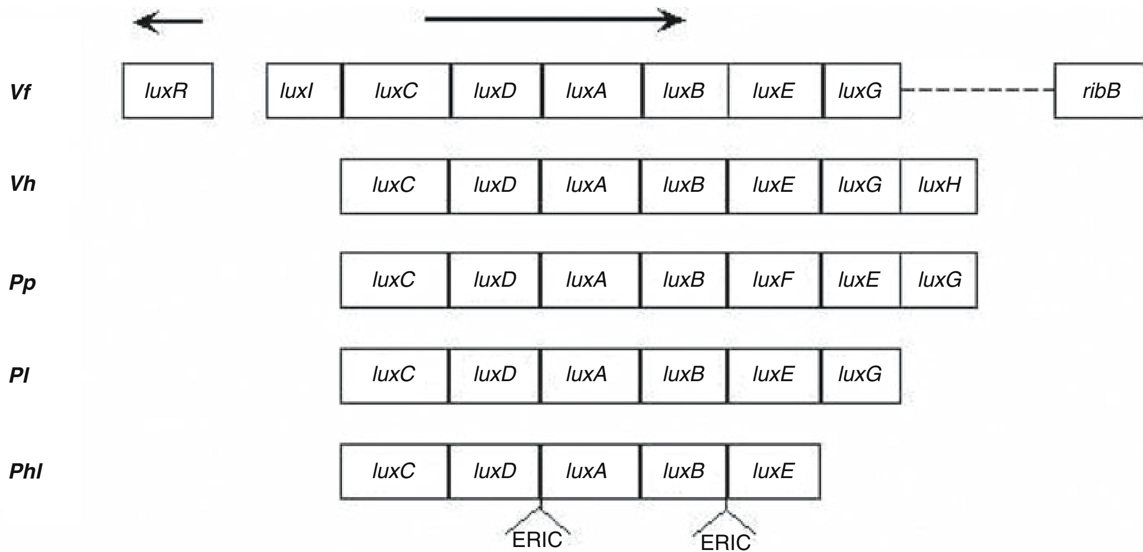


Fig. 3. Organization of the luminescence genes in luminous bacteria. Abbreviations and key references are: Vf, *V. fischeri* (Baldwin et al., 1989; Callahan and Dunlap, 2000; Devine et al., 1988a; Engebrecht et al., 1983; Engebrecht and Silverman, 1984; Engebrecht and Silverman, 1987; Foran and Brown, 1988; Swartzman et al., 1990a); Vh, *V. harveyi* (Cohn et al., 1985; Johnston et al., 1986; Johnston et al., 1989; Miyamoto et al., 1988a; Miyamoto et al., 1988b; Miyamoto et al., 1989; Swartzman et al., 1990b); Pp, *P. phosphoreum* (Soly et al., 1988); Pl, *P. leiognathi* (Baldwin et al., 1989; DeLong et al., 1987; Illarionov et al., 1990; Meighen, 1991; Meighen and Dunlap, 1993); Phl, *Ph. luminescens* (Frackman et al., 1990; Frackman et al., 1990; Johnston et al., 1990; Szittner and Meighen, 1990; Xi et al., 1991); and ERIC, enteric repetitive intergenic consensus sequence (Meighen and Szittner, 1992; Forst and Nealson, 1996). Additional regulatory genes in *V. fischeri* and regulatory genes in *V. harveyi* have been identified, as shown in Figs. 4 and 5. Regulatory genes controlling *luxCDAB(F)EG* expression in other species have not yet been identified. Arrows indicate direction of transcription, and genes are not drawn to scale.

et al., 1996; Stevens et al., 1994; Stevens and Greenberg, 1997), are contiguous with the *lux* operon. The *luxI* gene is part of the *lux* operon, whereas *luxR* is upstream and divergently expressed (Fig. 3). In *V. harveyi*, the *luxR* gene, which is not homologous to *V. fischeri luxR*, is not linked to the *lux* operon (Showalter et al., 1990; Swartzman et al., 1992). In other species, the *lux* regulatory genes have not been identified, but they apparently are unlinked to the *lux* operon. Additional *lux* regulatory genes have been identified in *V. fischeri* and *V. harveyi* and are described below.

Luminescence Autoinduction and Quorum-Sensing. Many luminous bacteria exhibit a distinctive pattern of luciferase synthesis and light production in laboratory culture, previously called "autoinduction" and now referred to as "quorum sensing." In *V. fischeri* and *V. harveyi*, expression of the *lux* operon, i.e., luciferase synthesis and luminescence, initially low in early exponential phase cultures, induces strongly as cultures attain the high cell densities associated with late exponential to early stationary phases of growth (Hastings and Greenberg, 1999; Dunlap, 2000). Early analyses of the "phases of luminescence" in culture (Baylor, 1949; Farghaly, 1950) were followed by the demonstration that luciferase synthesis is inducible and that complete medium contained a compound inhibitory to induction (Nealson et al., 1970; Eberhard, 1972). During growth, cells of *V. fischeri* and *V. harveyi* were found to release into the medium species-specific secondary metabolites, called "autoinducers." These compounds accumulate in the growth medium in a cell-density dependent manner, and once they attain threshold concentrations they induce luciferase synthesis (Nealson et al., 1970; Eberhard, 1972; Nealson, 1977a; Nealson and Hastings, 1979b; Ulitzur and Hastings, 1979; Rosson and Nealson, 1981). The cell density-dependent nature of autoinduction led to the coining of the term quorum sensing (Fuqua et al., 1996; Greenberg, 1997).

Analysis of autoinduction reached a notable fruition in the 1980s with the identification of autoinducer signal molecules and *lux* regulatory genes. The first autoinducer, 3-oxo-hexanoyl-HSL (3-oxo-C6-HSL), and the first *lux* regulatory genes, *luxI* (encoding 3-oxo-C6-HSL synthase; Schaefer et al., 1996) and *luxR* (encoding acyl-HSL receptor/transcriptional activator) were identified in *V. fischeri* (Eberhard et al., 1981; Engebrecht et al., 1983; Engebrecht and Silverman, 1984), followed by identification of 3-hydroxybutanoyl-HSL (3-OH-C4-HSL) and a

nonhomologous *luxR* gene in *V. harveyi* (Cao and Meighen, 1989; Showalter et al., 1990). From that base of knowledge, quorum sensing systems that are chemically and genetically homologous to those of *V. fischeri* and *V. harveyi* have been identified over the past decade in many species of nonluminous Gram-negative bacteria, including many terrestrial species and several pathogens of animals and plants. Quorum sensing plays important roles in the biology of these bacteria by regulating a wide variety of different kinds of genes, including those for the production of extracellular enzymes, plasmid transfer, antibiotic synthesis and biofilm formation, as well as luminescence (Fuqua et al., 1996; Dunlap, 1997; Swift et al., 1999). Thus, quorum sensing not only is common to luminous bacteria, but also widespread and evolutionarily conserved among Gram-negative bacteria.

Despite the importance of quorum sensing as a regulatory paradigm in Gram-negative luminous bacteria, it should be pointed out that apparently not every luminous bacterium can autoinduce luminescence. Certain strains identified as *P. leiognathi* lack the lag in luminescence and luciferase synthesis in batch culture that is characteristic of autoinduction (Katznelson and Ultizur, 1977); it is possible that expression of the *lux* system in these strains is independent of acyl-HSLs and that luciferase synthesis is essentially constitutive. These considerations highlight the likelihood that luminescence and quorum sensing had separate evolutionary origins, discussed below.

QUORUM SENSING CONTROL OF LUMINESCENCE IN *V. FISCHERI* AND *V. HARVEYI*. Intensive study of *V. fischeri* and *V. harveyi* over the past 20 years has developed the luminescence systems in these two species as prototypes for quorum sensing in bacteria (Bassler, 1999; Dunlap, 1997; Dunlap, 2000; Fuqua et al., 1994; Greenberg, 1997; Hastings and Greenberg, 1999). New and fundamental information, nonetheless, continues to accumulate on how these bacteria control light production and use quorum sensing. Indeed, the simple view that "quorum sensing signals accumulate in a cell density-dependent manner and trigger transcription of genes for the luminescence enzymes," though still entirely valid, has been replaced. The newer view is that quorum-sensing control of luminescence in these two species is remarkably complex, and that the mechanisms (Figs. 4 and 5) have intriguing genetic homologies and disparities.

In *V. fischeri*, luminescence is controlled by two quorum-sensing signals that coordinate a complex regulatory circuitry (Fig. 4). Major components of the quorum-sensing mechanism are: LuxI, the 3-oxo-C6-HSL synthase; LuxR, the transcriptional regulatory protein, which requires 3-oxo-C6-HSL for activity; GroEL, which is necessary for production of active LuxR; and AinS, octanoyl-HSL (C8-HSL) synthase (Engebrecht et al., 1983; Engebrecht and Silverman, 1984; Schaefer et al., 1996; Adar et al., 1992; Dolan and Greenberg, 1992; Adar and Ulitzur, 1993; Hanzelka et al., 1999; Kuo et al., 1994; Gilson et al., 1995; Kuo et al., 1996). A cell density-dependent accumulation of 3-oxo-C6-HSL, a membrane-permeant compound (Kaplan and Greenberg, 1985), triggers induction of *lux*operon expression by binding to LuxR, apparently a membrane-associated protein (Kolibachuk and Greenberg, 1993), forming a complex that facilitates the association of RNA polymerase with the *lux* operon promoter (Stevens and Greenberg, 1997). This activation initiates a positive feedback loop for synthesis of 3-oxo-C6-HSL (e.g., Eberhard et al., 1991), and LuxR/3-oxo-C6-HSL negatively autoregulate *luxR* expression (Dunlap and Greenberg, 1988; Dunlap and Ray, 1989b). The C8-HSL, which apparently interferes with 3-oxo-C6-HSL binding to LuxR, operates to limit premature *lux* operon induction (Eberhard et al., 1986; Kuo et al., 1996). Expression of *luxR* is activated by both cAMP and CRP (Dunlap and Greenberg, 1985b; Dunlap and Greenberg, 1988; Dunlap, 1989a; Dunlap and Kuo, 1992c), which also have other regulatory effects (Shadel and Baldwin, 1991) and thereby provide overall control over quorum sensing. Under anaerobic conditions, which are permissive of luciferase synthesis (Eberhard et al., 1979), a regulator of fumarate and nitrate reduction (Fnr) contributes to *lux* operon expression (Müller-Bretkreutz and Winkler, 1993). Recently, a homolog of the *V. harveyi* *luxO* gene was identified in *V. fischeri*. As is the case in *V. harveyi*, LuxO in *V. fischeri* apparently functions as a repressor of luminescence (Miyamoto et al., 2000). For details of*lux*gene regulation in *V. fischeri*, see Dunlap (2000).

Luminescence regulation in *V. harveyi* has several features in common with *V. fischeri*. Like *V. fischeri*, *V. harveyi* uses two different quorum-sensing signals, 3-OH-C4-HSL and Vh AI-2, an unidentified compound; two different genes (*luxLM* and *luxS*) direct synthesis of these respective signals. Luminescence in both species requires cAMP-CRP, and in both it is dependent on a transcriptional activator protein LuxR, although these proteins are not homologous (Bassler et al., 1993; Bassler et al., 1994b; Chen et al., 1985; Eberhard, 1972; Martin et al., 1989; Nealson et al., 1970; Nealson et al., 1972; Miyamoto et al., 1988b; Miyamoto et al., 1990; Miyamoto et al., 1994; Showalter et al., 1990; Swartzman et al., 1990a; Swartzman and Meighen, 1993; Ulitzur and Yashphe, 1975). Fur-

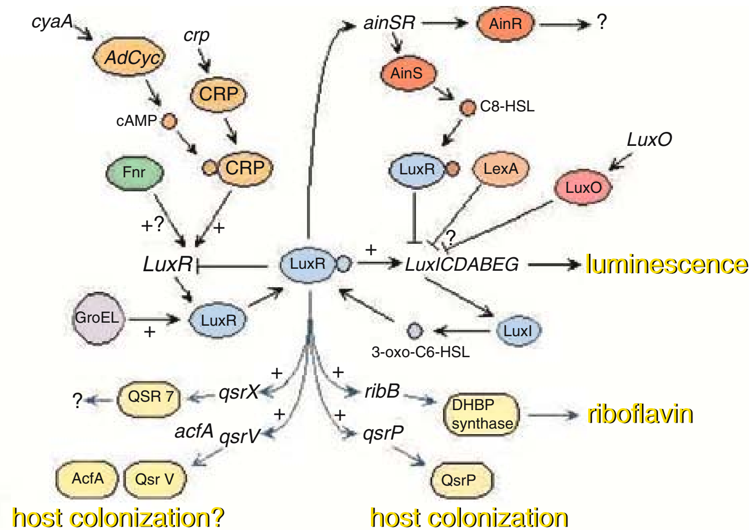


Fig. 4. Model for quorum-sensing control of luminescence and other genes of the quorum-sensing regulon of *V. fischeri*. Depicted in the upper portion of the figure are regulatory genes, proteins, effectors and positive and negative regulatory circuitry controlling *luxR*/*luxICDABEG* expression (summarized from Ulitzur and Dunlap, 1995; Dunlap, 2000; see also Lin et al., 2000). Abbreviations: AdCyc, adenylate cyclase; 3-oxo-C6-HSL, 3-oxo-hexanoyl-homoserine lactone; C8-HSL, octanoyl-homoserine lactone. Key elements of *lux* regulation are described in the text. The weak activation of *lux* operon expression by C8-HSL/LuxR is depicted as repression, due to the apparent competitive inhibition by C8-HSL of the interaction between 3-oxo-C6-HSL and LuxR (Kuo et al., 1996). The lower portion of the figure indicates genes downstream of the *lux* operon that are coordinately controlled with the *lux* operon, positively by LuxR/3-oxo-C6-HSL and negatively by LuxR/C8-HSL (Callahan and Dunlap, 2000).

thermore, the *lux* operons of these bacteria are similar in structure (Miyamoto et al., 1988a; Swartzman et al., 1990b), although regulatory genes apparently are not present in the region immediately upstream of *luxC* (Miyamoto et al., 1988b; Fig. 3). The recent identification of *ribB*, the *V. fischeri* homolog of *V. harveyi luxH*, which like the *lux* operon genes is controlled by LuxR and acyl-HSLs (Callahan and Dunlap, 2000) further demonstrates the overall similarity of *lux* genes in these two species.

A striking counterpoint to the general similarities in the *lux* operons and physiological control of luminescence in these bacteria is the qualitative difference in quorum sensing in *V. harveyi*. Expression of the *lux* operon in *V. harveyi* is regulated by a quorum-sensing phosphorelay signal transduction mechanism. The mechanism involves two separate two-component phosphorelay paths, each involving a transmembrane sensor/kinase, LuxN and LuxQ, responsive to a separate quorum-sensing signal (Fig. 5). The *luxLM* genes are necessary for synthesis of the 3-OH-C4-HSL signal. In the absence of 3-OH-C4-HSL, LuxN operates as a kinase, phosphorylating LuxU, a signal integrator, which in turn passes the phosphate on to LuxO; the phosphorylated LuxO represses the *lux* operon. In the presence of 3-OH-C4-HSL, the activity of LuxN is shifted from kinase to phosphatase, which draws phosphate from LuxU and thereby from LuxO; the dephosphorylated LuxO no longer represses *lux* operon expression. A similar activity is carried out by a second, as yet unidentified signal (Vh AI-2), which requires LuxS for its production. Operating via LuxP, a putative periplasmic protein, Vh AI-2 mediates the kinase/phosphatase activity of LuxQ, which in turn, like LuxN, feeds phosphate to or draws it from LuxO. Previously thought to directly repress *lux* operon expression, LuxO may operate indirectly, by controlling a negative regulator of luminescence. Expression of *luxO* itself is subject to repression by LuxT (Bassler, 1999; Bassler et al., 1994a; Cao and Meighen, 1989; Freeman and Bassler, 1999a; Freeman and Bassler, 1999b; Lilley and Bassler, 2000; Lin et al., 2000; Surete et al., 1999b). In a manner possibly analogous to LuxR in *V. fischeri*, LuxR in *V. harveyi* is autoregulatory and responsive to 3-OH-C4-HSL (Cao et al., 1995; Chatterjee et al., 1996; Miyamoto et al., 1996). The phosphorelay signal transduction mechanism of *V. harveyi* appears to differ substantially from the *V. fischeri* quorum-sensing paradigm of direct acyl-HSL/receptor protein activation of *lux* operon expression.

Despite this difference, evidence is growing that the quorum-sensing systems of these two species have significant overlaps at the genetic level. The first indication of genetic overlap was

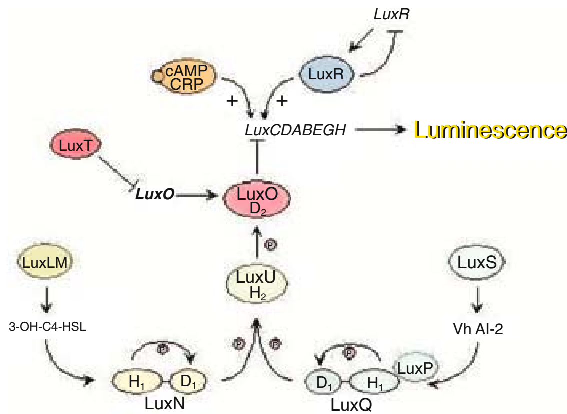


Fig. 5. Model for quorum-sensing control of luminescence in *V. harveyi*. Regulatory inputs positively (cAMP-CRP and LuxR) and negatively (LuxO) controlling the *lux* operon and negatively controlling *luxO* are shown. The circled P indicates the intramolecular phosphate transfer and the separate phosphorylation/dephosphorylation signal transduction circuits mediated by the quorum sensing signals 3-OH-C4-HSL and Vh AI-2. H_1 and D_1 refer to the conserved histidine and aspartate residues of the sensor kinase and response regulator domains, respectively, of LuxN and LuxQ. H_2 refers to a histidine residue of LuxU, which integrates signals from LuxN and LuxQ, and D_2 refers to an aspartate residue of LuxO, to which the signal is then transduced. The phosphoryl flow is from H_1 to D_1 of LuxN, and from H_1 to D_1 of LuxQ, to H_2 of LuxU to D_2 of LuxO. In the absence of the quorum-sensing signals, LuxN and LuxQ, the depicted phosphoryl flow leads to phosphorylation of LuxO, which may directly or indirectly repress the *lux* operon. Phosphorylase activity of LuxN and LuxQ is activated by the accumulation of the quorum-sensing signals, leading to dephosphorylation and inactivation of LuxO. Not shown are the membrane-permeant nature of the quorum-sensing signals and the membrane association of LuxN, LuxQ, and LuxP. Modified from Bassler (1999).

the finding that the C-terminal half of the *V. fischeri* AinS protein is 34% identical to the *V. harveyi* LuxM protein, and the N-terminal portion of *V. fischeri* AinR (encoded by *ainR*, a gene downstream of *ainS*) is 38% identical to the N-terminal portion of *V. harveyi* LuxN (Gilson et al., 1995). Whether AinR itself, possibly with C8-HSL, plays a role in *lux* regulation (Gilson et al., 1995; Kuo et al., 1994) has not been established. The recent identification in *V. fischeri* of *luxO* is another example. The deduced amino acid residue sequence of *V. fischeri* LuxO is approximately 70% identical to that of *V. harveyi* (Miyamoto et al., 2000). Furthermore, a gene immediately downstream of *luxO* in *V. fischeri* is likely to be a homolog of *V. harveyi luxU*. These homologies suggest that *V. fischeri* (like *V. harveyi*) uses a phosphorelay system for quorum sensing. Consequently, the extent of actual differences between the quorum-sensing mechanisms in *V. fischeri* and *V. harveyi* is not yet clear.

Nonetheless, the qualitative difference in the quorum sensing systems of *V. fischeri* and *V. harveyi* is surprising. One could reasonably anticipate that the mechanisms regulating light production in these two species would be very similar because these bacteria are closely related evolutionarily, and are metabolically and physiologically very similar. Furthermore, whereas *V. fischeri* and *V. harveyi* have ecological differences, the differences do not seem to provide a compelling rationale for the qualitative difference in*lux*regulation. *Vibrio fischeri*, a more temperate-water species, colonizes light organs of monocentrid fish and certain sepiolid squids, whereas *V. harveyi*, a species more abundant in warmer waters and able to utilize more sole carbon and energy sources for growth in laboratory culture, has not been found as a light organ symbiont. However, both species can be isolated from seawater, sediments, gut tracts of marine animals, and from infected crustaceans—habitats in which they commonly are found together (Baumann and Baumann, 1981). This ecological commonality suggests that the physiological and ecological importance of quorum sensing would be very similar in the two species. The mechanistic differences outlined above, however, even with genetic overlaps, indicate that the two systems are substantially different and that the difference might result from subtle ecological differences. An alternative possibility, however, is that the *lux* operons were acquired separately by *V. fischeri* and *V. harveyi*, under different circumstances. For example, selective pressures and chromosomal locations for *lux* operons may have differed at the times of acquisition, accounting for the different qualities of the regulatory mechanisms. Lateral transfer of the *lux* operon is discussed below. Regardless, the presence of multiple cross-acting quorum-sensing systems in *V. fischeri* and *V. harveyi* most likely indicates the importance in both species of sensing and responding to complex and changing conditions in a variety of different habitats.

Quorum-sensing Regulated Genes "Downstream" of *lux*. An emerging area in luminous bacteria biology is the identification of non-Lux activities controlled by quorum sensing. Quorum sensing is known to control various activities in nonluminous bacteria (Dunlap, 1997; Swift et al., 1999), but studies of quorum sensing in luminous bacteria have focused exclusively on luminescence until very recently. Studies of *V. harveyi* led to the first demonstration of quorum-sensing-regulated activities other than luminescence in luminous bacteria. In *V. harveyi*, the production of the fatty acid storage product poly-β-hydroxybutyrate is controlled in a cell density-dependent manner by 3-OH-C4-HSL (Sun et al., 1994; Miyamoto et al., 1998). The use of acyl-

HSL inhibitors has revealed that production of exotoxins by *V. harveyi* is linked to quorum-sensing control of luminescence (Harris and Owens, 1999a; Harris et al., 1999b). Furthermore, LuxO controls not only luminescence but also cell morphology and siderophore production in *V. harveyi* (Lilley and Bassler, 2000). Consistent with these observations, homologs of *V. harveyi* LuxO, LuxR and LuxS have been identified in several nonluminous bacteria (Jobling and Holmes, 1997; Klose et al., 1998; McCarter, 1998; Sperandio et al., 1999; Surete et al., 1999b; Joyce et al., 2000; McDougald et al., 2000), indicating that, as is the case for *V. fischeri*, elements of the *V. harveyi* quorum-sensing system are widespread in Gram-negative bacteria. In *V. fischeri*, proteomic analysis of mutants defective in *luxR*, *luxI* and *ainS* recently revealed the presence of several quorum-sensing regulated genes "downstream" of the *lux* operon (Callahan and Dunlap, 2000; Fig. 4). These genes code for an apparently diverse array of proteins, including proteins contributing to the ability of *V. fischeri* to colonize its squid host, *E. scolopes*. The identification of non-*lux* genes in luminous bacteria controlled by quorum sensing and the characterization of quorum-sensing regulatory genes in nonluminous bacteria serves to demonstrate the generality of quorum sensing and to indicate that quorum sensing and luminescence are functionally separate activities.

Independent Evolutionary Origins of Quorum Sensing and Luminescence

Knowledge of quorum sensing developed out of studies of luminescence autoinduction in *V. fischeri* and *V. harveyi* during the 1970s and 1980s, as described above. Despite the phenomenological and historical linkage between them, however, luminescence and quorum sensing apparently have separate evolutionary origins. The following considerations lead to this view.

1) The existence of strains that apparently do not regulate luciferase synthesis in an autoinducible manner (Katznelson and Ulitzur, 1977) suggests that bacterial luminescence is not necessarily subject to quorum sensing control.

2) The physical linkage of the *lux* structural and regulatory genes found in *V. fischeri*, which if consistently present in other species would imply an evolutionary link between light production and its regulation, is not found in other bacteria, e.g., *V. harveyi*. Furthermore, other luminous bacteria (e.g., *V. harveyi*) apparently lack homologs of the *V. fischeri* LuxR and LuxI proteins, and are very different from either *V. fischeri* or *V. harveyi* in the way they regulate light production. For example, luminescence in *Photorhabdus*, which does not exhibit autoinduction (Forst and Nealson, 1996), apparently is controlled at the posttranscriptional level (Wang and Dowds, 1991; Hosseini and Nealson, 1995).

3) Many nonluminous bacteria use acyl-HSL-mediated quorum sensing, indicating that quorum sensing is not exclusive to luminous bacteria.

4) Quorum sensing controls several activities in nonluminous bacteria (Dunlap, 1997; Swift et al., 1999) and controls activities in luminous bacteria unrelated to luminescence (Callahan and Dunlap, 2000; Sun et al., 1994; Harris et al., 1999b; Lilley and Bassler, 2000), indicating regulatory significance beyond and unrelated to light production.

There appears to be no necessary functional or physical connection between quorum sensing and luminescence. In the absence of that connection, one can then ask why *luxCDABE* is under quorum sensing control in some species but apparently not in others. We postulate that this apparent discordance points to different origins of *lux* genes among the different luminous bacteria.

Origin and Lateral Transfer of the *lux* Genes

Evolutionary Origin of Bacterial Luminescence

The presence of naturally acquired genes necessary for producing light defines the luminous bacteria. The necessary genes *luxA* and *luxB*, encoding the luciferase subunits, and *luxC*, *luxD* and *luxE*, for the fatty-acid reductase subunits, are consistently found together as a cotranscribed unit *luxCDABE* (Fig. 3). Furthermore, the individual *lux* proteins have a high degree of sequence identity, 54–88% and 45–77% for the α- and the β-subunits of luciferases, respectively, and 57–80%, 59–74%, and 59–81% for the fatty acid reductase subunits, LuxC, LuxD and LuxE, respectively (Meighen and Dunlap, 1993). The reason for this conservation as a unit is not known; it might be necessary for efficient light production, perhaps by ensuring an interaction of luciferase and fatty acid reductase that facilitates substrate generation and processing. Conservation as a unit to permit coordinate regulation would not seem to be the reason because quorum sensing coordinately regulates several widely separated sets of genes in *V. fischeri* (Callahan and Dunlap, 2000). The possibility exists therefore that newly identified luminous bacteria will be found to have *luxAB* and *luxCDE* in separate chromosomal locations.

These considerations lead to questions on the evolutionary origin of bacterial luminescence.

Seliger (1987) proposed that bacterial luminescence arose under ecological selection, as a biochemical analog of Darwin's principle of functional change in structural continuity. A flavoprotein catalyzing fatty acid α-oxidation reactions with low chemiluminescent quantum yields is postulated to have mutated under hypoxic conditions to accept $FMNH_2$ as the flavin cofactor, generating a fortuitously high fluorescence yield, termed "protobioluminescence," via the 4a-hydroxy-FMNH product. This flavin-dependent, aldehyde-oxidizing protoluciferase produced sufficient light, and with an appropriate emission spectrum, to be detected by phototactic organisms. Responses to the light by visually cueing animals (e.g., to ingest luminous particles), enhanced the growth of the protobioluminescence emitter by introducing it into the animal's nutrient-rich digestive system, ensuring the emitter's survival and presumably leading to selection for more intense light output. It is possible that early evolutionary steps leading to protoluciferase involved oxygen detoxification activity that permitted early anaerobic organisms to survive an increasingly aerobic environment (McElroy and Seliger, 1962; Rees et al., 1998).

A single gene has been hypothesized to encode bacterial protoluciferase (O'Kane and Prasher, 1992). Although a single-subunit protoluciferase presumably would have differed somewhat from the modern-day luciferase α-subunit and therefore might have produced light, the inability of either of the modern-day α- or β-subunits alone to produce light in vitro or in vivo (Li et al., 1993) argues against the single-gene hypothesis. Alternatively, bacterial luminescence may have arisen following a gene duplication event postulated to have created *luxB* from *luxA* (Baldwin et al., 1979; O'Kane and Prasher, 1992; Meighen and Dunlap, 1993). The association of the fatty-acid reductase genes with *luxA* might have predated the *luxA* to *luxB* gene duplication event. Alternatively, the presence of ERIC sequences flanking *luxA* and *luxB* in *P. luminescens* (Meighen and Szittner, 1992) might mark an insertion of the *luxAB* genes into the fatty aldehyde reductase operon during the evolution of the bacterial luminescence system. Origins and functions of other luminescence proteins have been discussed elsewhere (O'Kane and Prasher, 1992; Meighen and Dunlap, 1993).

A marine origin for bacterial luminescence, though speculative, seems reasonable. Most species of luminous bacteria are marine (Table 1), luminescence appears to have arisen independently in various (mainly marine) phylogenetic groups (Hastings, 1995), and present-day luminous organisms are much more common in the ocean than in terrestrial and freshwater environments. Palmer and Colwell (1991) have interpreted the high level of nucleotide sequence identity for a region of *luxA* among *V. cholerae* and marine vibrios as indicating a common luminescent marine ancestor. However, a growing number of terrestrial luminous species are being identified (Fischer-Le Saux et al., 1999), so the possibility of a terrestrial origin for bacterial luminescence should not be ruled out.

Lateral Transfer

Despite the conservation of the *luxCDABE* genes in luminous bacteria, the presence of these genes is not monophyletic. Genera with luminous members include the closely related and physiologically similar *Vibrio* and *Photobacterium* and the more distantly related and physiologically distinct *Photorhabdus* and *Shewanella* (Fig. 2). Various evolutionary scenarios can be envisioned to account for the polyphyletic distribution of*lux*genes and to accommodate the presence of luminous and nonluminous species and strains in *Vibrio* and *Photobacterium*:

1) The *lux* genes may have been present in the ancestor that diverged into the lines leading to modern-day members of the Vibrionaceae, Enterobacteriaceae and Alteromonadaceae. The *lux* genes were then lost from many descendents but retained by some. If this scenario is correct, one might expect to find more species in the Enterobacteriaceae and Alteromonadaceae that carry *lux* genes.

2) Alternatively, the *lux* genes might have arisen later, within the line leading to modern-day members of the Vibrionaceae. These genes then may have been lost from several descendents, retained by some, and transferred relatively recently from a member or members of the Vibrionaceae to *Photorhabdus* and *S. hanedai* and *S. woodyi*. The presence of the *luxCDABE* genes in *Photorhabdus* species has been interpreted as an instance of lateral gene transfer (Forst et al., 1997). Furthermore, the chromosomal locations of the *luxCDABE* genes in two ecologically distinct strains of *Ph. luminescens* apparently differ (Meighen and Szittner, 1992), raising the possibility that lateral transfer to this species occurred more than once (Forst et al., 1997).

3) Also possible is that the *lux* genes did not arise indigenously in the ancestral line that diverged into the Vibrionaceae, Enterobacteriaceae and Alteromonadaceae (scenario 1) or later within the Vibrionaceae (scenario 2). Instead, they may have been acquired relatively recently by certain species and strains in the Vibrionaceae by lateral gene transfer from an

unknown source. The same source might have transferred *lux* genes to *Photorhabdus*, *S. hanedai* and *S. woodyi*, or these genes might have been acquired secondarily by lateral transfer from a member or members of the Vibrionaceae. The recent identification of luminous strains of *V. salmonicida* (Fidopiastis et al., 1999) and *P. angustum* (K. Kita-Tsukamoto et al., manuscript in preparation), species previously characterized as nonluminous, is consistent with all three scenarios. Mapping the chromosomal locations of the *lux* genes in *Vibrio* and *Photobacterium* would help differentiate among these scenarios. Similar chromosomal locations for the *lux* genes would tend to support an evolutionary origin in an ancestor of or within the Vibrionaceae lineage (scenarios 1 and 2), whereas different chromosomal locations, as seen in *Ph. luminescens* (Meighen and Szittner, 1992), would be more consistent with lateral transfer to members of the Vibrionaceae (scenario 3). In regard to this latter possibility, the differences in DNA flanking *luxCDABE* in different members of the Vibrionaceae, for example in *V. fischeri* and *V. harveyi*, are intriguing. An issue that complicates each of these scenarios, however, is the possible mobility of the *lux* genes among members of the Vibrionaceae, with losses and recent lateral transfer events accounting for or contributing to the modern-day presence of luminous and nonluminous species and strains in this family.

Physiological Functions of the Luminescence System

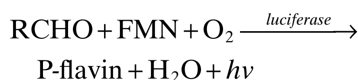
One the most interesting and long-standing questions about luminous bacteria is the physiological function of luminescence. In other words, "Why do bacteria produce light?" Despite extensive knowledge of the biochemistry and genetics of bacterial luminescence, the cellular role of luminescence in bacteria is not well understood. However, the benefit light production provides to bacteria has been variously hypothesized, and multiple ecological and physiological functions for luminescence seem likely.

Ecologically, in the realm of visually orienting animals, light production undoubtedly plays a role in the dissemination of luminous bacteria and may have been instrumental in the evolution of strongly luminous strains, as discussed above. The feeding of animals on luminous particles (decaying tissues, fecal pellets, and moribund animals infected by luminous bacteria) disperses the bacteria and brings them into the animal's nutrient-rich gut tract for further growth and dispersal (Nealson and Hastings, 1992). Bioluminescent symbiosis serves a similar role through a continual or diurnal release from the host animal, as mentioned above. Once bacteria developed the ability to emit light at levels that could be detected by animals, ecological interactions may then have selected for higher levels of light production (Seliger, 1987), fostering the development of progressively more specific, luminescence-based associations with animals, eventually leading to species-specific bioluminescent symbiosis. A high level of activity of the luminescence system might promote bacterial survival and growth in these associations, especially in bioluminescent symbiosis (Dunlap, 1984; Visick et al., 2000).

A strong case for a physiological role for bacterial luminescence can be made, despite the fact that the *lux*g enes are not essential for survival or growth of luminous bacteria, at least in laboratory culture (Kuo et al., 1994). Several genes are committed to the light-producing reaction, including structural genes for the luminescence proteins and regulatory genes controlling *lux* operon expression. Retention of these genes and the expenditure of energy for the synthesis and activity of their protein products (Dunlap and Greenberg, 1991b; Meighen and Dunlap, 1993) imply that the luminescence system carries out an activity beneficial to the cell. For example, luciferase activity can consume up to 20% of the oxygen taken up by luminous cells (Eymers and van Schouwenberg, 1937; Watanabe et al., 1975; Dunlap, 1985a; Makemson, 1986a). Furthermore, luminescence in many species is regulated, such that the *lux* genes are expressed under certain environmental conditions but not others, and *lux* regulation is deeply integrated into the physiologial response networks and gene regulatory circuitry (e.g., cAMP-CRP and quorum sensing) of the cell. Indeed, luminescence is just one of a suite of metabolic and physiological activities controlled by quorum sensing (Callahan and Dunlap, 2000). The coordinated expression of the *lux* genes with other sets of genes in response to the physiological state of the cell suggests that the luminescence system plays an integral physiological role in the biology of luminous bacteria.

Most attention to the question of that physiological role for bacterial luminescence has focused on oxygen. McElroy and Seliger (1962) proposed that light-emitting reactions arose evolutionarily as detoxifying reactions that removed oxygen and thereby allowed anaerobic organisms to survive. This hypothesis has been developed further from the perspective that luciferin substrates for luciferase are the evolutionary core of bioluminescent systems (Rees et al., 1998). The luminescence reaction, as a terminal oxidase or secondary respiratory chain that is active when oxygen or iron levels are too low for the cytochrome system to operate, would allow

reduced coenzymes to be reoxidized, thereby permitting cells under microaerobic conditions, such as in animal gut tracts, to continue to metabolize growth substrates (Nealson and Hastings, 1979b; Ulitzur et al., 1981; Makemson and Hastings, 1982; Seliger, 1987). Consistent with this possibility, luciferase activity can partially complement the lack of cytochromes (Makemson and Hastings, 1986b). In bioluminescent symbiosis, the luciferase reaction has been proposed to help protect cells from host-generated reactive oxygen species (ROS; Visick et al., 2000). Alternatively, the physiologically important function of the luciferase reaction may be the production of FP390 (P-flavin binding protein), including its prosthetic group, Q (P)-flavin (Kasai, 1997), according to the following reaction scheme:



$$\mathrm{RCHO + FMN + O_2 \xrightarrow{luciferase} P\text{-}flavin + H_2O + } h\nu$$

The protein FP_{390} functions as a substitute for flavodoxin, at high salt concentrations where flavodoxin is less active. Flavodoxin, e.g., FldA from *V. fischeri* (Kasai, 1999), functions to reactivate oxidatively inactivated cobalamin-dependent methionine synthase (CDMS; Hoover and Ludwig, 1997). It follows that bacteria would not produce light under conditions of low ionic strength because under these conditions cells would use flavodoxin for reoxidation of CDMS in lieu of producing FP390 (Farghaly, 1950; Kasai, 1997). It is tempting to speculate that the postulated relationship between conditions of high ionic strength, synthesis of P-flavin, and light production might account for the apparently exclusive occurence of luminous bacteria in habitats of relatively high ionic strength, i.e., seawater, brackish water, and tissues of marine, freshwater and terrestrial animals.

In each of the above cases, light production is an incidental though ecologically important byproduct of the luciferase reaction, and not its primary physiological function. An alternative to incidental light production is the recent proposal that bacterial luminescence serves as an internal light source for a photoreactivation-like repair of damaged DNA (Czytz et al., 2000). Studies of UV survival of *V. harveyi lux* mutants and an *E. coli lexA* mutant carrying the *V. harveyi lux* genes (Czytz et al., 2000) suggest that damaged DNA would be the "missing photoreceptor" for bacterial luminescence. It is intriguing that the *V. fischeri lux* operon *lux* box is similar to the *E. coli* LexA protein-binding site (Ulitzur and Kuhn, 1988; Devine et al., 1988b; Baldwin et al., 1989; Shadel et al., 1990a) and that various SOS-response-inducing and DNA-intercalating agents stimulate luminescence in bacteria (Weiser et al., 1981; Ulitzur and Dunlap, 1995). The strong link between oxidative stress and DNA damage (Hemnani and Parihar, 1998) is consistent with this role. Possibly, then, the luciferase reaction carries out the dual physiological functions of ROS detoxification and photoreactivation-like DNA damage repair. The induction of *lux* gene expression at high population density and the coordinate stimulation of superoxide dismutase (Colepicolo et al., 1992) suggest that DNA damage and oxidative stress become more significant in bacteria as nutrients are exhausted and growth begins to slow.

Isolation, Cultivation and Identification of Luminous Bacteria

Detailed information on the isolation, cultivation and phenotypic characterization of luminous bacteria can be found in Nealson (1978a), Baumann et al. (1984b), Baumann and Baumann (1981), and Farmer and Hickman-Brenner (1992). Methods and information not otherwise referenced here were introduced to the author by K. H. Nealson and E. P. Greenberg during summer courses at the Marine Biological Laboratory at Woods Hole, Massachusetts.

Isolation

Light-emitting bacteria can be isolated from most marine habitats, through direct plating of samples or by enrichment. For direct plating, 0.1–0.2 ml of coastal seawater is spread on nutritionally complete agar plates, such as Seawater Complete (SWC) agar (Nealson, 1978a; see below). Open-ocean water contains fewer bacteria, so cells from 10 ml to 1 liter are concentrated by filtration (pore size 0.2–0.45 mM), and then the filter is placed on SWC agar or a similar medium. Sediments and gut tracts contain higher numbers of bacteria and therefore usually are diluted 1,000 fold or more before spreading 0.1 to 0.2 ml. Media prepared with 4% agar (Baumann et al., 1984b) helps limit the spreading of swarming and gliding bacteria. Various crustaceans (e.g., gammarid and caprellid amphipods) are suitable sources for luminous bacteria, as they can become infected with luminous bacteria and develop a strong luminescence before and for several hours after dying. In a dark room, after dark-adapting for 12–15 min), one can pick out the infected, luminous crustaceans from collected seaweed. In a lighted room, the exoskeleton of the animal is punctured to obtain the hemolymph, which is streaked onto a suitable agar medium. The plates can be incubated at ambient or cool temperatures and are observed after 12–24 h for luminous colonies, which are then picked and streaked to obtain pure cultures.

Enrichments of marine luminous bacteria can be made from fresh fish (such as mackerel and flounder), other marine animals, and previously frozen fish. The entire animal or portions are placed in a tray and half covered with seawater, allowing part of the animal's body to be submerged and part to be exposed to air. This enrichment is then incubated at cool temperatures and observed daily in a darkened room. Luminous spots develop on the exposed portions of the animal within one to several days, depending on the temperature, and these are picked and steaked onto a suitable agar medium. Use of 4% agar (Baumann et al., 1984b) is recommended to limit the spreading of gliding and swarming bacteria.

Picking of luminous spots and luminous colonies is made easier by working in a darkened room with a red lamp on variable control. The intensity the lamp and angle of illumination can be adjusted so that luminous colonies are bluish and nonluminous colonies stand out as orange-red. Sterile toothpicks are convenient for picking luminous colonies.

Storage

Storing luminous bacteria on agar slants or in agar stabs for more than a week is not recommended inasmuch as dim and dark variants can easily arise with some species and survival can be poor. Similarly, survival under refrigeration is poor for some species. Lyophylization or storage in liquid nitrogen may be an option if appropriate equipment is available (Baumann et al., 1984b). Storage at ultra-low temperature, e.g., −75°C to −80°C, in a cryoprotective medium, however, works well for all species examined. An effective cryoprotective medium for luminous bacteria is filter-sterilized Deep Freeze Medium (2X DFM), prepared with 1% w/v yeast extract, 10% dimethyl sulfoxide (DMSO), 10% glycerol and 0.2M K_2HPO_4/NaH_2PO_4 (pH 7.0). E. F. DeLong recommended 2X DFM, originally developed by R. Rodriquez for storing yeast. For permanent storage of luminous bacteria, a dense culture is prepared by growing the strain to be stored for 12–18 h in a complete liquid medium, adding 0.5 ml each of the culture and 2X DFM to cryovials, briefly vortexing to mix, allowing the mixture to stand for 10 min before placing the vial into the ultra-low temperature freezer. Commercial containers that allow a slow rate of cooling work well as does quick freezing in an ethanol bath kept in the ultra-low temperature freezer or chilled with dry ice. Cultures of luminous bacteria stored in this manner retain viability apparently indefinitely when the tubes are kept at constant ultra-low temperature.

Cultivation

Most complete marine media, whether prepared with artificial or natural seawater to supply appropriate levels of Na^+, Ca^{2+} and Mg^{2+}, support the growth of luminous bacteria from most habitats. Nealson (1978a) listed and compared various formulations for complete and minimal media. A commonly used complete medium is SWC, prepared with natural seawater diluted to 70% or 75% with distilled water to minimize precipitation, 5 g per liter of tryptone or peptone, 3 g per liter of yeast extract and 3 ml per liter of glycerol, and with 1.5 g per liter of agar for solid medium. Traditionally, SWC has been buffered with 50 mM Tris or HEPES, or 1 g per liter of solid calcium carbonate has been incorporated into the agar medium to control acid production (Nealson, 1978a). Acid production in SWC apparently results, however, from the presence of glycerol, and elimination of this component avoids the problem (Dunlap et al., 1995) with no major effect on growth or luminescence. An easily prepared complete medium contains 10 g per liter of tryptone, 5 g per liter of yeast extract, 70% natural or artificial seawater, and 1.5% agar for solid medium. Artificial seawater can be prepared according to the formulation of MacLeod, as described by Nealson (1978a), or for routine culture work, a commercial aquarium marine salt mix can be used. Procedures for preparing minimal media have been described by Nealson (1978a).

Identification

A combination of phenotypic and genotypic traits is useful for the identification of luminous bacteria. Taxonomy of the marine luminous bacteria and their relationships to other marine enterobacteria were established during the 1970s and early 1980s through the use of an array of diagnostic physiological and molecular traits (Reichelt and Baumann, 1973; Reichelt et al., 1976; Baumann and Baumann, 1981). Using as few as 10–25 phenotypic traits, one can identify with good accuracy many of the commonly encountered species of marine luminous bacteria (Nealson, 1978a; Baumann and Baumann, 1981; Hastings and Nealson, 1981). Genotypic traits, specifically*lux*genes and 16S rRNA (Haygood, 1990; Haygood et al., 1992; Haygood and Distel, 1993b; Wimpee et al., 1991; Nealson et al., 1993), complement these diagnostic characters and can be particularly useful for rapid identification.

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Prokaryotes (2006) 2:893–955
DOI: 10.1007/0-387-30742-7_28

CHAPTER 1.28

Bacterial Toxins

VEGA MASIGNANI, MARIAGRAZIA PIZZA AND RINO RAPPUOLI

Introduction

Toxins were the first bacterial virulence factors to be identified and were also the first link between bacteria and cell biology. Cellular microbiology was, in fact, naturally born a long time ago with the study of toxins, and only recently, thanks to the sophisticated new technologies, has it expanded to include the study of many other aspects of the interactions between bacteria and host cells. This chapter covers mostly the molecules that have been classically known as toxins; however, the last section also mentions some recently identified molecules that cause cell intoxication and have many but not all of the properties of classical toxins. Tables 1 and 2 show the known properties of all bacterial toxins described in this chapter, while Figure 1 shows the subunit composition and the spatial organization of toxins whose structures have been solved either by X-ray crystallography or by quick-freeze deep-etch electron microscopy.

Abbreviations: SEA–SEI, staphylococcal enterotoxin A through I; TSST-1, toxic shock syndrome toxin 1; SPEA, B and C, streptococcal pyrogenic enterotoxins A, B and C; ETA and B, exfoliative toxins A and B; MHC, major histocompatibility complex; Vβ or Vγ, T-cell-receptor variable domains; LukF, leucocidin F; PA, protective antigen; RTX, repeats-in-toxin; CryIA, CytB, G_i, G_s, G_o, G_t, G_{olf}, GTP-binding proteins; MAPKK1 and 2, mitogen-activated protein kinases 1 and 2; EF2, elongation factor 2; Rho, Rac and Cdc42, GTP-binding proteins that control assembly of actin stress fibers; IL2, 4 and 5, interleukins 2, 4 and 5; TeNT, tetanus neurotoxin; VAMP, vesicle-associated membrane protein; BoNT, botulism neurotoxin; SNAP, synaptosome-associated protein; YOP, *Yersinia* outer-membrane proteins; AvrRxv, plant-pathogen virulence protein; Ipa, invasion-plasmid antigen; ICE, interleukin-converting enzyme; Sop, *Salmonella* outer-membrane protein; Tir, translational initiation region; CagA, cytotoxin-associated gene A; YpkA, *Yersinia* protein kinase A.

For abbreviations, refer to the footnote in Table 1.

Toxins have a target in most compartments of eukaryotic cells. For simplicity, the toxins are divided into three main categories (Fig. 2): 1) those that exert their powerful toxicity by acting on the surface of eukaryotic cells simply by touching important receptors, by cleaving surface-exposed molecules, or by punching holes in the cell membrane, thus breaking the cell permeability barrier (panel 1); 2) those that have an intracellular target and hence need to cross the cell membrane (these toxins need at least two active domains, one to cross the eukaryotic cell membrane and the other to modify the toxin target) (panel 2); and 3) those that have an intracellular target and are directly delivered by the bacteria into eukaryotic cells (panel 3).

Toxins Acting on the Cell Surface

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

Toxins Acting on the Immune System (Superantigens)

Superantigens (Fig. 2, panel 1) are bacterial and viral proteins that share the ability to activate a large fraction of T-lymphocytes. They are bivalent molecules that have been shown to simultaneously bind two distinct molecules, the major histocompatibility complex (MHC) and the T-cell receptor variable domains (Vβ or Vγ; Kotzin et al., 1993; Fig. 3). Binding of these molecules to MHC class II requires no prior processing and occurs outside the antigen-binding groove. This results in the activation of between 2–15% of all T cells, ultimately leading to T-cell proliferation, the production of a variety of cytokines, and expression of cytotoxic activity.

Bacterial superantigens, also known as pyrogenic toxins, comprise a class of secreted proteins mostly produced by *Staphylococcus aureus* and *Streptococcus pyogenes* (Bohach et al., 1990; Alouf and Muller-Alouf, 2003). So far, they include the group of staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and

Table 1. Classes of toxins described in the text, their features and activity.

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
Toxins acting on the cell surface	Immune system (Superantigens)	SEA-SEI, TSST-1, SPEA, SPEC, SPEL, SPEM, SSA, and SMEZ	*Staphylococcus aureus and Streptococcus pyogenes*	Binding to MHC class II molecules and to Vβ or Vγ of T cell receptor	T cell activation and cytokines secretion	SEB SEC2, SEC3, SED, SEH TSST1, SPEA SPEC
		MAM	*Mycoplasma arthritidis*	Binding to MHC class II molecules and to Vβ or Vγ of T cell receptor	Chronic inflammation	+
		YPMa	*Yersinia pseudotuberculosis*	Binding to MHC class II molecules and to Vβ or Vγ of T cell receptor	Chronic inflammation	–
		SPEB	*S. pyogenes*	Cysteine protease	Alteration in immunoglobulin-binding properties	+
		ETA, ETB, and ETD	*S. aureus*	Trypsin-like serine proteases	T-cell proliferation, intraepidermal layer separation	ETA, ETB
	Surface molecules	BFT enterotoxin	*Bacteroides fragilis*	Metalloprotease, cleavage of E-cadherin	Alteration of epithelial permeability	–
		AhyB	*Aeromonas hydrophyla*	Elastase, metalloprotease	Hydrolization of casein and elastine	–
		Aminopeptidase	*Pseudomonas aeruginosa*	Elastase, metalloprotease	Corneal infection, inflammation and ulceration	–
		ColH	*Clostridium histolyticum*	Collagenase, metalloprotease	Collagenolytic activity	–
		Nhe	*Bacillus cereus*	Metalloprotease and collagenase	Collagenolytic activity	–
	Cell membrane			Cell membrane permeabilization	Cell death	
	Large pore-forming toxins	PFO	*C. perfringens*	Thiol-activated cytolysin, cholesterol binding	Gas gangrene	+
		SLO	*S. pyogenes*	Thiol-activated cytolysin, cholesterol binding	Transfer of other toxins, cell death	–
		LLO	*Listeria monocytogenes*	Induction of lymphocyte apoptosis	Membrane damage	–
		Pneumolysin	*S. pneumoniae*	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–
	Small pore-forming toxins	Alveolysin	*B. alvei*s	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–

	ALO	*B. anthracis*	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–
	α-Toxin	*S. aureus*	Binding of erythrocytes	Release of cytokines, cell lysis, apoptosis	+
	PVL leukocidin (LukS-LukF)	*S. aureus*	Cell membrane permeabilization	Necrotic enteritis, rapid shock-like syndrome	LukF
	γ-Hemolysins (HlgA-HlgB and HlgC-HlgB)	*S. aureus*	Cell membrane permeabilization	Necrotic enteritis, rapid shock-like syndrome	HlgB
	β-Toxin	*C. perfringens*	Cell membrane permeabilization	Necrotic enteritis, neurologic effects	–
RTX toxins	Hemolysin II	*B. cereus*	Cell membrane permeabilization	Hemolytic activity	–
	CytK	*B. cereus*	Cell membrane permeabilization	Necrotic enteritis	–
	HlyA	*E. coli*	Calcium-dependent formation of transmembrane pores	Cell permeabilization and lysis	–
Membrane-perturbing toxins	ApxI, ApxII, and ApxIII	*A. pleuropneumoniae*	Calcium-dependent formation of transmembrane pores	Lysis of erythrocytes and other nucleated cells	–
	LtxA	*A. actinomycetem-comitans*	Calcium-dependent formation of transmembrane pores	Apoptosis	–
	LktA	*P. haemolytica*	Calcium-dependent formation of transmembrane pores	Activity specific versus ruminant leukocytes	–
Other pore-forming toxins	δ-Hemolysin	*S. aureus*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	–
	Aerolysin	*A. hydrophila*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+
	AT	*C. septicum*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+ (model)
Insecticidal toxins	PA	*B. anthracis*	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+
	HlyE	*E. coli*	Perturbation of the lipid bilayer	Osmotic lysis of cells lining the midgut	+
	CryIA, CryIIA, CryIIIA, etc	*Bacillus thuringiensis*	Destruction of the transmembrane potential	Osmotic lysis of cells lining the midgut	CryIA, CryIIIA

(*Continued*)

Table 1. *Continued*

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
		CytA, CytB	*B. thuringiensis*	Destruction of the transmembrane potential	Osmotic lysis of cells lining the midgut	CytB
		BT toxin	*B. thuringiensis*	Destruction of the transmembrane potential	Cytocidal activity on human cells	–
Toxins acting on intracellular targets	Protein synthesis	DT	*Corynebacterium diphtheriae*	ADP-ribosylation of EF-2	Cell death	+
		PAETA	*P. aeruginosa*	ADP-ribosylation of EF-2	Cell death	+
		SHT	*S. dysenteriae*	N-glycosidase activity on 28S RNA	Cell death, apoptosis	+
	Signal transduction	PT	*Bordetella pertussis*	ADP-ribosylation of G_i	cAMP increase	+
		CT	*Vibrio cholerae*	ADP-ribosylation of G_s	cAMP increase	+
		LT	*E. coli*	ADP-ribosylation of G_s	cAMP increase	+
		α-Toxin (PLC)	*C. perfringens*	Zinc-phospholipase C, hydrolase	Gas gangrene	+
		Toxins A and B (TcdA and TcdB)	*C. difficile*	Monoglucosylation of Rho, Rac, Cdc42	Breakdown of cellular actin stress fibers	–
		Adenylate cyclase (CyaA)	*B. pertussis*	Binding to calmodulin ATP→cAMP conversion	cAMP increase	–
		Anthrax edema factor (EF)	*B. anthracis*	Binding to calmodulin ATP→cAMP conversion	cAMP increase	+
		Anthrax lethal factor (LF)	*B. anthracis*	Cleavage of MAPKK1 and MAPKK2	Cell death, apoptosis	+
		Cytotoxin necrotizing factors 1 and 2 (CNF1, 2)	*E. coli*	Deamidation of Rho, Rac and Cdc42	Ruffling, stress fiber formation.	CNF1 (catalytic domain)
		DNT	*Bordetella species*	Transglutaminase, deamidation or polyamination of Rho GTPase	Ruffling, stress fiber formation	–
		CDT	*Several species*	DNA damage, formation of actin stress fibers via activation of RhoA	Cell-cycle arrest, cytotoxicity, apoptosis	–
	Cytoskeleton structure	Toxin C2 and related proteins	*C. botulinum*	ADP-ribosylation of monomeric G actin	Failure in actin polymerization	–

		Lymphostatin	*E. coli*	Block of interleukin production	Chronic diarrhea	–
		Iota toxin and related proteins	*C. perfringens*	Block of interleukin production	Chronic diarrhea	+ (C2I)
	Intracellular trafficking	TeNT	*C. tetanii*	Cleavage of VAMP/ synaptobrevin	Spastic paralysis	+ (Hc domain)
		BoNT-B, D, G and F neurotoxins	*C. botulinum*	Cleavage of VAMP/ synaptobrevin	Flaccid paralysis	BoNT-B
		BoNT-A, E neurotoxins	*C. botulinum*	Cleavage of SNAP-25	Flaccid paralysis	BoNT-A
		BoNT-C neurotoxin	*C. botulinum*	Cleavage of syntaxin, SNAP-25	Flaccid paralysis	–
		Vacuolating cytotoxin VacA	*H. pylori*	Alteration in the endocytic pathway	Vacuole formation, apoptosis	–
		NAD glycohydrolase	*S. pyogenes*	Keratinocyte apoptosis	Enhancement of GAS proliferation	–
Toxins injected into eukaryotic cells	Mediators of apoptosis	IpaB	*Shigella*	Binding to ICE	Apoptosis	–
		SipB	*Salmonella*	Cysteine proteases	Apoptosis	–
		YopP/YopJ	*Yersinia species*	Cysteine protease, blocks MAPK and NFkappaB pathways	Apoptosis	–
	Inositol phosphate metabolism	SopB	*Salmonella species*	Inositol phosphate phosphatase, cytoskeleton rearrangements	Increased chloride secretion (diarrhea)	–
		IpgD	*S. flexneri*	Inositol phosphate phosphatase, cytoskeleton rearrangements	Increased chloride secretion (diarrhea)	–
	Cytoskeleton	ExoS	*P. aeruginosa*	ADP-ribosylation of Ras, Rho GTPase	Collapse of cytoskeleton	+ (GAP domain)
		C3 exotoxin	*C. botulinum*	ADP-ribosylation of Rho	Breakdown of cellular actin stress fibers	+
		EDIN-A, B and C	*S. aureus*	ADP-ribosylation of Rho	Modification of actin cytoskeleton	EDIN-B
		SopE	*S. typhimurium*	Rac and Cdc42 activation	Membrane ruffling, cytoskeletal reorganization, proinflammatory cytokines production	+
		SipA	*S. typhimurium*	Rac and Cdc42 activation	Membrane ruffling, cytoskeletal reorganization, proinflammatory cytokines production	+

(*Continued*)

Table 1. *Continued*

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
		IpaA	*Shigella species*	Vinculin binding	Depolymerization of actin filaments	–
		YopE	*Yersinia species*	GAP activity towards RhoA, Rac1 or Cdc42	Cytotoxicity, actin depolymerization	+
		YopT	*Yersinia species*	Cysteine protease, cleaves RhoA, Rac, and Cdc42 releasing them from the membrane	Disruption of actin cytoskeleton	–
		VirA	*Shigella flexneri*	Inhibition of tubulin polymerization	Microtubule destabilization and membrane ruffling	–
	Signal transduction	YpkA	*Yersinia species*	Protein serine/threonine kinase	Inhibition of phagocytosis	–
		YopH	*Yersinia species*	Tyrosine phosphatase	Inhibition of phagocytosis	+
		Tir	*E. coli EPEC*	Receptor for intimin	Actin nucleation and pedestalformation	–
		CagA	*H. pylori*	Tyrosine phosphorylated	Cortactin dephosphorylation	–
		YopM	*Yersinia species*	Interaction with PRK2 and RSK1 kinases	Cytotoxicity	+
		SptP	*S. typhimurium*	Inhibition of the MAP kinase pathway	Enhancement of Salmonella capacity to induce TNF-alpha secretion	+
		ExoU	*P. aeruginosa*	Lysophospholipase A activity	Lung injury	–
Toxins with unknown mechanism of action		Zot	*V. cholerae*	?	Modification of intestinal tight junction permeability	–
		Hemolysin BL (HBL)	*B. cereus*	Hemolytic, dermonecrotic and vascular permeability activities	Food poisoning, fluid accumulation and diarrhea	–
		BSH	*L. monocytogenes*	?	Increased bacterial survival and intestinal colonization	–

Abbreviations: SEA-SEI, staphylococcal enterotoxins; TSST, toxic shock syndrome toxin; SPE, streptococcal exotoxin; SSA, streptococcal superantigen; SMEZ, streptococcal mitogenic exotoxin z; MAM, *Mycoplasma* arthritidis mitogen; YPMa, *Y. pseudotuberculosis*-derived mitogen; ETA and ETB, exfoliative toxins; ColH, collagenase; Nhe, nonhemolytic entertoxin; PFO, perfringolysin O; SLO, streptolysin O; LLO, listeriolysin O; ALO, anthrolisin O; AT, α-toxin; PA, protective antigen; DT, diphtheria toxin; PAETA, *Pseudomonas aeruginosa* exotoxin A; SHT, Shiga toxin; PT, pertussis toxin; CT, cholera toxin; LT, heat-labile enterotoxin; DNT, dermonecrotic toxin; CDT, cytolethal distending toxin; TeNT, tetanus neurotoxin; RTX, repeats in the structural toxin; Hly, hemolysin; Cry, crystal; BoNT, botulinum neurotoxin; Ipa, invasion plasmid antigen; Sip, *Salmonella* invasion protein; EDIN, epidermal cell differentiation inhibitor; Sop, *Salmonella* outer protein; Ipg, invasion plasmid gene; Yop, *Yersinia* outer protein; GAP, GTPase-activating protein; GAS, group A *Streptococcus*; Vir, virulence protein; YpkA, *Yersinia* protein kinase A; Tir, translocated intimin receptor; EPEC, enteropathogenic *E. coli*; CagA, cytotoxin-associated gene A; SptP, *Salmonella* protein tyrosine phosphatase; VAMP, vesicle-associated membrane protein; ICE, interleukin-1β-converting enzyme; SNAP, synaptosome-associated protein; MAPKK, mitogen-activated protein kinase kinase; Zot, zonula. occludens toxin; and BSH, bile salt hydrolase.

Table 2. Toxins classified according to their enzymatic activities.

Toxin	Substrate	Effect
Glucosyl-transferases		
Clostridium difficile toxins A and B	Rho/Ras GTPases	Breakdown of cytoskeletal structure
Deamidases		
E. coli CNF1	Rho, Rac and CdC42	Stress fiber formation
Bordetella DNT	Rho	Stress fiber formation
ADP-ribosyltransferases		
DT	Elongation factor EF-2	Cell death
PAETA	Elongation factor EF-2	Cell death
PT	G_i, G_o and transducin	cAMP increase
CT	G_s, G_t and G_{olf}	cAMP increase
E. coli LT	G_s, G_t and G_{olf}	cAMP increase
Clostridium botulinum C2	Actin	Failure in actin polymerization
P. aeruginosa ExoS	Ras	Collapse of cytoskeleton
Clostridium botulinum C3	Rho	Breakdown of cellular actin stress fibers
N-Glycosidases		
Shiga toxin	Ribosomal RNA	Stop of protein synthesis
Metalloproteases		
Bacillus anthracis LF	Macrophages	Disruption of normal homoeostatic functions
Clostridium tetanii TeNT	VAMP/synaptobrevin	Spastic paralysis
C. botulinum BoNTs	VAMP/synaptobrevin, SNAP-25	Flaccid paralysis

Abbreviations: CNF1, cytotoxin necrotizing factor 1; DNT, dermonecrotic factor; DT, diphtheria toxin; PAETA, *Pseudomonas aeruginosa* exotoxin A; PT, pertussis toxin; CT, cholera toxin; LT, heat-labile enterotoxin; ExoS, exoenzyme S; LF, lethal factor; TeNT, tetanus neurotoxin; BoNT, botulinum neurotoxin; VAMP, vesicle associated membrane protein; and SNAP-25, synaptosome-associated protein of 25kDa.

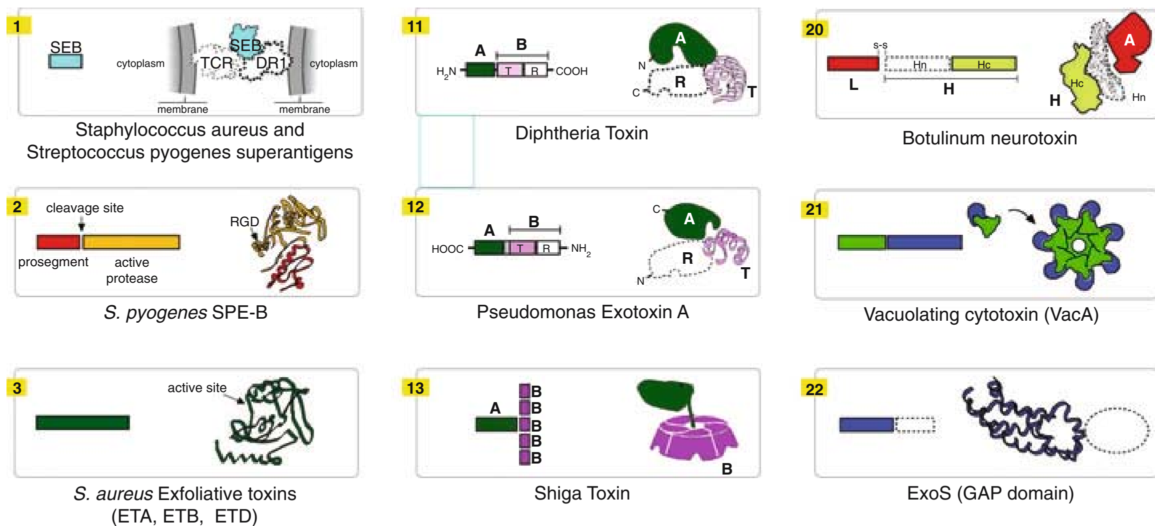

Fig. 1. Structural features of bacterial toxins. (Left) Scheme of the primary structure of each toxin. For the A/B toxins, the domain composition is also shown. The A (or S1 in PT) represents the catalytic domain, whereas the B represents the receptor-binding domain. The A subunit is divided into the enzymatically active A1 domain and the A2 linker domain in Shiga toxin, CT, *Escherichia coli* LTI and LTII, and PT. The B domain has either five subunits, which are identical in Shiga toxin, CT, and *E. coli* LTI and LTII and different in size and sequences in PT, or two subunits (the translocation [T] and the receptor-binding [R] subunits) in DT, Pseudomonas exotoxin A, botulinum toxin, and tetanus toxin. (Right) Schematic representation of the three-dimensional (3D) organization of each toxin. For *Staphylococcus* enterotoxin B, the protein is shown in the ternary complex with the human class II histocompatibility complex molecule (DR1) and the T-cell antigen receptor (TCR). For *Salmonella* SptP, the structure is shown in the transition state complex with the small GTP binding protein Rac1. Similarly, toxin SopE is represented in complex with its substrate Cdc42. In the case of *E. coli* CNF1 and *Pseudomonas* ExoS, only one domain has been crystallized. In the case of SipA, a 3D reconstruction of SipA bound to F-actin filaments is also reported. For all toxins, the schematic representation is based on the X-ray structure, except that for VacA, whose structure has been solved by quick-freeze, deep-etch electron microscopy.

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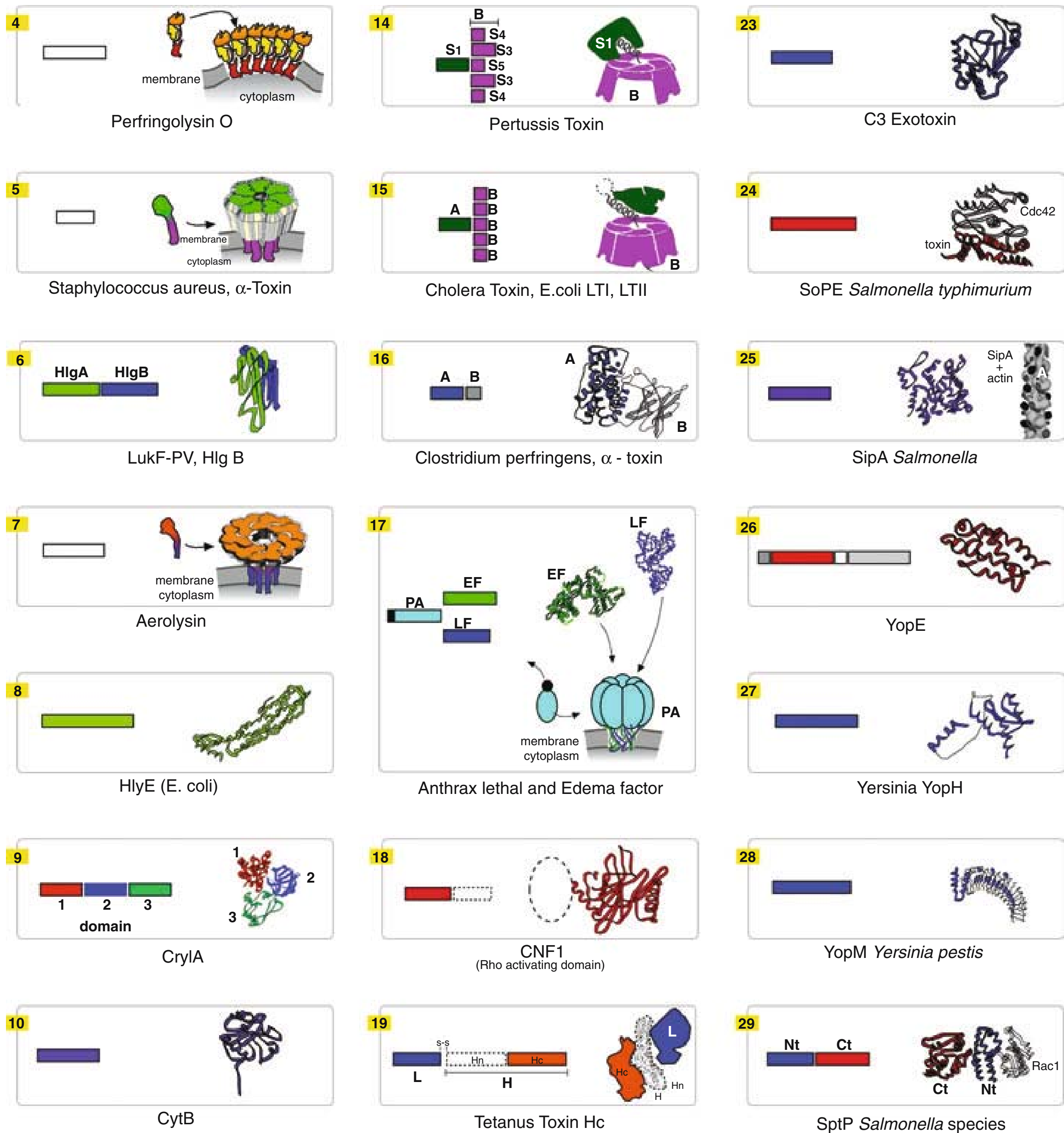


Fig. 1. *Continued.*

SEI), exfoliative toxins (ETA and ETB), the toxic shock syndrome toxin-1 (TSST-1; Dinges et al., 2000), the streptococcal pyrogenic enterotoxins (SPEA and SPEC; Papageorgiou et al., 1999) and streptococcal superantigen SSA (Sundberg and Jardetzky, 1999).

These toxins play an important role in diseases such as the staphylococcal toxic shock syndrome induced by TSST-1 (Schlievert et al., 1981), vomiting and diarrhea caused by staphylococcal enterotoxins, and the exanthemas caused by the pyrogenic streptococcal exotoxins. Furthermore, these toxins have been linked to the pathogenesis of several acute or chronic human disease states such as the Kawasaki syndrome (Leung et al., 1993), which is the leading cause of acquired heart disease among children in the United States, and to the pathogenesis of other life-threatening events such as food poisoning (Blackman and Woodland, 1995).

In addition to their functional similarities, the staphylococcal enterotoxins share a number of genetic and biochemical characteristics, as well as similar primary (Schlievert et al., 1995) and 3D structures (Swaminathan et al., 1992; Prasad et al., 1993; Papageorgiou et al., 1995; Schad et al., 1995). The genes for these toxins are generally carried on plasmids, bacteriophage chromosomes, or other heterologous genetic elements (Lindsay et al., 1998; Zhang et al., 1998), and all

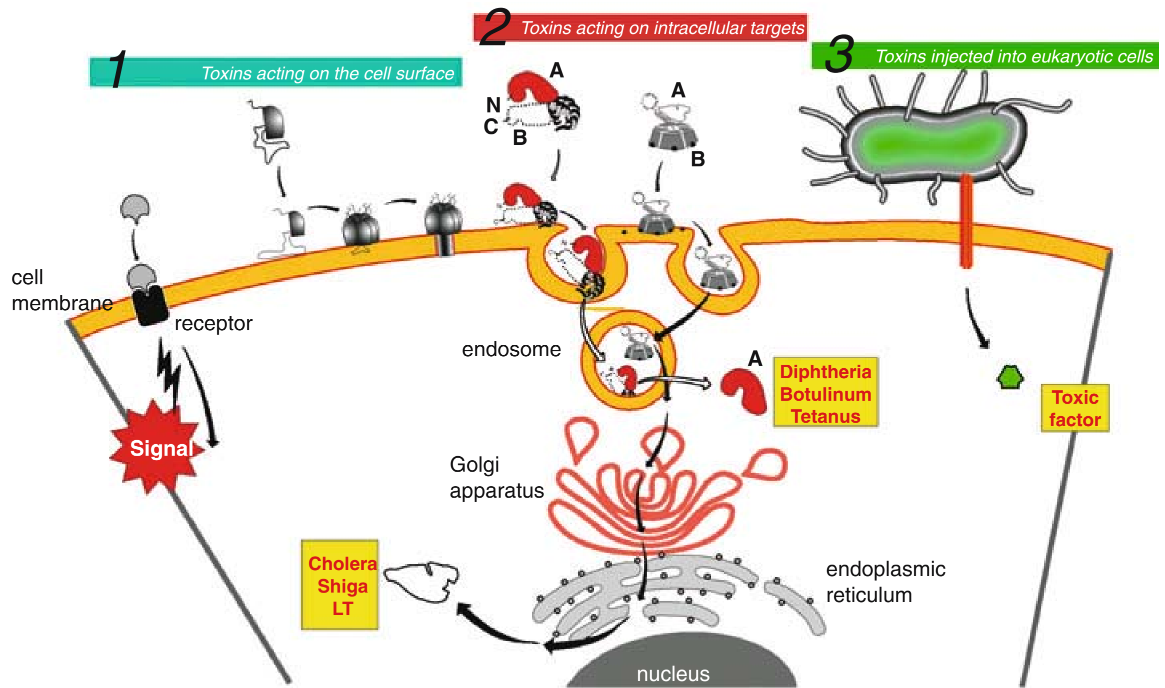


Fig. 2. Schematic representation of the three groups of bacterial toxins. Group 1 toxins act either by binding receptors on the cell membrane and sending a signal to the cell or by forming pores in the cell membrane, perturbing the cell permeability barrier. Group 2 toxins are A/B toxins, composed of a binding domain (B subunit) and an enzymatically active effector domain (A subunit). Following receptor binding, the toxins are internalized and located in endosomes, from which the A subunit can be transferred directly to the cytoplasm by using a pH-dependent conformational change or can be transported to the Golgi and the endoplasmic reticulum (ER), from which the A subunit is finally transferred to the cytoplasm. Group 3 toxins are injected directly from the bacterium into the cell by a specialized secretion apparatus (type III or type IV secretion system).

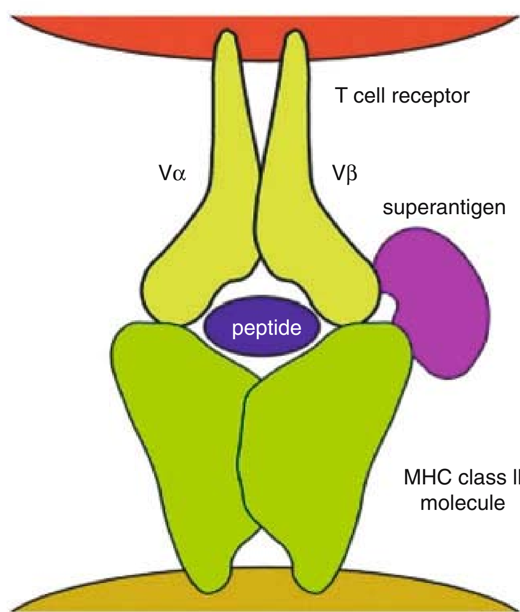


Fig. 3. Schematic representation of the interaction of a superantigen with a major histocompatibility complex (MHC) class II molecule and T-cell receptor.

of them are translated into a precursor protein containing an amino terminal signal sequence that is cleaved during export from the cell. The mature products are small nonglycosylated polypeptide molecules with molecular weights ranging from 20 kDa to 30 kDa and are moderately stable to chemical inactivation, proteolysis and denaturation by boiling.

Staphylococcal and streptococcal superantigens share 20–80% sequence similarity (Fig. 4); in particular, staphylococcal SEA is more related to SEE and SED, whereas SEB has greater homology with SEC, TSST-1, and streptococcal superantigens SPEA and SSA. The overall homology found in the staphylococcal enterotoxins has been suggested to stem from duplication of a gene encoding a common "ancestral" toxin (Iandolo, 1989).

Computer analysis of the *S. pyogenes* genome has revealed the presence of novel superantigen genes, and among them the one coding for the mitogenic exotoxin Z (SMEZ). This toxin is particularly similar to the SPE-C group of superantigens and, although present in all group A streptococci (GAS) strains, it shows extensive

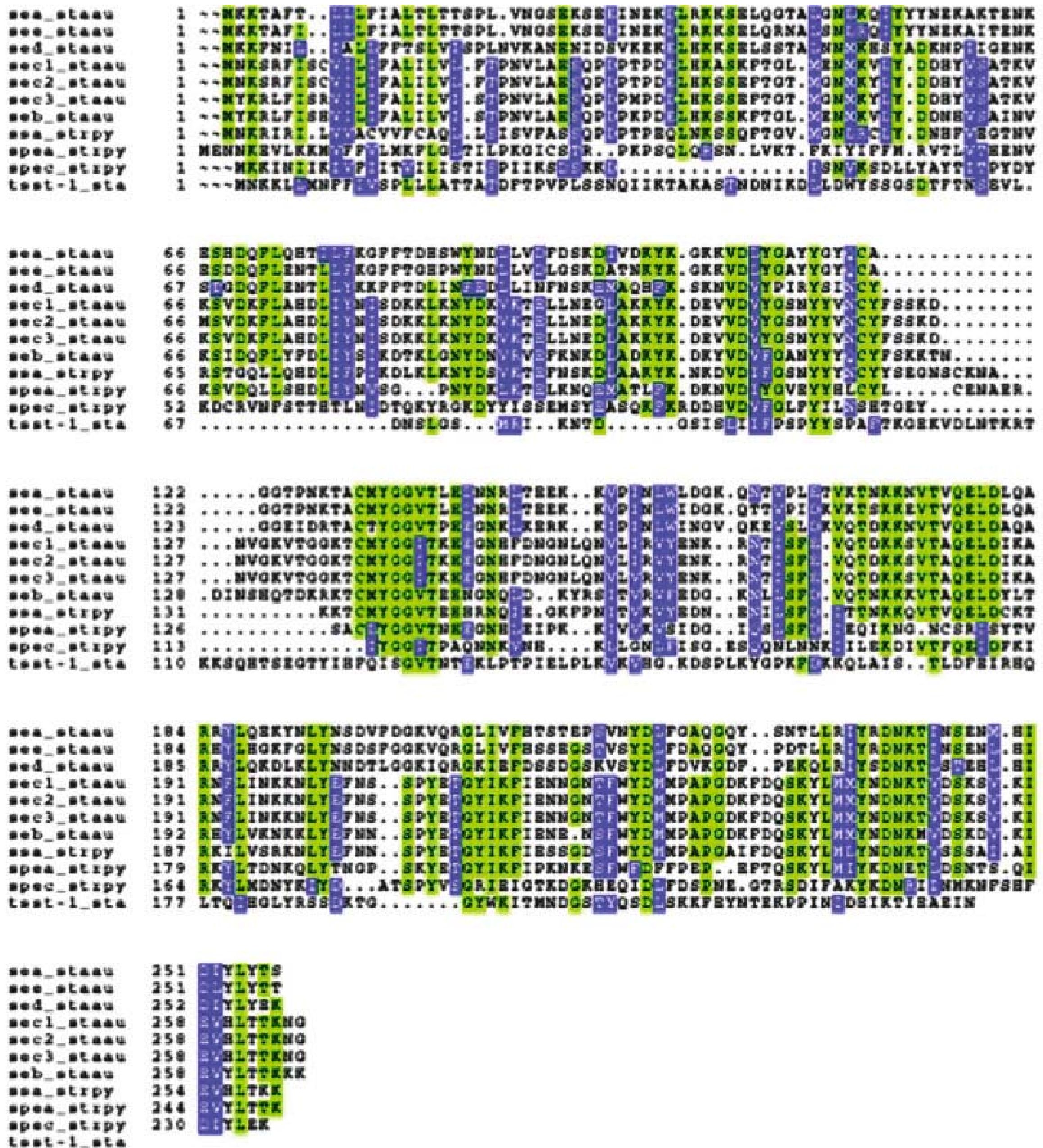


Fig. 4. Multiple sequence alignment of staphylococcal and streptococcal superantigens. Green indicates identity, whereas blue stands for amino acid similarity.

allelic variation. Further genetic characterization has shown that SMEZ is the most potent bacterial superantigen so far discovered and that it strongly contributes to the immunological effects of GAS both in vitro and in vivo by eliciting a robust cytokine production (Unnikrishnan et al., 2002).

Three novel streptococcal genes (*spe-g*, *spe-h* and *spe-j*) have been identified from the *Streptococcus pyogenes* M1 genomic sequence, while a fourth novel gene (*smez-2*) was isolated from the strain 2035. Of these, SMEZ-2, SPE-G and SPE-J are most closely related to streptococcal pyrogenic exotoxin SPEC, whereas SPE-H is more similar to the staphylococcal toxins than to any other streptococcal toxin (Proft et al., 1999).

Finally, other pyrogenic toxin superantigens recently discovered by genome mining include proteins SPEL and SPEM produced by several isolates of *S. pyogenes* of the M18 serotype. The corresponding genes are contiguous and coded within a bacteriophage. Both toxins were shown to be lethal in different animal models and to directly participate in the host-pathogen inter-

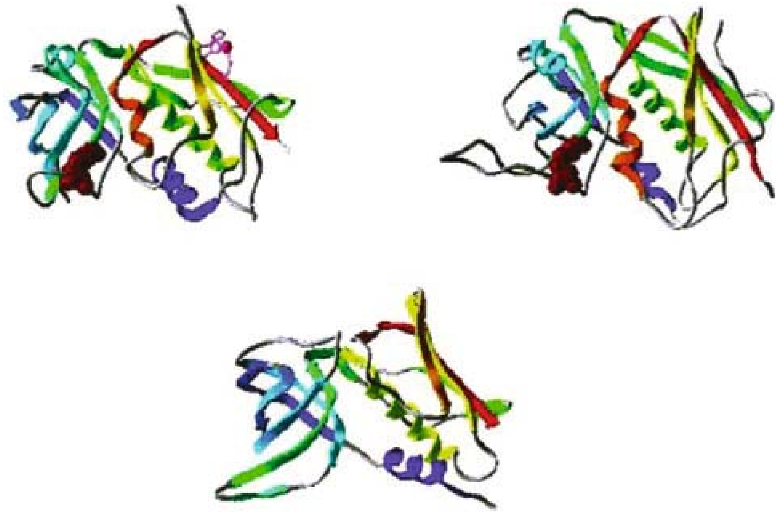


Fig. 5. Comparison of the X-ray structures of SEA (*left*), SEB (*right*) and TSST-1 (*below*). The colors follow the secondary structure succession where the N-terminus is blue, the C-terminus is red, and the long central helix is pale yellow. The zinc atom and the coordination site are colored pink and the cysteines involved in the disulfide bond are dark-red.

action in some acute rheumatic fever (ARF) patients (Proft et al., 2003).

Crystallographic structures are currently available for most of the described staphylococcal and streptococcal superantigens, such as SEA (Schad et al., 1995), SEB (Swaminathan et al., 1992), SEC2 (Papageorgiou et al., 1995), SEC3 (Fields et al., 1996), SED (Sundstrom et al., 1996), TSST-1 (Prasad et al., 1993; Prasad et al., 1997), SPEA (Papageorgiou et al., 1999), SPEB (Kagawa et al., 2000), SPEC (Roussel et al., 1997) and SSA (Sundberg et al., 1999). However, primary sequence homology among superantigens does not assure homology in their secondary and tertiary structures, and vice versa; in fact SEA, SEB, SEC and TSST-1, despite their low level of sequence similarity, all fold into very similar 3D structures. Below are the X-ray structures of SEA, SEB and TSST-1 that share a very similar fold despite low levels of sequence similarity that range from less than 20% identity in the case of SEA and TSST-1, to 33% in the case of SEA and SEB.

All of these toxins have a characteristic two-domain fold composed of a β-barrel at the *N*-terminus and a β-grasp at the C-terminus connected by a long α-helix that diagonally spans the center of the molecule (Fig. 5). Moreover, all of these toxins are characterized by a central disulfide bond (with the exception of TSST-1, which has no cysteines) and by a Zn^{+2} coordination site which is believed to be involved in MHC class II binding (Abrahmsen et al., 1995).

The presence of two zinc-binding sites in SpeC indicates different modes in the assembly of the MHC-superantigen-T-cell receptor (TcR) trimolecular complex.

The crystal structures of SEB and TSST-1 in complex with an MHC class II molecule, and those of SEC2/SEC3 in complex with a TcR Vβ chain have been solved (Li et al., 1998; Fields et al., 1996). As an example, the complex between SEB and the Vβ domain of a TcR is reported (Fig. 6).

Superantigen molecules have also been identified in other pathogens, where they represent important virulence determinants.

MaM is a T-cell mitogen produced by *Mycoplasma arthritidis*, which contributes to the acute and chronic inflammatory disease mediated by this organism (Cole and Atkin, 1991). The recently determined X-ray structure of MaM in complex with HLA-DR1 has revealed that this protein has a fold and a mode of binding, which are entirely different from those of the known pyrogenic superantigens (Zhao et al., 2004; Fig. 7).

Another superantigenic toxin is the YPMa produced by a subset of *Yersinia pseudotuberculosis* strains. This 14.5-kDa protein was originally purified from bacterial lysates and found to exert a mitogenic activity on human peripheral blood

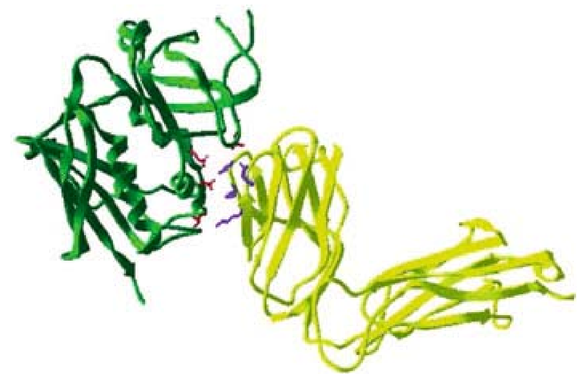


Fig. 6. Crystal structure of the complex between SEB (green) and TcR (yellow). The residues involved in hydrogen bonds between the two molecules have side-chains colored in red and blue, respectively.

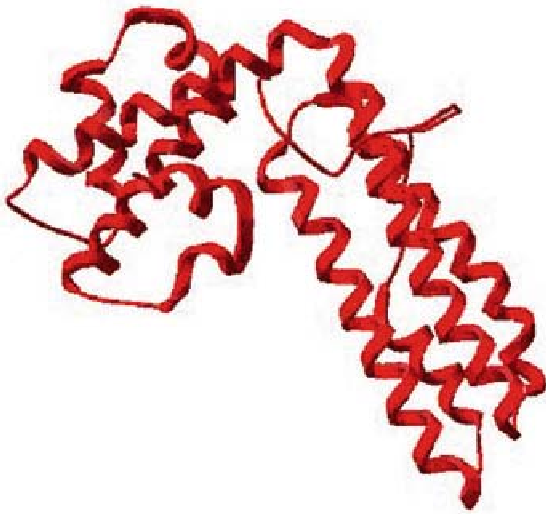


Fig. 7. Three-dimensional structure of MaM superantigen.

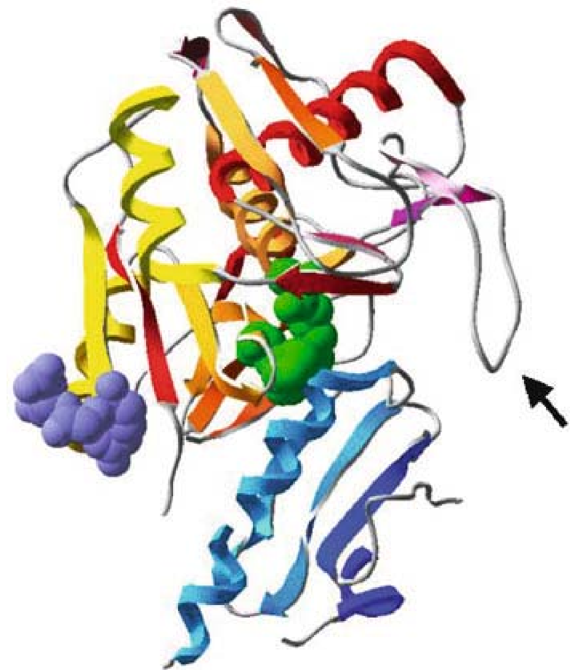


Fig. 8. The three-dimensional structure of the precursor form of streptococcal cysteine protease SpeB. The prosegment (blue) and active protease (yellow-orange) are indicated with different color scales. The solvent-exposed Arg-Gly-Asp (RGD) motif is violet, and the active site (Cys-47-His-195-Trp-212) is buried by the prosegment and is colored in green. The highly conserved finger loop is also indicated (arrow).

mononuclear cells. Although the precise role of this protein is currently unknown, the data show that YPMa contributes to the virulence of *Y. pseudotuberculosis* in systemic infection in mice (Carnoy et al., 2000).

Other toxins that have long been known as superantigens are the streptococcal pyrogenic exotoxin B (SPEB), a virulence factor with cysteine protease activity produced by all isolates of group A streptococci, and the exfoliative toxins A and B produced by *S. aureus* (Fig. 2, panels 2 and 3).

Although these proteins strongly contribute to the virulence of the corresponding microorganism, their role as mitogenic factors has been disproved when it was shown that all the nonrecombinant forms were in fact contaminated with trace amounts of the SMEZ superantigen (Unnikrishnan et al., 2002).

SPEB appears to contribute to *S. pyogenes* pathogenesis in several ways, including proteolytic cleavage of human fibronectin and vitronectin, two abundant extracellular matrix proteins involved in maintaining host tissue integrity. SPEB causes a cytopathic effect on human endothelial cells and represents a critical virulence factor in human infection and in mouse models of invasive disease. Despite low levels of sequence similarity, this toxin can be considered as a structural homologue of the papain superfamily that also includes the mammalian cathepsins B, K and L (Kagawa et al., 2000). Like other proteases, the enzyme SpeB is produced as an inactive precursor (zymogen) of 40 kDa which, following autolytic cleavage of the N-terminal 118 residues, is converted to the mature, active 27.6-kDa protease. The catalytic site lacks the Asn residue generally present in the catalytic Cys-His-Asn triad, which is in this case substituted by a Trp. The structure also reveals the presence of a surface-exposed integrin-binding Arg-Gly-Asp (RGD) motif that is a feature unique to SpeB among cysteine proteases and is linked to the pathogenesis of the most invasive strains of *S. pyogenes* (Stockbauer et al., 1999). Sequence analysis performed on more than 200 streptococcal isolates has revealed an overall limited structural variation in SPEB, with the entire active site being completely conserved. Interestingly, the prominent finger loop that extends from the N-terminal domain (Fig. 8) is also invariant, suggesting that antibodies directed against this region could be effective therapeutic agents.

The exfoliative toxins ETA and ETB of *Staphylococcus aureus* are produced during the exponential phase of growth and excreted from colonizing staphylococci before being absorbed into the systemic circulation. They have been recognized as the causative agents in staphylococcal scalded skin syndrome, an illness characterized by specific intraepidermal separation of the layers of skin between the stratum spinosum and the stratum granulosum (Ladhani et al., 1999). The two ETs are about 40% identical, with no apparent sequence homology to other bacterial

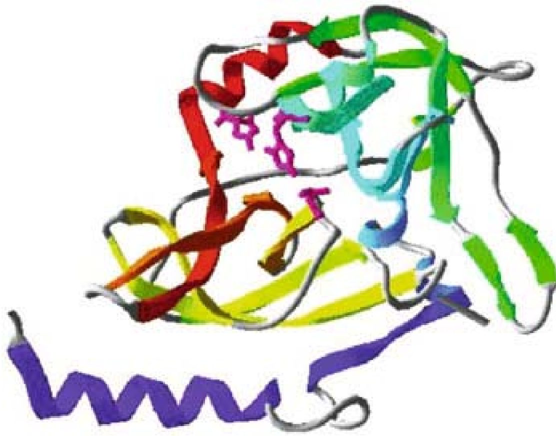


Fig. 9. Crystal structure of exfoliative toxin A (ETA) of *Staphylococcus aureus*. The three residues of the catalytic triad responsible for the serine protease activity are colored in magenta.

toxins. Both superantigens have been proved to act as serine proteases, and this enzymatic activity could be one of the mechanisms hypothesized as the cause of epidermal separation. In fact, at least in the case of ETA (Fig. 8), substitution of the active site serine residue with cysteine abolishes its ability to produce the characteristic separation of epidermal layers but not its ability to induce T-cell proliferation (Redpath et al., 1991). The two ETs are about 40% identical, with no apparent sequence homology to other bacterial toxins. The overall structures of ETA and ETB are similar to that of the chymotrypsin-like serine protease family of enzymes, with the catalytic triad being composed of His-57, Asp-102 and Ser-195 (Vath et al., 1997, 1999).

Recently, a novel member of the exfoliative group of toxins has been discovered in *S. aureus*. This protein, termed "ETD," is encoded within a pathogenicity island, which also contains the genes for a serine protease and the edin-B gene. When injected in neonatal mice as recombinant protein, ETD has been shown to induce exfoliation of the skin with loss of cell-to-cell adhesion in the upper part of the epidermis (Yamaguchi et al., 2002).

Toxins Acting on Surface Molecules

Bacteroides fragilis enterotoxin (BFT) is a protein of 186 residues that is secreted into the culture medium. The toxin has a zinc-binding consensus motif (HEXXH), characteristic of metalloproteases and other toxins such as tetanus and botulinum toxins. In vitro, the purified enterotoxin undergoes autodigestion and can cleave a number of substrates including gelatin, actin, tropomyosin and fibrinogen. When added to cells in tissue culture, the toxin cleaves the 33-kDa extracellular portion of E-cadherin, a 120-kDa transmembrane glycoprotein (responsible for calcium-dependent cell-cell adhesion in epithelial cells) that also serves as a receptor for *Listeria monocytogenes*. In vitro, BFT does not cleave E-cadherin, suggesting that the membrane-embedded form of E-cadherin is necessary for cleavage.

BFT causes diarrhea and fluid accumulation in ligated ileal loops. In vitro, it is nonlethal but causes morphological changes such as cell rounding and dissolution of tight clusters of cells. The morphological changes are associated with F-actin redistribution. In polarized cells, BFT is more active from the basolateral side than from the apical side, decreases the monolayer resistance, and causes dissolution of some tight junctions and rounding of some of the epithelial cells, which can separate from the epithelium. In monolayers of enterocytes, BFT increases the internalization of many enteric bacteria such as *Salmonella*, *Proteus*, *E. coli* and *Enterococcus* but decreases the internalization of *L. monocytogenes* (Sears, 2001).

BFT belongs to a large family of bacterial metalloproteases that usually cleave proteins of the extracellular matrix. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* elastases (aminopeptidase and AhyB) and *Clostridium histolyticum* collagenase (ColH) are the best-known examples (Yoshihara et al., 1994; Cascon et al., 2000; Cahan et al., 2001).

Lately, a novel member of this family of protein toxins has been identified in *Bacillus cereus*. The protein, termed "Nhe" (nonhemolytic enterotoxin), is a 105-kDa metalloprotease, which shares homologies to the above-mentioned elastases and collagenases. Biochemical characterization has shown that Nhe possesses both gelatinolytic and collagenolytic activities (Lund and Granum, 1999).

Toxins Acting on the Cell Membrane

Protein toxins forming pores in biological membranes occur frequently in Gram-positive and Gram-negative bacteria (Braun and Focareta, 1991). Pore-forming toxins, also known as "lytic factors," work by punching holes in the plasma membrane of eukaryotic cells, thus breaking the permeability barrier that keeps macromolecules and small solutes selectively within the cells (Sugawara et al., 1997; Gilbert, 2002; Fig. 2, panel 1). Because erythrocytes have often been used to test the activity of these toxins, some of them are also called "hemolysins"; however, whereas erythrocytes appear to be very good targets in vitro, they are never the main physiological targets of this class of proteins in vivo (Tomita et al., 1997).

The pathogenicity of the toxin-producing organisms in eukaryotes is clearly related to the toxins they produce. Furthermore, pore-forming toxins represent the most potent and versatile tool with which invading microbes damage the host cell (Bhakdi et al., 1994). Cell permeabilization exerted by the toxic activity of these proteins generally results in release of cytokines, activation of intracellular proteases, induction of apoptosis, and finally, death of the eukaryotic cell (Alouf and Geoffrey, 1991).

To generate channels and holes in the cell membrane, this class of toxins must be able to fold in a characteristic amphipathic structure typical of porins (Weiss et al., 1991; Cowan et al., 1992), with one side facing the internal hydrophilic cavity, and the other side interacting with the lipid chains or the nonpolar segments of integral membrane proteins.

Most of the toxins are produced or stored in a protoxin inactive form. The activation step varies from the cleavage of an N_{term} acidic peptide as in the case of melittin, to a C_{term} proteolytic cleavage as in aerolysin (van der Goot et al., 1992); in the particular case of the Gram-negative hemolysins (cytolysins), these toxins are usually synthesized as precursor proteins, then covalently modified to an acylated, active form and finally secreted via specific export systems, which differ for various types of hemolysins (Issartel et al., 1991; Stanley et al., 1994). All such steps increase the affinity for the membrane, which appears to be essential for activity.

A large proportion of these proteins are produced by Gram-positive bacteria and can be divided into large pore-forming and small pore-forming toxins on the basis of the dimension of the holes produced on the plasma membrane and also of the kind of interaction that they establish with the eukaryotic receptor. In addition, the pore-forming, repeats-in-toxin (RTX) family of toxins includes a large group of Ca^{+2}-dependent hemolysins (secreted by both Gram-positive and Gram-negative bacteria), which are characterized by a conserved glycine- and aspartate-rich motif of nine amino acids (Welch, 1991; Coote, 1992). Given their predominant role on cellular membranes, we have included in this section also the so-called "membrane perturbing toxins" and the insecticidal toxins produced by *Bacillus thuringensis*.

Large Pore-Forming Toxins

This class of cytolysins (Fig. 2, panel 2) comprises more than 20 family members, which are generally secreted by taxonomically diverse species of Gram-positive bacteria and which have the common property of binding selectively to cholesterol on the eukaryotic cell membrane (Alouf and Geoffrey, 1991). Each toxin consists of a single 50- to 80-kDa polypeptide chain, and they are characterized by a pretty remarkable sequence similarity, also suggesting possible similar 3D structures. These proteins are produced by *Streptococcus pyogenes*, *S. pneumoniae*, *Bacillus*, a variety of *Clostridia*, including *Clostridium tetanii* and *C. perfringens*, and *Listeria*.

To date, the best characterized are perfringolysin O (PFO), a virulence factor of *Clostridium perfringens*, which causes gas gangrene (Rossjohn et al., 1997), streptolysin O, secreted by *Streptococcus pyogenes* (Kehoe et al., 1987), alveolysin, produced by Bacillus alvei (Geoffroy et al., 1990), and pneumolysin, the major causative agent of streptococcal pneumonia and meningitis (Rossjohn et al., 1998).

In addition to its role as a cytolysin, listeriolysin O (LLO), which is an essential virulence factor of *Listeria monocytogenes* (Gedde et al., 2000) has also been shown to induce lymphocyte apoptosis with rapid kinetics (Carrero et al., 2004).

These toxins share a similar mechanism of action, which consists of an interaction of monomeric toxin with target cells via cholesterol (their receptor), followed by oligomerization and insertion into the host cell membrane; this process ultimately results in serious membrane damage with formation of large pores with diameters exceeding 150 Å. All these toxins contain a common motif (boxed in Fig. 10), which is located approximately 40 amino acids from the carboxy terminus; this motif includes a Cys residue, which if oxidized abolishes the toxin's lytic activities. Lytic activity can be restored only upon addition of reducing agents such as thiols. However, despite their designation as "thiol-activated cytolysins," thiol activation is clearly not an important property of this group of toxins (Billington et al., 2000). Interestingly, the membrane-bound receptor, cholesterol, plays an important role in the oligomerization step as well as in membrane insertion and pore formation (Alouf and Geoffrey, 1991).

Crystallographic data are available only for the thiol-activated cytolysin (perfringolysin O; PFO; Fig. 1, panel 4) of *Clostridium perfringens* (Rossjohn et al., 1997). Nevertheless, given the high degree of sequence conservation (Fig. 10) detected within this class of protein toxins (ranging from the 43% identity of PFO and listeriolysin, to the 72% identity of PFO and alveolysin), this structure can be considered the prototype of the entire family (Fig. 11).

PFO is an unusually elongated rod-shaped molecule mainly composed of β-sheets; the monomer is made of four discontinuous domains, indicated with different colors in the picture. Domain 1 (green) has an α/β structure

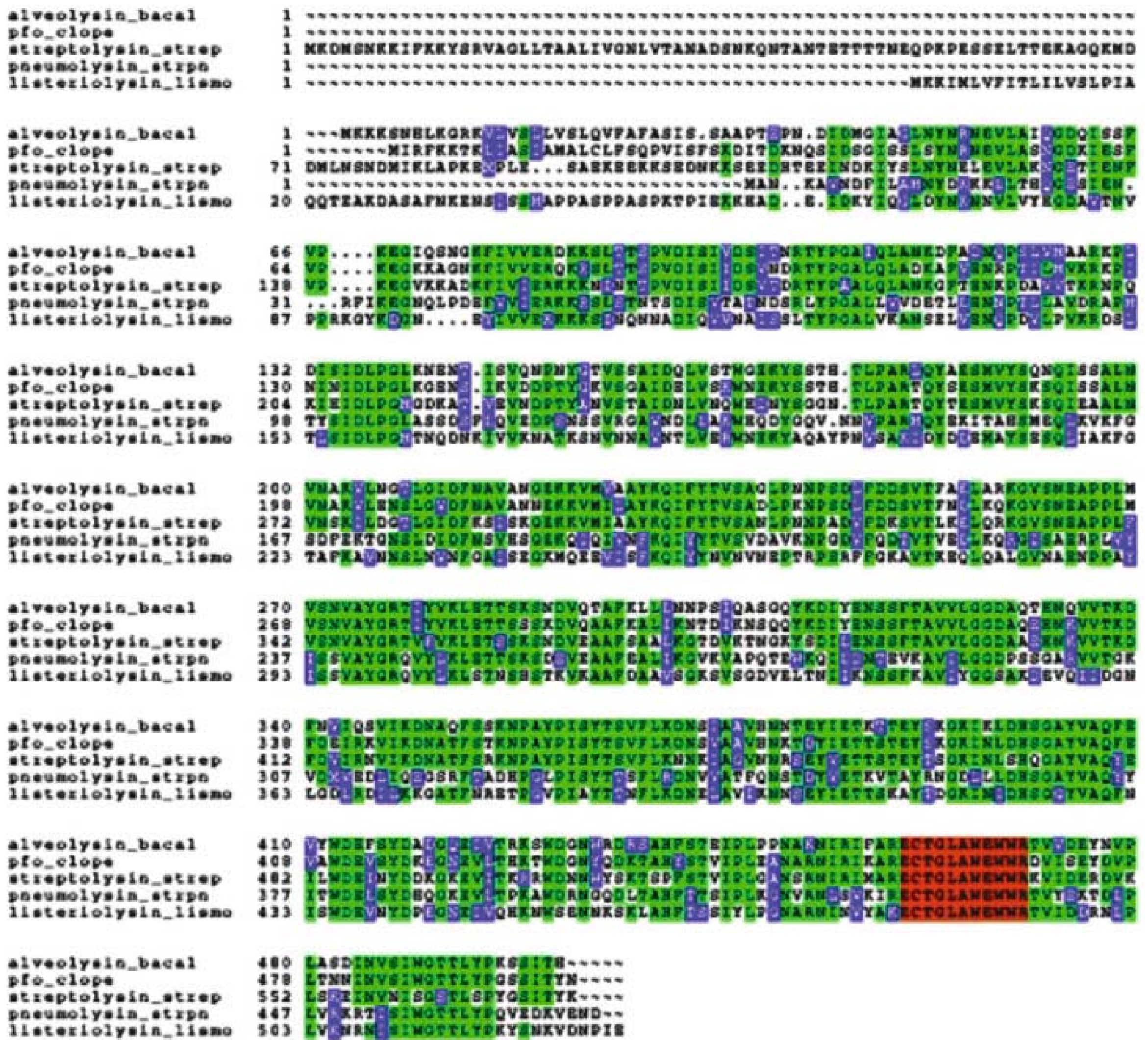


Fig. 10. Multiple sequence alignment of proteins belonging to the class of large-pore-forming toxins. Green indicates identity, whereas blue stands for amino acid similarity.

containing a seven-stranded antiparallel β-sheet. Domain 2 (blue) consists mainly of four β-strands, while domain 3 (yellow) is comprised of an α/β/a structure. Finally, domain 4 (red) is folded into a compact β-sandwich consisting of multiple-stranded sheets.

The mechanism of membrane insertion is not clear; in fact, no canonical transmembrane domains can be identified along the primary structure and no significant patches of hydrophobic residues can be mapped on the surface of the molecule. Nevertheless, a model of the membrane-bound state, which takes into account the interaction with the cholesterol receptor as the first step for penetration of the hydrophobic bilayer core, has been proposed on the basis of electron microscopy and other experimental data. Several chemical modifications and mutagenesis studies have suggested the cholesterol-binding site to be located at the tip of domain 4 (Fig. 12), and in particular, it has been mapped within the highly conserved, Trp-rich segment (Michel et al., 1990; Hill et al., 1994). Proteolysis studies have further demonstrated that domain 4 is also the membrane-spanning domain, although the distribution of charged and hydrophobic residues on the β-sheet of this region is not compatible with an insertion into the lipid bilayer. From these studies, it has emerged that only the tip of the β-barrel domain D4 is responsible for membrane insertion and that a major conformational rearrangement takes place during pore formation (Shepard et al., 1998; Shatursky et al., 1999).

Taken together, these observations suggest a model of oligomer insertion. After the toxin binds to the cholesterol molecule, the aliphatic side chains neutralize the charged resi-

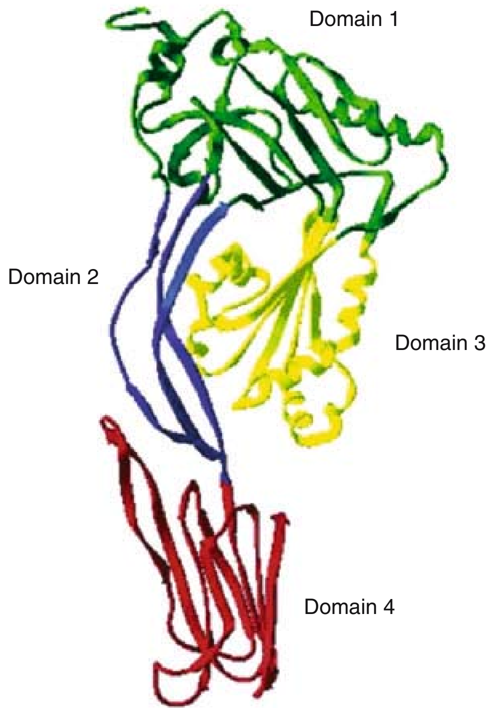


Fig. 11. Crystallographic structure and domain organization of perfringolysin O (PFO) produced by *Clostridium perfringens*.

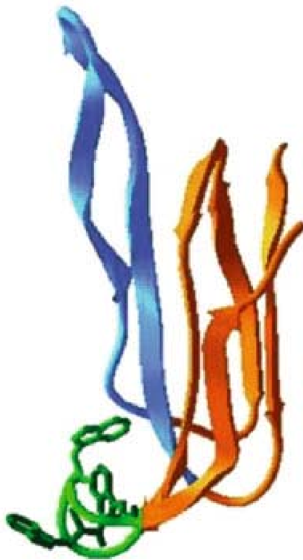


Fig. 12. Graphical representation of domain 4 of perfringolysin. The Trp-rich loop along with tryptophan side-chains are colored in green. In blue is the β-sheet probably involved in membrane insertion.

dues present on the β-sheet (blue) of domain 4 and then trigger membrane penetration. Consistent with this model is the hypothesis that the highly hydrophobic Trp-rich loop

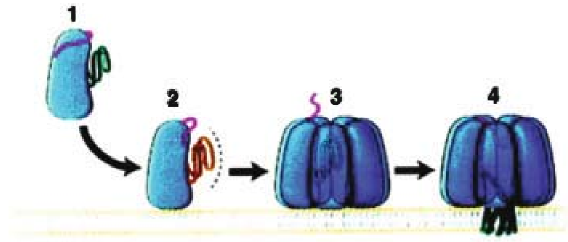


Fig. 13. General mechanism of assembly for small-pore-forming toxins: the stem region is initially folded against in the body of the water-soluble monomer; upon binding to the membranes and oligomerization, it subsequently undergoes conformational rearrangement and promotes insertion into the lipid bilayer.

could lead to and promote the final penetration step.

Furthermore, on the basis of recent data, a mechanism has been proposed whereby insertion into the bilayer occurs only after PFO monomers have assembled into a pre-pore state. Monomer-monomer interactions therefore not only promote insertion, but cooperative interactions between PFO monomers appear to be required to drive transmembrane insertion and β-barrel formation (Hotze et al., 2002). Recently, a protein belonging to this class of cytolysins has been identified in *Bacillus anthracis* and named "anthrolysin O" (ALO). This putative toxin is able to bind erythrocytes and could have a role in the virulence of anthrax (Shannon et al., 2003).

Small Pore-Forming Toxins

The family of small-pore-forming toxins acts by creating very small pores (1–1.5 nm of diameter) in the membrane of host cells, thus allowing their selective permeabilization to solutes with a molecular mass less than 2 kDa. Alpha toxin (α-hemolysin) is the prototype of a group of pore-forming toxins produced by most pathogenic strains of *Staphylococcus aureus* (Gray and Kehoe, 1984a; Song et al., 1996; Gouaux, 1998; Fig. 1, panel 5); other members of this family include leukotoxins, such as leukocidin F (LukF), leukocidin S (LukS), Panton-Valentine leukocidin (PVL) and γ-hemolysin (Prèvost et al., 1995; Tomita and Kamio, 1997; Olson et al., 1999; Pedelacq et al., 1999; Cooney et al., 1993) and the β-toxin of *Clostridium perfringens* (Steinthorsdottir et al., 2000; Tweten, 2001; Magahama et al., 2003). These staphylococcal and streptococcal proteins are secreted as water-soluble monomers and assemble on the surface of susceptible cells to form heptameric transmembrane channels of approximately 1 nm in diameter (Finck-Barbancon et al., 1993; Sugawara et al., 1997; Fig. 13).

Fig. 14. Multiple sequence alignment of proteins belonging to the family of small pore-forming toxins. Green and blue stand for amino acid identity and similarity, respectively.

The monomers have molecular weights of 33 kDa and are related in sequence and function (Fig. 14).

These toxins bind to human erythrocytes, monocytes, platelets, lymphocytes and endothelial cells, causing (at high concentrations) membrane rupture and cell lysis and death. Alpha-toxin has been recently shown to be the major mediator of caspase activation and apoptosis (Haslinger et al., 2003).

The structure of the transmembrane pore of staphylococcal α-toxin has been solved and has confirmed the heptameric structure of the oligomer (Song et al., 1996; Fig. 15). The complex is mushroom-shaped and measures 100 Å in height and up to 100 Å in diameter; the aqueous channel forms the transmembrane pore and spans the length of the entire complex ranging from 14 Å to 46 Å in diameter.

Each protomer (Fig. 16) is mainly composed of β-strand elements; two of these in particular constitute the stem domain, which contributes to the formation of the transmembrane pore in the heptameric form of the complex; a glycine-rich

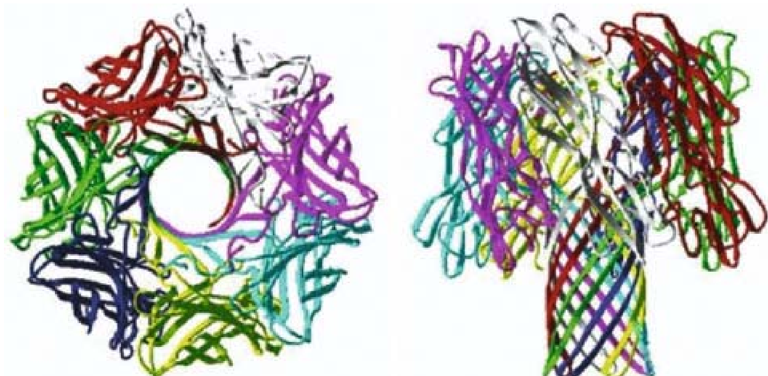


Fig. 15. Top and side views of the heptameric complex of a-toxin; each monomer is represented here with a different color (see Fig. 1, panel 5).

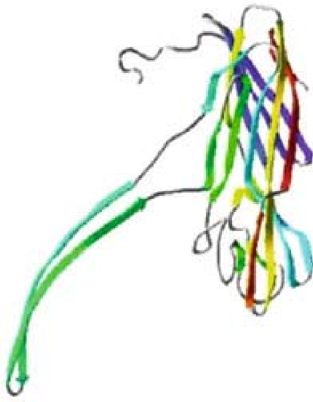


Fig. 16. Structure of the monomer of *S. aureus* α-toxin; the stem domain involved in pore formation protrudes outside of the core of the structure.

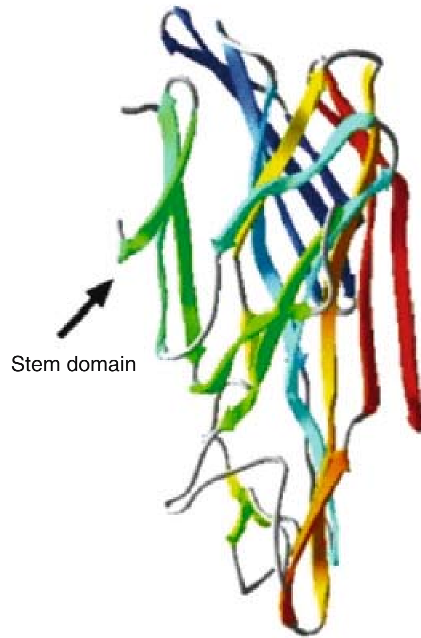


Fig. 17. Crystallographic structure determined for the protomer of toxin LukF; the glycine-rich, stem domain is in this case folded against the main body of the structure.

segment that is probably involved in solvent interaction characterizes this domain.

Leukotoxins and γ-hemolysin (HγII) should be grouped together, inasmuch as they form two types of bi-component complexes (LukF+LukS and LukF+HγII) that exhibit leukotoxic and hemolytic activity, respectively (Tomita and Kamio, 1997). Panton-Valentine leukocidin (PVL) is a closely related toxin carried by 2% of clinically isolated *S. aureus* strains and is also composed of type F and S components (Prèvost et al., 1995). The components of each protein class are produced as nonassociated, water-soluble proteins that undergo conformational changes and form oligomeric complexes after recognition of their cell targets, a process leading to transmembrane-pore formation and, ultimately, to cell death. The resultant transmembrane channels (estimated diameter 8 Å) are mainly permeable to divalent cations. Recently, fluorescence microscopy experiments have been performed to elucidate the mechanism of membrane insertion of the γ-hemolysin complex. This study shows that the three cooperative stages (dimer-dimer interaction, single pore assembly, and aggregation of pores) enhance the efficiency of assembly of oligomeric pores (Nguyen et al., 2003).

As representative of this class of bi-component toxins, consideration is given the X-ray structure of the Luk-F protomer (Olson et al., 1999; Fig. 17), which has been solved at a 1.90 Å resolution. The superposition of this monomer with that of α-toxin shows that the core structures are very similar despite the relatively low primary sequence identity (32%); nevertheless, a conformational change has affected the region of the glycine-rich stem domain, which appears in this case as a compact β-sheet folded against the body of the structure.

From a structural point of view, in contrast to a wide range of bacterial and insect toxins that utilize α-helices to perturb or penetrate the bilayer, these pore-forming toxins (members of an emerging family of proteins) can be defined by their use of bilayer-spanning antiparallel β-barrels instead.

Since the initial discovery of the first small pore-forming toxins, the number of these proteins has grown to include several members, among which are the recently identified hemolysin II (HlyII), and cytotoxin K (CytK) of *Bacillus cereus*, implicated in necrotic enteritis (Lund et al., 2000; Hardy et al., 2001; Miles et al., 2002).

RTX Toxins

Escherichia coli hemolysin (HlyA) is a 110-kDa protein, which can be considered as the prototype of a class of pore-forming toxins mainly produced by Gram-negative bacterial pathogens (Felmlee et al., 1985; Welch, 1991). This well-represented family includes a large number of calcium-dependent cytolysins known as RTX toxins, which are produced by different genera of Enterobacteriaceae and Pasteurellaceae. Characterized by the presence of a conserved repeated glycine- and aspartate-rich motif of nine amino acids, these cytolysins have multiple calcium-binding sites essential for function (Felmlee and Welch, 1988).

The toxin is encoded by four genes, one of which, *hlyA*, encodes the 110-kDa hemolysin.

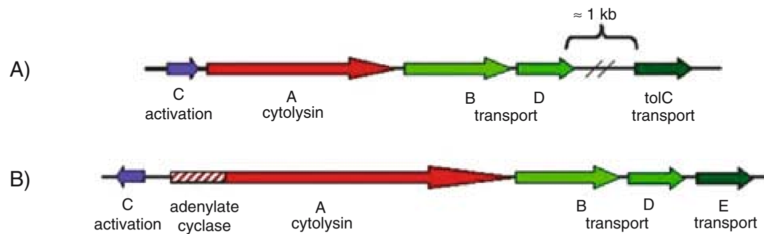


Fig. 18. Schematic representation of the genetic organization of RTX determinants; the genes encoding the Hly, Lkt, Aalt and Hpp proteins are organized in the same fashion, as illustrated in panel A, whereas the genes involved in synthesis and secretion of adenylate cyclase/hemolysin of *B. pertussis* display a somewhat different organization (panel B).

The other genes are required for its posttranslational modification (*hlyC*) and secretion (*hlyB* and *hlyD*). The four genes are found in a very limited number of *E. coli* clonal types, and can be sometimes located on transmittable plasmids (Smith and Halls, 1967). To give an idea of the level of toxicity associated with *hlyA* gene product, when non-hemolytic strains of *E. coli* are transformed with recombinant plasmids encoding the hemolysin, the transformants (in rodent models of peritonitis) are 10-fold to a 1000-fold more virulent than the parental strains. The receptor-binding domain of HlyA has been recently mapped (Cortajarena et al., 2003).

Other members of this class of RTX proteins include the adenylate cyclase/hemolysin of *Bordetella pertussis* (CyaA; Glaser et al., 1988), the ApxI-II and III hemolysins from Actinobacillus pleuropneumoniae (Maier et al., 1996), and the leukotoxins of *A. actinomycetemcomitans* (LtxA; Korostoff et al., 1998; Henderson et al., 2003) and of *Pasteurella haemolytica* (LktA; Chang et al., 1987; Wang et al., 1998).

Although a remarkable level of primary structure similarity can be detected among this group of toxins (20–60% identity), nevertheless they differ in host cell specificity and seem to adopt diverse mechanisms for cellular damage (Frey et al., 2002).

The synthesis and secretion of RTX toxins involve the participation of at least five different gene products; the organization of the five genes is very similar (Fig. 18, panel A), with the exception of B. pertussis bifunctional adenylate cyclase/hemolysin, where all five (*cyaC*, *A*, *B*, *D* and *E*) are found together (Glaser et al., 1988; Barry et al., 1991; Fig. 18, panel B); for the other family members, in fact, four of the genes are encoded within a single operon, whereas the fifth gene is located approximately 1 kb downstream (Welch and Pellett, 1988; Wandersman and Delepelaire, 1990).

The activation process performed by HlyC on HlyA ultimately results in the acquired capacity of HlyA to bind target cells; this activation involves proteolytic processing and posttranslational acylation, as well as binding of Ca^{+2} ions to the repeated domain.

Membrane-Perturbing Toxins

δ-Toxin or δ-hemolysin is secreted into the medium by *S. aureus* strains at the end of the exponential phase of growth. It is a 26-amino-acid peptide (MAQDIISTIGDLVKWIIDTVNKFTKK) that has the general structure of soap with a nonpolar segment followed by a strongly basic carboxy-terminal peptide. The peptide has no structure in aqueous buffers but acquires an a-helical structure in low-dielectric-constant organic solvents and membranes. The α-helix has a typical amphipathic structure, which is necessary for the toxin to interact with membranes. The toxin binds nonspecifically parallel to the surface of any membrane without forming transmembrane channels. At high concentration, the peptide self-associates and increases the perturbation of the lipid bilayer that eventually breaks into discoidal or micellar structures. Interestingly, mellitin, which is also a 26-amino-acid lytic peptide produced by *S. aureus*, has no sequence homology with δ-toxin but has identical distribution of charged and nonpolar amino acids. These toxins are active in most eukaryotic cells. Cells first become permeable to small solutes and eventually swell and lyse, releasing cell intracellular content.

Recent data have demonstrated that δ-hemolysin insertion is strongly dependent on the peptide-to-lipid ratio, suggesting that association of a critical number of monomers on the membrane is required for activity. The peptide appears to cross the membrane rapidly and reversibly and cause release of the lipid vesicle contents during this process.

Other Pore-Forming Toxins

Additional members of this class of β-barrel, channel-forming toxins include aerolysin of *Aer-*

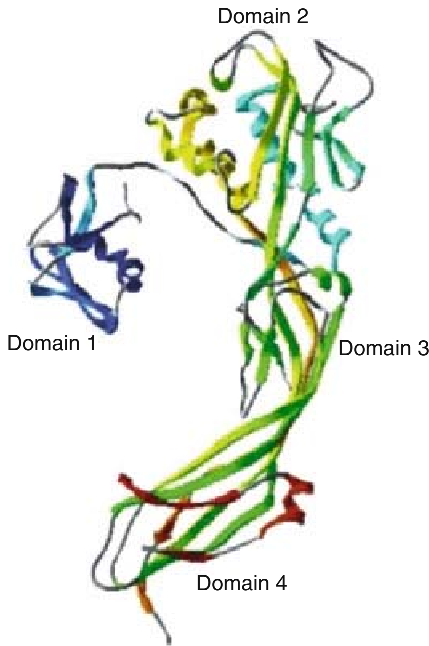


Fig. 19. X-ray structure of aerolysin toxin of *Aeromonas hydrophila*; the four domains are indicated; in particular, domain 1 clearly protrudes outside of the main body of the structure.

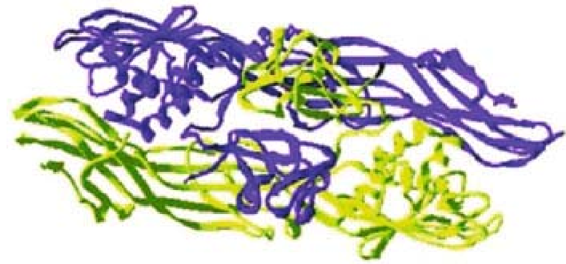


Fig. 20. Structure of the dimer of aerolysin and interaction between the two first domains.

omonas hydrophila (Parker et al., 1994; Rossjohn et al., 1998), and the closely related a-toxin of *Clostridium septicum* (Ballard et al., 1995), the anthrax toxin protective antigen PA of *Bacillus anthracis* (Petosa et al., 1997; Wesche et al., 1998), and the HlyE pore-forming toxin produced by pathogenic *E. coli*.

AEROLYSIN AND ALPHA-TOXIN (AT). Aerolysin (Fig. 1, panel 7) is mainly responsible for the pathogenicity of Aeromonas hydrophila, a bacterium associated with diarrheal diseases and wound infections (Altwegg and Geiss, 1989; Fivaz et al., 2001). It is secreted as a 52-kDa protoxin that is proteolytically cleaved into a 25-residue carboxy-terminal peptide and a 48-kDa active protein. Like other functionally related toxins, aerolysin changes its topology in a multistep process from a completely water-soluble form to a membrane-soluble heptameric transmembrane channel (ca. 1.5 nm in diameter) that destroys sensitive cells by breaking their permeability barriers.

Proaerolysin is a dimer in solution as well as in the crystal form (van der Goot et al., 1993; Parker et al., 1994); four structural domains characterize the monomer (Fig. 19).

In the structure of the dimer, the position of domain 1 appears to be stabilized by contacts with domain 1 of the other monomer, resulting in a very strict interaction of the two (Fig. 20).

Domain 4 is characterized by an amphipatic β-barrel structure, which is responsible for membrane insertion of the final complex. In fact, oligomerization is an essential step in channel formation and it seems to precede membrane insertion. A model has been suggested for the entire process; it assumes that proaerolysin approaches the target cell as a water-soluble, hydrophilic dimer which, once concentrated on the surface of the target cell, binds to the receptor; subsequent proteolytic cleavage would cause dimer dissociation and oligomerization. This would ultimately result in an exposure of the hydrophobic region of the toxin and thus in membrane penetration.

Clostridium septicum AT is a channel-forming protein that is an important contributor to the virulence of the organism. Recent data have proved that this toxin, like aerolysin, binds to glycosylphosphatidylinositol (GPI)-anchored protein receptors. Furthermore, AT is also active against *Toxoplasma gondii* tachyzoites. Toxin treatment causes swelling of the parasite endoplasmic reticulum thus providing the first direct evidence that α-toxin is a vacuolating toxin (Ballard et al., 1995; Gordon et al., 1999). Recently, based on the available crystal structure of aerolysin, a molecular model of the membrane spanning domain of AT has been generated (Melton et al., 2004).

ANTHRAX PROTECTIVE ANTIGEN (PA). Anthrax protective antigen (PA; Fig. 1, panel 17) is one of the three components of the anthrax toxin complex secreted by *Bacillus anthracis*, which also includes the edema factor (EF) and the lethal factor (LF; Brossier et al., 2000; Collier and Young, 2003). Whereas EF and LF are responsible for the toxic activity, PA can be considered as the receptor-binding domain for two distinct A subunits, which are in turn EF and LF. The three subunits are encoded on a plasmid and are synthesized and secreted independently. Once on the host cell surface, PA needs a proteolytic activation to form a membrane-inserting heptamer through which EF and LF can be translocated (Klimpel et al., 1992; Milne and Collier, 1993; Milne et al., 1994; see Fig. 37 for the mechanism of action). The monomer is mainly constituted by antiparallel

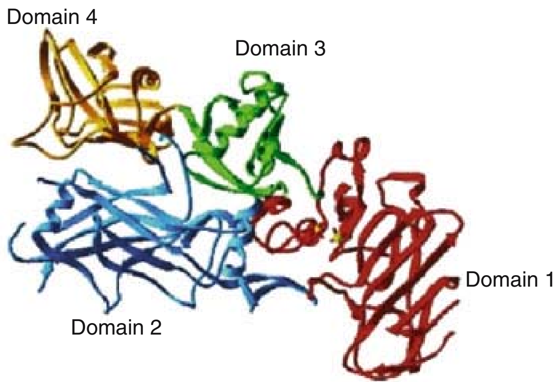


Fig. 21. X-ray structure of *Bacillus anthracis* protective antigen PA. The four structural domains are indicated by different colors. The two cysteines present in domain 1 are colored in yellow.

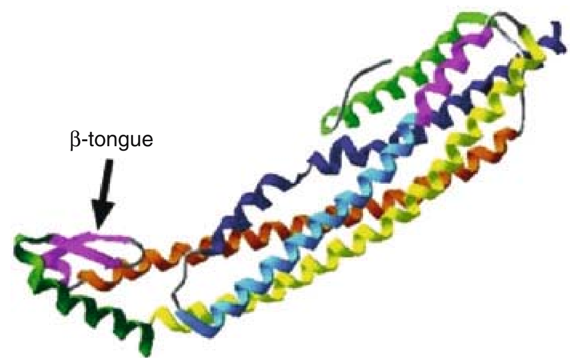


Fig. 22. X-ray structure of *E. coli* HlyE cytolysin. Two hydrophobic domains are present at the extremities of the α-helical bundle (colored in magenta).

β-sheets and contains four functional domains (Fig. 21). The crystallographic structure has revealed how PA can be assembled into heptamers and has suggested how some of the domains can undergo pH-driven conformational change.

Domain 1 (red) contains two Ca^{+2} ions (yellow) and the cleavage site for proteolytic activation; domain 2 (cyan) is the heptamerization domain and is implicated in membrane insertion; domain 3 (green) has an unknown function, whereas domain 4 (yellow) is for receptor-binding. Given its ability to promote the translocation of many heterologous proteins, PA is being evaluated as a general protein delivery system (Leppla et al., 1999).

ESCHERICHIA COLI HLYE. *Escherichia coli* produces a novel pore-forming toxin HlyE (Fig. 1, panel 8), which is completely unrelated to the *E. coli* hemolysin HlyA of the RTX family (Reingold et al., 1999; Wallace et al., 2000). Nevertheless, sequence comparison studies confirm the presence of highly homologous toxins in other pathogenic organisms such as *Salmonella typhi* and *Shigella flexneri* (these orthologs display 92–98% identity to HlyE). This observation suggests that HlyE could be the prototype of a new family of HlyE-like hemolysins specific for Gram-negative bacteria.

This new class of pore-forming toxins form cation-selective water-permeable pores (25–30 Å in diameter); the channel formation could be either part of a mechanism for iron acquisition by the bacterial cell, or it may promote bacterial infection by killing immune cells and causing tissue damage (Ludwig et al., 1999).

The crystal structure of HlyE has been solved (Wallace et al., 2000; Fig. 22).

The toxin has an elongated shape characterized by a four-helix (A–D) bundle topology with each helix approximately 70–80 Å long. Two predicted hydrophobic domains have been identified on the primary sequence: both are located at the extremities of the molecule, one being mainly composed of a short β-hairpin (β-tongue) folded between the third and fourth helices of the main bundle, and the other consisting of the C-terminal end (magenta) of helix B (cyan).

The precise mechanism of HlyE oligomerization to form the final transmembrane pore is at the moment unknown; nevertheless, the first step involves a process of dimerization of two HlyE molecules that pack in a head-to-tail fashion burying the two hydrophobic patches against each other. Electron microscopy experiments have led to a model of channel formation in which the possible oligomer topology is that of an octameric complex, and the β-tongue domain is primary responsible for interaction with the membrane.

Insecticidal Toxins

The class of insecticidal proteins, also known as δ-endotoxins, includes a number of toxins produced by species of *Bacillus thuringiensis*. These exert their toxic activity by making pores in the epithelial cell membrane of the insect midgut (Hofte and Whiteley, 1989; Knowles, 1994).

δ-Endotoxins form two multigenic families, *cry* and *cyt*; members of the cry family are toxic to insects of Lepidoptera, Diptera and Coleoptera orders (Hofmann et al., 1988), whereas members of the cyt family are lethal specifically to the larvae of Dipteran insects (Koni and Ellar, 1994). The insecticidal toxins of the cry family are synthesized by the bacterium as protoxins with molecular masses of 70–135 kDa; after ingestion by the susceptible insect, the protoxin is cleaved by gut proteases to release the active toxin of 60–70 kDa (Drobniewski and Ellar, 1989). In this form, they bind specifically and with high affinity to protein

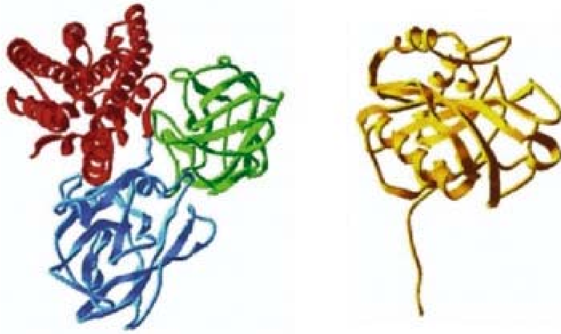


Fig. 23. Comparison of X-ray structures determined for representatives of the cry and cyt families of insecticidal δ-endotoxins: CryIA (left panel) is organized in three structural domains, whereas CytB (right panel) is a single-domain globular protein.

receptors and create channels 10–20 Å wide in the cell membrane. This subgroup includes several toxins (CryIA, CryIIIA, CryIV, CryV, etc.), whereas the only proteins so far characterized that belong to the cyt are CytA and CytB (Koni and Ellar, 1993; 1994).

Three-dimensional (3D) structures determined for members of the two families show that the folding of these toxins is entirely different. As representative of the two families, consideration is given to the structures of CryIA (Fig. 1, panel 9; Grochulski et al., 1995) and CytB (Li et al., 1996; Figs. 1 [panel 10] and 23), which share more than 39% sequence identity, suggesting an overall similar folding of the corresponding 3D structures.

The CryIA toxin is a globular protein composed of three distinct (but closely packed) domains connected by single linkers: domain 1 is totally α-helical, domain 2 consists of three antiparallel β-sheets and two short α-helices, and domain 3 is a β-sandwich. On the other hand, CytB (also a globular protein) is composed of a single domain with α/β architecture. The molecular mass of the protoxin is in this case only 30 kDa.

The region of CryIA, which has been associated with receptor–binding, maps within a loop of domain 2, whereas domain 1 has been shown to be responsible for membrane insertion and pore formation (Martens et al., 1995); this notion is strongly supported by the high structural similarity between the domain 1 of CryIA and that of CryIIIA to the pore-forming domains of colicin A and diphtheria toxin, both composed of helical bundles (Cabiaux et al., 1997; Duche et al., 1999). Conversely, in the case of the CytB/A, the model that has been proposed for the channel formation is based on a β-barrel structure.

Because they are toxic to several species of insects, δ-endotoxins have been formulated into commercial insecticides, and these insecticides have been used for more than three decades. Recently, Lepidoptera-specific toxin genes have also been used to engineer insect-resistant plants (Christov et al., 1999).

Very recently, a novel crystal protein produced by *B. thuringiensis* has been identified. This toxin (BT) is noninsecticidal and nonhemolytic, but has strong cytocidal activity against various human cells. Its amino acid sequence has little homology with the other known insecticidal toxins, suggesting that BT might belong to a new group of *Bacillus thuringiensis* crystal toxins (Ito et al., 2004).

Toxins Acting on Intracellular Targets

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

The group of toxins with an intracellular target (A/B toxins) contains many toxins with different structures that have only one general feature in common: they are composed of two domains generally identified as “A” and “B.” The A domain is the active portion of the toxin; it usually has enzymatic activity and can recognize and modify a target molecule within the cytosol of eukaryotic cells. The B domain is usually the carrier for the A subunit; it binds the receptor on the cell surface and facilitates the translocation of A across the cytoplasmic membrane (Fig. 2, panel 2). Depending on their target, these toxins can be divided into different groups that act on protein synthesis, signal transduction, actin polymerization, and vesicle trafficking within eukaryotic cells.

Toxins Acting on Protein Synthesis

These toxins are able to cause rapid cell death at extremely low concentrations. Two ADP-ribosylating bacterial proteins (see also the section ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity) are actually known to belong to this class of toxins: diphtheria toxin (DT) of *Corynebacterium diphtheriae* (Pappenheimer, 1977; Collier et al., 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; Gray et al., 1984b; Wick et al., 1990). Both display their toxic activity by transferring the ADP-ribose moiety to a posttranslationally modified histidine residue of the cytoplasmic elongation factor 2 (EF2) of eukaryotic cells (Brown and Bodley, 1979; Van Ness et al., 1980). This reaction leads to the formation of a completely inactive EF2-ADP-ribose complex, which ultimately results in inhibition of protein

synthesis and cell death. From the biochemical point of view, the two toxins have a similar size, a signal peptide and disulfide bridges, and both are produced in iron-depleted medium. Nevertheless, they show a completely different amino acid composition and bind different cell receptors. In addition, Shiga toxin is another protein that exerts its toxic activity by interfering with protein synthesis.

DIPHTHERIA TOXIN. This toxin (DT; Fig. 1, panel 11) is a 535-amino acid polypeptide that is secreted into the growth medium by strains of toxinogenic *Corynebacterium diphtheriae*, and the polypeptide sequence is encoded by a lysogenic bacteriophage. Biosynthesis is regulated by an iron-binding protein, and proceeds only in the absence of iron (Qiu et al., 1995; Ding et al., 1996). The toxin is synthesized as a single polypeptide chain that is subsequently cleaved into two fragments, A and B of 21 kDa and 37 kDa, respectively (Pappenheimer, 1977).

From the functional point of view, three separate domains (C, T and R) are seen in the crystallographic structure of DT. The catalytic domain (C) entirely corresponds to the A subunit, whereas the translocation domain (T) and the carboxy-terminal, receptor-binding domain (R) are contained in fragment B (Choe et al., 1992; Bennett and Eisenberg, 1994).

From the structural point of view, the C domain (residues 1–191) has an α+β structure, the receptor-binding domain is a flattened β-barrel with a jelly-roll-like topology, whereas the translocation domain T (residues 201–384) consists in nine helices, two of which may participate in the pH-triggered membrane insertion. The molecule contains four cysteines and two disulfide bridges: one joins fragment C to fragment T and the other is contained within fragment R (Fig. 24).

Although the toxicity of DT is entirely due to the enzymatic activity carried on by fragment A (Fig. 25), fragment B is absolutely required for the cell intoxication process.

After secretion from *Corynebacterium diphtheriae*, the toxin binds to the DT receptor and is internalized by receptor-mediated endocytosis. In the endosome, the acidic environment triggers a conformational change of the B subunit that exposes the hydrophobic regions of the T domain allowing the interaction with the endosomal membrane and the translocation of the amino-terminal catalytic domain C across the membrane to the cytosol. According to a recent model, the A subunit of DT is able to cross the endosomal membrane making use of a metastable transmembrane domain, which has also been identified (Wolff et al., 2004). The toxin receptor is the heparin-binding, epidermal growth factor (EGF)-like precursor (Naglich et al., 1992; Hooper and Eidels, 1995) that is present in most mammalian cells; nevertheless, the receptors of murine cells contain a few amino acid substitutions that make rodents insensitive to DT.

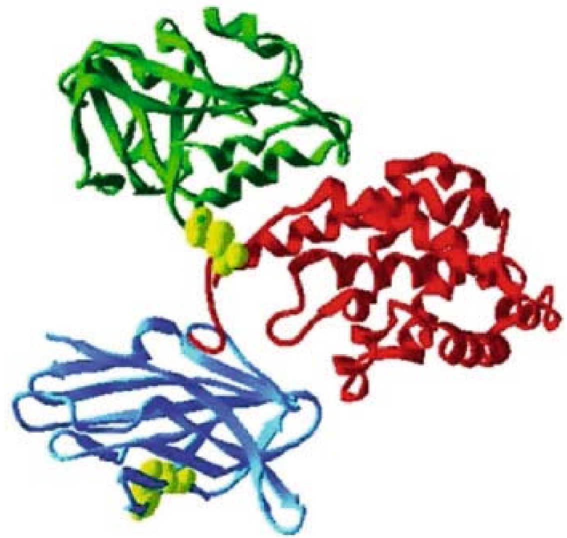


Fig. 24. X-ray structure of diphtheria toxin. The three functional domains are indicated with different colors: the catalytic domain C is green, the translocation domain T is red and the receptor-binding domain R is cyan. The two disulfide bridges are colored yellow.

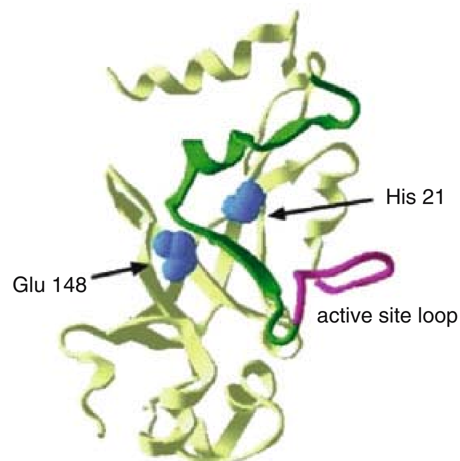


Fig. 25. Crystal structure of the isolated catalytic domain of diphtheria toxin. The scaffold of the enzymatic cleft is green, and the two described catalytic residues are blue. The "active-site loop" is represented here in the "closed" conformation.

Diphtheria toxin is one of the most potent bacterial toxins: in vitro experiments have shown that a single molecule of the enzymatically active fragment A is by itself able to kill one eukaryotic cell (Yamaizumi et al., 1978).

Biochemical and mutagenesis studies have greatly contributed to the understanding of structure-function relationships and to the mapping of the catalytic residues. In particular,

His-21 has been mutagenized with a number of different residues and has been found to be essential for catalysis (Papini et al., 1989; Blanke et al., 1994); in fact, some activity was maintained only when Asn replaced His. In a similar manner, Glu-148 was identified as an active-site residue by photoaffinity labeling experiments with nicotinamide adenine dinucleotide (NAD; Carroll et al., 1985) and subsequent site-directed mutagenesis studies; in this case, not even a conservative substitution with Asp could be possible without complete loss of activity (Tweten et al., 1985). Whereas the possible function for His-21 could be that of maintaining the integrity of the active-site pocket, Glu-148 is likely to be involved in the interaction with the upcoming substrate molecule. Later, crystallographic data confirmed and extended the experimental observations, and added a number of other important residues to the list of the catalytic ones.

A very important step in the elucidation of the mechanism of enzymatic activity has been the determination of the crystal structure for the complex of diphtheria toxin with NAD (Bell and Eisenberg, 1997). Upon the addition of NAD to nucleotide-free DT crystals, a significant structural change affects the region encompassing residues 39–46. This portion of the C domain constitutes a mobile loop that becomes disordered after the formation of the complex. The best hypothesis to explain this observation is that NAD enters the cavity upon displacement of the mobile loop, which is then made available for the recognition and binding of the acceptor substrate EF-2. This would explain why DT recognizes EF-2 only after NAD has bound (see the section A Common Structure of the Catalytic Site in this Chapter).

Detoxified diphtheria toxin has been used in the formulation of a vaccine against toxinogenic strains of *Corynebacterium diphtheriae* (Porro et al., 1980; Rappuoli, 1983).

PSEUDOMONAS AERUGINOSA EXOTOXIN A. This exotoxin (PAETA; Fig. 1, panel 12) is a 66-kDa single-chain protein that inhibits protein synthesis (by a mechanism of action identical to that of DT) in eukaryotic cells by catalyzing the transfer of the ADP-ribosyl moiety of oxidized NAD onto elongation factor 2 (Brown and Bodley, 1979; Van Ness et al., 1980; Gray et al., 1984b; Wick et al., 1990; see the section ADP-ribosylating Toxins in this Chapter). Exotoxin A is the most toxic of the proteins secreted by the opportunistic pathogen *Pseudomonas aeruginosa*, having an LD_{50} of 0.2 mg upon intraperitoneal injection into mice. Secreted in the supernatant as an enzymatically inactive proenzyme; this toxin must undergo structural alteration to be able to perform its ADP-ribosylating activity.

According to X-ray crystallography (Allured et al., 1986; Li et al., 1995; 1996b), the molecule can be divided into three functional domains. The receptor-binding domain I binds to the ubiquitous α2-macroglobulin receptor of eukaryotic cells, thus initiating receptor-mediated endocytosis. This domain is composed primarily of antiparallel β-structure and is arranged in two noncontiguous regions that encompass residues 1–252 (Ia) and 365–399 (Ib), respectively. Domain II maps within amino acids 253–364, is composed mostly of hydrophobic α-helices, and mediates the translocation of the enzymatically active carboxy-terminal domain III (residues 400–613) to the cytosol of infected cells. Furthermore, it has been shown that for domain III to be functional, a specific proteolytic cleavage at residue 280 of domain II is needed.

Genetic studies based on the expression of mutated forms of the exotoxin A gene in *E. coli* have confirmed these functional assignments. In fact, whereas deletion of domain Ia results in nontoxic, enzymatically active molecules that cannot bind the cells, deletions in domain II give rise to molecules that bind to the cells, are enzymatically active, but are not toxic; finally, deletions or mutations in domain III result in enzymatically inactive molecules (Siegall et al., 1989). To become active, the PAETA toxin requires an intracellular furin-mediated proteolytic cleavage to generate a 37-kDa C-terminal fragment that is then translocated to the cytoplasm to reach the EF2 target (Inocencio et al., 1994).

By using a fluorescence resonance energy transfer approach, the mechanism of interaction between ExoA and its substrate EF has been studied, showing that the binding is strongly dependent on the pH. Furthermore, the finding that EF-2 bound to GDP or GTP is still recognized by ExoA shows how adaptable this toxin is in ADP-ribosylating its substrate.

In particular, mutational analysis affecting the last five residues at the carboxy-terminus of the enzymatic domain resulted in complete loss of cytotoxicity; this segment (Arg-Glu-Asp-Leu-Lys, REDLK) closely resembles the KDEL motif that is a well-defined endoplasmic reticulum retention sequence and that has also been found at the C-terminus of other ADP-ribosyltransferases such as cholera toxin and heat-labile enterotoxin of *E. coli* (Chaudhary, 1990). It has been postulated that the sequence REDLK may be a recognition signal required for entry of the ADP-ribosylation domain of PAETA into the cytosol. Four disulfide bonds are present in the structure, but all of them are confined to the portion of exotoxin A that is not required for enzymatic activity.

Photoaffinity labeling experiments have identified Glu-553 as an active-site residue; substitu-

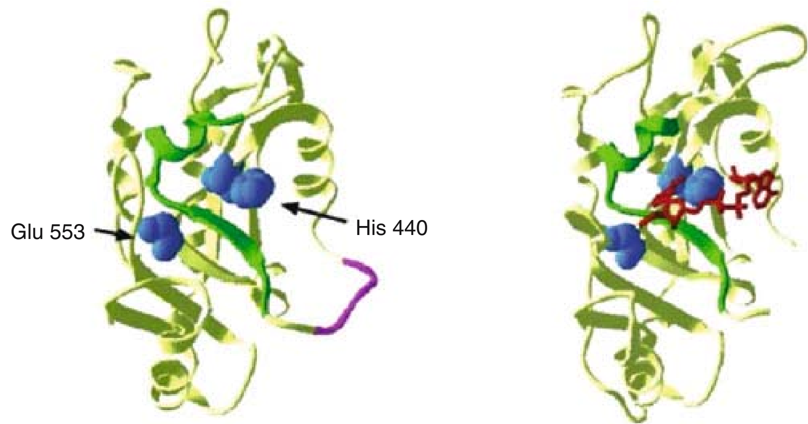


Fig. 26. Comparison of exotoxin A crystal structures in the absence (left panel) and in the presence (right panel) of the ligand (in red). The active site residues are shown in blue. The loop (when present) is colored in magenta (see Fig. 1, panel 12).

tion of this residue with any other amino acid, including the closely related Asp, decreased the enzymatic activity by a factor of 1000 (Douglas and Collier, 1990). In a similar manner, experiments of site-directed mutagenesis on His-440 led to molecules with a severely reduced cytotoxic activity, thus suggesting an important role for this residue in the reaction (Han and Galloway, 1995).

The crystal structure of the catalytic domain has been recently solved both in the isolated conformation and in the presence of an NAD analog (β-methylene thiazole-4-carboxamide adenine dinucleotide; β-TAD; Li et al., 1995; 1996b; Fig. 26). Comparison of the two structures shows that the major difference resides in the new conformation of the loop 458–463, which appears to be displaced by ligand binding; displacement of this loop from the active-site cleft could be an essential step allowing entrance and correct positioning of the NAD molecule during the enzymatic reaction.

Given the potent lethal activity, the catalytic domain of exotoxin A has been widely used for the construction of fusion proteins with cell-binding domains specific for tumor cells or other types of dangerous cells. So far, nucleotides encoding domain I have been replaced by sequences encoding interleukin (IL) 2, IL-6 and T-cell antigen CD4. These fusion molecules are promising candidates for the treatment of arthritis and allograft rejection (PAETA-IL2), acquired immune deficiency syndrome (PAETA-CD4), and other diseases (Chaudhary et al., 1987, 1988; Siegall et al., 1988; Ogata et al., 1989; Baldwin et al., 1996; Mori et al., 1997).

SHIGA TOXIN. This toxin (SHT; Fig. 1, panel 13), also known as "verotoxin," is the key virulence factor produced by *Shigella dysenteriae*, the pathogen responsible for the most severe forms of dysentery in humans (Kozlov et al., 1993). Shiga toxin is the prototype of a family of closely related bacterial protein toxins (Shiga-like toxins), also produced by certain strains of *E. coli* responsible for hemorrhagic colitis (Karmali et al., 1988).

From its 3D structure (Fraser et al., 1994), it is possible to recognize this protein as belonging to the class of A/B bacterial toxins, which consist of an enzymatic A subunit associated with a B domain binding to specific cell-surface receptors. The A subunit bears the enzymatic activity and is thus responsible for toxicity; like *Pseudomonas aeruginosa* exotoxin A and diphtheria toxin of *Corynebacterium diphtheriae*, SHT has an effect on protein synthesis, and in particular, by means of its *N*-glycosidase activity, it is able to depurinate a specific adenosine of ribosomal RNA and stop protein synthesis in the target cell (Endo et al., 1988). The catalytic subunit is composed of two regions, A1 and A2, and like many other bacterial protein toxins, it needs to be activated by proteolytic cleavage. Fragment A2 has an α-helical structure and is noncovalently linked to the B domain (Fig. 27). Interestingly, its primary structure displays a notable similarity to chain A of ricin, a plant toxin that also shares the same enzymatic function acting on the same substrate (Katzin et al., 1991).

This domain displays an overall organization which is very similar to that of the corresponding receptor-binding subunits of the ADP-ribosyltransferases cholera toxin and heat-labile enterotoxin LT of *E. coli*, all formed by five identical protomers which assemble into the final ring-like structure of the B oligomer (Fig. 28). The B-subunit of Shiga toxin has been demonstrated as a powerful vector for carrying attached peptides into cells for intracellular transport studies and for medical research (Hagnarelle et al., 2003).

Upon binding of verotoxin to its receptor (globotrialosylglyceramide, Gb) on the surface of a eukaryotic cell (Cohen et al., 2000), the toxin is internalized by receptor-mediated endocytosis and is transported to the Golgi and to the endoplasmic reticulum, from which the A subunit is

translocated to the cytoplasm, where it can gain access to the ribosomal target. Numerous recent studies have shown that Shiga toxins trigger programmed cell death signaling cascades in intoxicated cells. The mechanisms of apoptosis induction by these toxins are newly emerging, and the toxins may signal apoptosis in different cells types via different mechanisms (Cherla et al., 2003).

Toxins Acting on Signal Transduction

Signal transduction is an essential mechanism for the survival of any living organism. In eukaryotic cells, signals received from the outside stimulate receptors on the cell surface and are subsequently transmitted across the cell membrane mainly using two types of mechanism: 1) tyrosine phosphorylation of the cytoplasmic portion of the receptor which initiates a cascade of intracellular signaling events; and 2) modification of a receptor-coupled GTP-binding protein that transduces the signal to various enzymes which respond with the release of secondary messengers such as cyclic AMP (cAMP), inositol triphosphate, and diacylglycerol; accumulation of these products alter the normal equilibrium of the cell and thus provoke malfunction and death.

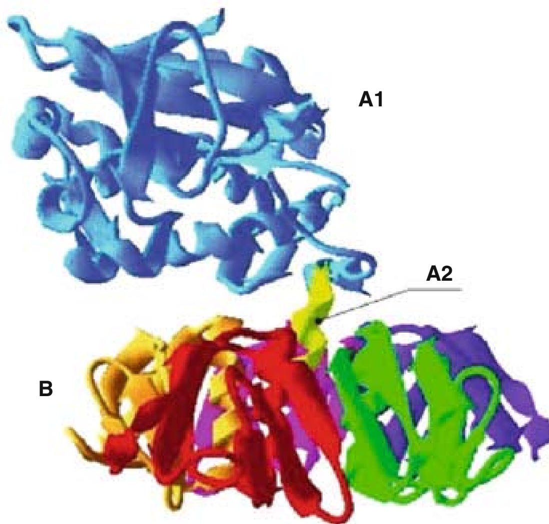


Fig. 27. Three-dimensional structure of Shiga holotoxin. The A subunit is distinguished between A1 (blue) and A2 (yellow), whereas the receptor-binding domain B has different colors for the five monomers.

Pertussis Toxin

This toxin (PT; Fig. 1, panel 14) is a protein of 105 kDa released into the extracellular medium by *Bordetella pertussis*, the etiological agent of whooping cough. It belongs to the A/B class of ADP-ribosylating toxins and is composed of five distinct subunits, named "S1" through "S5," where S4 is present in two copies in the final oligomer. The genes encoding for the five monomers of pertussis toxin are organized into an operon structure (Locht et al., 1986; Fig. 29) and contained within a chromosomic DNA fragment of approximately 3200 base pairs.

Interestingly, the genes coding for S2 and S3 share a 75% similarity (67%, if calculated from S2 and S3 gene products at the amino acid level), suggesting a common evolutionary origin for the two sequences, possibly because of gene duplication.

The five subunits are independently secreted into the periplasmic space, where the toxin is assembled and then released in the culture

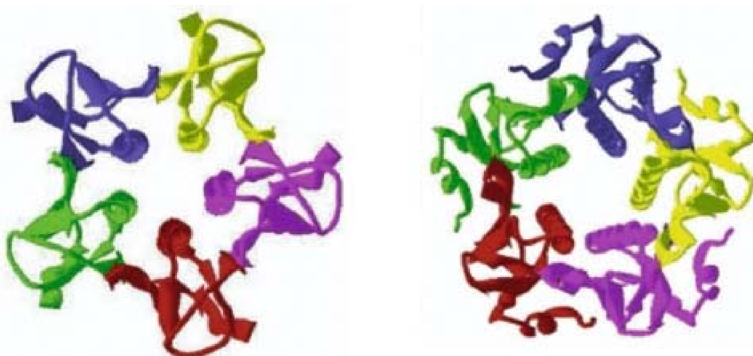


Fig. 28. Bottom view of the B subunit of Shiga toxin (left panel) in comparison to the B subunit of *E. coli* LT (right panel).

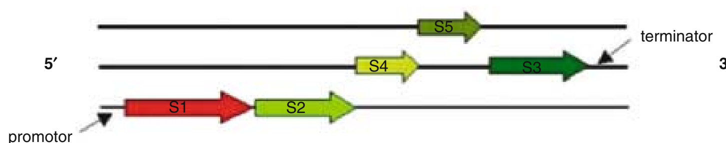


Fig. 29. Schematic representation of the genetic organization of the open reading frames (ORFs) coding for the five subunits of pertussis toxin.

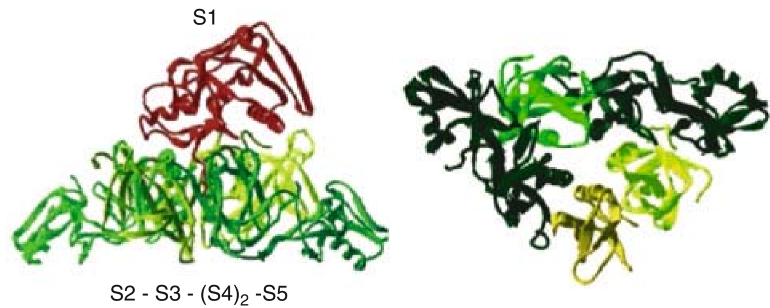


Fig. 30. Three-dimensional structure of the pertussis holotoxin. Left panel: side view of the intact holotoxin; right panel: bottom view of the receptor-binding domain. Each subunit is colored accordingly to the corresponding genes as represented in Fig. 29.

medium by a specialized type IV secretion apparatus (Covacci and Rappuoli, 1993a; Weiss et al., 1995). Subunit S1 represents the enzymatically active domain A, which is totally responsible for the toxicity, whereas the pentamer S2-S3-$(S4)_2$-S5 constitutes the receptor-binding domain B (Fig. 30).

The A domain acts on eukaryotic cells by ADP-ribosylating their GTP-binding proteins, and specifically it transfers an ADP-ribose group to a cysteine residue located in the carboxy-terminal region of the α-subunit of many G proteins such as G_i, G_o and transducin (Katada et al., 1983; West et al., 1985); G_s which has a tyrosine residue in place of the cysteine is not a valid substrate for PT. The consequence of ADP-ribosylation is the uncoupling of G-proteins from their receptors which results in an alteration of the response of eukaryotic cells to exogenous stimuli and thus in a variety of in vivo phenotypes, such as leukocytosis, histamine sensitization, and increased insulin production (Sekura, 1985). Conversely, the most interesting activity displayed by PT in vitro is the observed change in cell morphology in Chinese hamster ovary (CHO) cells (Hewlett et al., 1983).

The B domain is a nontoxic oligomer that binds the receptors on the surface of eukaryotic cells and allows the toxic subunit S1 to reach its intracellular target proteins through a mechanism of receptor-mediated endocytosis, likely following a mechanism of retrograde transport through the Golgi apparatus. The importance of the Golgi localization of pertussis toxin for the S1-dependent ADP-ribosylation of G-proteins was investigated employing Brefeldin A (BFA) treatment to disrupt Golgi structures. This treatment completely blocked the pertussis toxin ADP-ribosylation activity of cellular G-proteins, therefore indicating that retrograde transport to the Golgi network is a necessary prerequisite for cellular intoxication (el Baya et al., 1997). In CHO cells, the PT receptor has been shown to be a high-molecular weight glycoprotein that binds the B oligomer through a branched-mannose core containing *N*-acetylglucosamine (Sekura, 1985). In contrast to the other ADP-ribosyltransferases, where the enzymatically active domain A mediates all the toxic activities, PT possesses other nonlethal activities (such as a mitogenic activity on T cells), which are mediated exclusively by the receptor-binding domain B (Tamura et al., 1983). The active site of pertussis toxin is structurally homologous to the active sites of other ADP-ribosylating toxins. This aspect will be described in the section ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity in this Chapter.

Pertussis toxin plays a central role in the pathogenesis of whooping cough and in the development of protective immunity against reinfection. For this reason, the role of many residues of S1 has been tested by site-directed mutagenesis to produce nontoxic mutants of the toxin to be used as vaccines. The minimal region still enzymatically active is constituted by amino acids 4–179 of S1 subunit (Pizza et al., 1988; Cieplak et al., 1988; Fig. 31), and it is within this fragment that many mutations have been

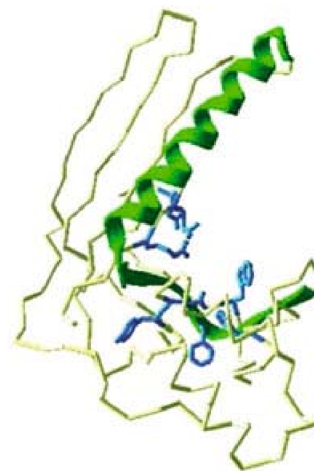


Fig. 31. Crystal structure of the wildtype S1 subunit of pertussis toxin. The scaffold of the enzymatic cleft is represented as a green ribbon, whereas the rest of the molecule is in pale yellow carbon trace representation. Residues proved to be essential for activity by means of site-directed mutagenesis are represented with side chains and are colored in blue.

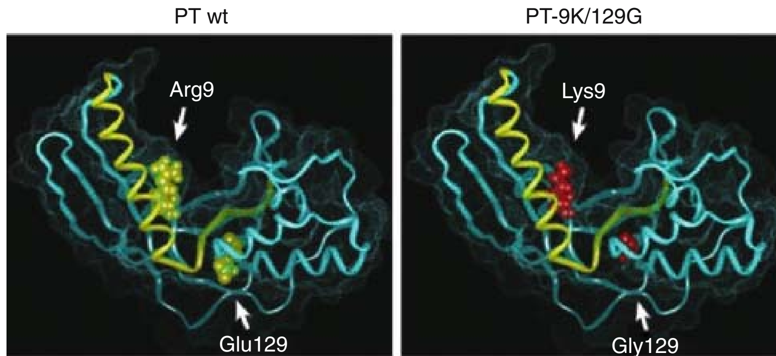


Fig. 32. X-ray representation of the wildtype pertussis toxin (left panel) and of the double mutant 9K/129G (right panel). The catalytic cleft is colored in yellow, whereas the mutated residues are in red.

designed and analyzed for activity. In particular, Arg-9, Asp-11, Arg-13, Trp-26, His-35, Phe-50, Glu-129 and Tyr-130 were found to be essential for enzymatic activity and, when replaced with other residues, the toxicity was reduced to levels of about 1%; nevertheless, none of the single-amino acid mutants were completely devoid of toxicity.

The most successful mutant contains in fact two amino acid substitutions: Arg-9/Lys and Glu-129/Gly (PT-9K/129G; Fig. 32). This mutant is structurally identical to the wildtype but is completely nontoxic and has been used for the construction of an acellular vaccine against pertussis. This vaccine has been extensively tested and has been shown to induce protection from disease (Pizza et al., 1989; Rappuoli, 1997).

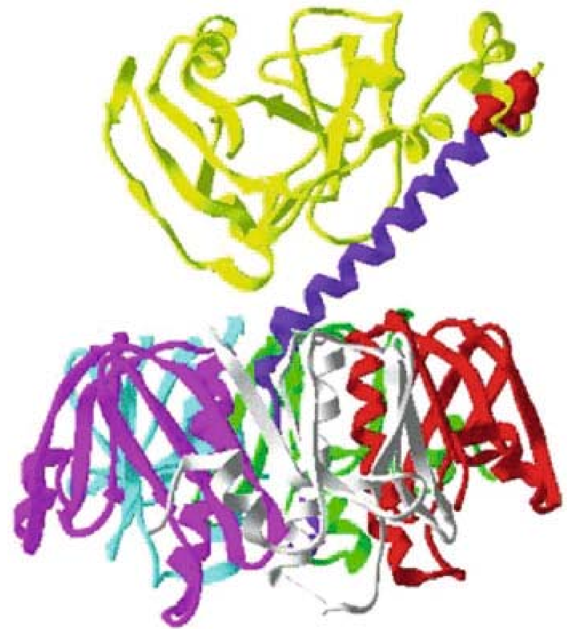


Fig. 33. X-ray structure of heat-labile enterotoxin LT of *E. coli*. The catalytic domain A1 is yellow, the linker domain A2 is blue, and the five monomers of the B subunit are all represented in different colors.

Cholera Toxin and Heat-Labile Enterotoxin

Cholera toxin (CT) and *E. coli* heat-labile enterotoxins (LT-I and LT-II) share an identical mechanism of action and homologous primary and 3D structures (Dallas and Falkow, 1980; Spicer et al., 1982; Sixma et al., 1991; Figs. 1 [panel 15] and 33). The CT is produced by *Vibrio cholerae* (the etiological agent of cholera), whereas LT-I and LT-II are produced by enterotoxigenic strains of *E. coli* (ETEC) isolated from humans with traveler's diarrhea, from pigs (LT-I), or from food (LT-II; Seriwatana et al., 1988). The two toxins belong to the class of ADP-ribosylating toxins and are organized in an AB_5 architecture, where the B domain is a pentamer which binds the receptor on the surface of eukaryotic cells, and domain A bears the enzymatic activity and is thus responsible for toxicity (Holmgren, 1981; Moss and Vaughan, 1988). Both the A and B subunits of CT and LT are synthesized intracellularly as precursor proteins which, after removal of the leader peptide and translocation across the cytoplasmic membrane, assemble in the periplasmic space to form the final AB_5 complex. While *V. cholerae* exports the CT toxin into the culture medium, LT remains associated to the outer membrane bound to lipopolysaccharide (LPS; Horstman et al., 2002). The corresponding genes of CT and LT are organized in a bicistronic operon and are located on a filamentous bacteriophage and on a plasmid, respectively (So et al., 1978).

The A subunit (Fig. 34, left panel) is a 27-kDa monomer composed of a globular structure and linked to the B domain by a trypsin-sensitive loop and a long α-helix, which inserts inside the core of the B pentamer thus anchoring the two subunits. For full activity, the A subunit needs to be proteolytically cleaved and reduced at the disulfide bridge between cysteines 187 and 199 to give two fragments: the enzymatic subunit A1 and the linker fragment A2 (Lai et al., 1981).

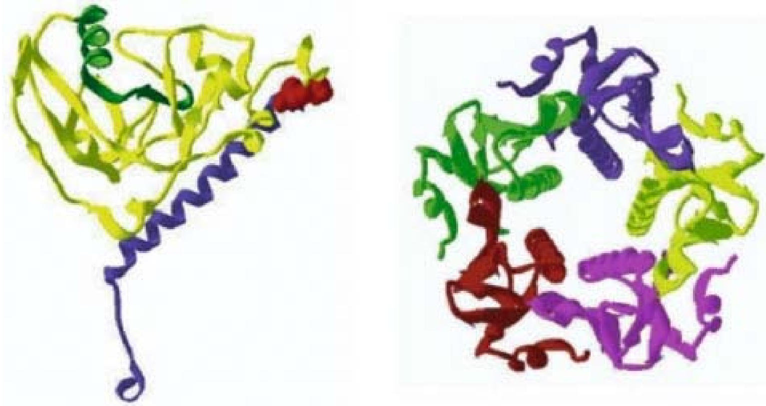


Fig. 34. Left side: front view of the catalytic A subunit, with the toxic moiety A1 in pale green and the linker domain A2 in violet; cysteines 187 and 199 involved in the disulfide bridge are red. Right side: bottom view of the pentameric receptor-binding domain B.

Whereas in cholera toxin the proteolytic process is performed during biosynthesis by an endoprotease (Booth et al., 1984), in the case of LT, it occurs by extracellular processes; in both cases, the reduction is thought to take place at the surface of the target cell.

The enzymatically active domain A binds NAD and transfers the ADP-ribose group to an Arg residue located within the central portion of several GTP-binding proteins such as G_s, G_t and G_{olf}. Upon ADP-ribosylation of G_s, in particular, the adenylate cyclase is permanently activated, causing an abnormal intracellular cAMP accumulation, which in turn alters ion transport and thus is the main reason for the toxic effects (Field et al., 1989a, 1989b).

A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with 20-kDa guanine-nucleotide binding proteins, known as "ADP-ribosylation factors" (ARFs; Tsai et al., 1988; Moss and Vaughan, 1991). After receptor binding, the holotoxins are internalized and undergo retrograde transport through the Golgi to the endoplasmic reticulum (ER). Recent studies show that both A and B subunits move together from the cell surface into the ER, and this depends on the B-subunit binding to ganglioside GM1. The KDEL motif in the A2 chain does not appear to affect retrograde transport, but slows recycling of the B-subunit from ER to distal Golgi stacks. Specificity for GM1 in this trafficking pathway is shown by the failure of the *E. coli* type II toxin LTIIb that binds ganglioside GD1a to concentrate in lipid rafts, enter the ER, or induce toxicity. These results show that the B subunit carries the A1 chain from cell surface into the ER where they dissociate, and that a membrane lipid with strong affinity for lipid rafts provides the dominant sorting motif for this pathway (Fujinaga et al., 2003). In the ER, the A1-chain of the CT unfolds and enters the cytosol by a process termed "retro-translocation." Upon entering the cytosol, the A1-chain rapidly refolds, binds ARF and induces toxicity (Lencer et al., 1995). The B subunits persist in the Golgi and are subsequently degraded.

The exact localization of the ARF-binding site is still unknown, but it has emerged from recent studies that the two domains (the NAD-binding and ARF-binding) are independent and located in different regions of the A domain (Stevens et al., 1999).

When the toxins are released in the intestine during infection, the major consequence is intestinal fluid accumulation and watery diarrhea (also typical symptoms of the diseases; Holmgren, 1981).

The B domain (Fig. 34, right) is composed of five identical subunits (each 11.5 kDa) that are arranged in a symmetric shape around a central pore inside which the C-terminal portion of the catalytic domain (A2) is inserted (Sixma et al., 1991, 1993). Their secondary structure consists predominantly of two three-stranded antiparallel β-sheets, a short N-terminal helix, and a long central helix. Although still well conserved in terms of quaternary structure, CT and LT B domains have a lower degree of primary sequence homology than the corresponding A domains. Interestingly, the B subunit of LT-II, although maintaining a conserved structure, lacks any sequence homology with the corresponding B domains of CT and LT-I (Domenighini et al., 1995).

In addition to their function as receptor-binding domains and as carriers of the toxic moieties, the B subunits possess specific biological activities such as induction of apoptosis of CD8+ and CD4+ T cells (Truitt et al., 1998) and the property to function as potent mucosal adjuvants (Xu-Amano et al., 1994). This feature has been extensively used to develop mucosal vaccines against cholera and ETEC infection, and to induce a mucosal response also against the other antigens used.

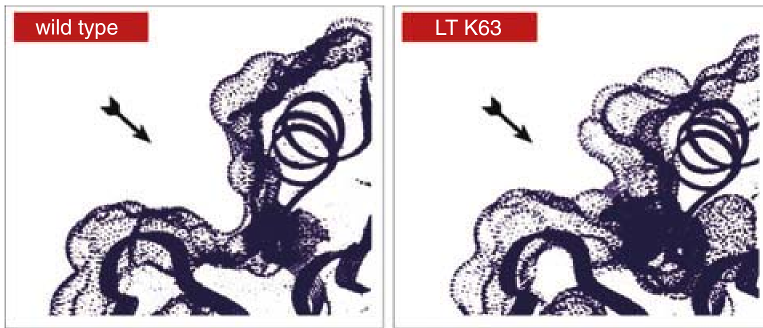


Fig. 35. Three-dimensional structure of the enzymatic cavity of the wildtype LT (left) and of the mutant LT-K63 (right). The arrows point out how much a single amino acid substitution can affect the dimension of the pocket and thus the entrance of NAD.

To produce molecules suitable for these pharmacological applications but completely devoid of toxic activity, more than fifty mutant derivatives have been constructed both for the A and B subunits. Among those which completely inactivate the toxin, the best characterized are LT-K63 (Fig. 35), LT-K97 and LT-K7, all in the vicinity of the catalytic domain, and for which the 3D structures have also been determined (Merritt et al., 1995; Van den Akker et al., 1995, 1997). In the case of LT-K63 (and the corresponding CT-K63), where the wildtype Ser in position 63 is substituted with a Lys, the mutated proteins are enzymatically inactive and nontoxic, either in vitro and in vivo, but are otherwise indistinguishable from the wildtype. In fact, they are still able to bind the receptor and the ARFs (Stevens et al., 1987), and the crystal structure and that of wildtype LT are almost perfectly superimposable except for the catalytic site, where the bulky side-chain of Lys-63 fills the catalytic pocket thus making it unsuitable for NAD entrance and binding (Giannelli et al., 1997; Douce et al., 1998).

Another interesting mutant is LT-K97, where the substitution Val/Lys introduces a salt bridge between Lys-97 and the carboxylate of Glu-112, thus making it unavailable to further interactions. This observation suggests a dominant role of this glutamic acid in the enzymatic reaction.

Mutations affecting the B domain lead often to products that can no longer bind to eukaryotic receptors, as is the case of LTB-D33, which contains a glycine-to-aspartic acid substitution in position 33. These types of mutants have been found to be almost completely nonimmunogenic at mucosal surfaces, suggesting that an intact receptor-binding site is necessary not only for binding but also for immunogenicity and adjuvanticity (Guidry et al., 1997).

Clostridium perfringens Alpha-Toxin

This toxin (Fig. 1, panel 16) is the most important toxin produced by *Clostridium perfringens* and is responsible for gas gangrene or clostridial myonecrosis (Stevens et al., 1987; Florez-Diaz et al., 2003). It plays a key role in the spread of the infection either by suppressing host immune responses, triggering the release of inflammatory mediators, or causing changes in intracellular calcium levels. Specific mutants of *C. perfringens* that do not produce the toxin are unable to cause disease, and vaccination with a genetically engineered toxoid has been shown to induce protection against gas gangrene (Williamson and Titball, 1993).

This virulence factor is a 370-amino acid zinc metalloenzyme that also displays phospholipase C (PLC) activity (Leslie et al., 1989); nevertheless, not all the bacterial PLCs act as virulence determinants, therefore this enzymatic activity is not sufficient for toxicity.

Alpha-toxin is capable of binding to mammalian cell membrane and cleaving membrane-bound phosphatidylcholine (or sphingomyelin) to produce phosphocholine and diacylglycerol (or ceramide). The reaction product diacylglycerol, which is a leukotriene precursor, is believed to be the responsible of the subsequent lethal effects.

The crystal structure of α-toxin has been recently solved (Naylor et al., 1998; Fig. 36), indicating the presence of two distinct domains in the molecule. Whereas the N-terminus is mainly organized as a globular α-helical domain that contains the active site, the β-sandwich C-terminal subunit is involved in membrane binding and shows strong structural analogy to eukaryotic calcium-binding C2 domains. A flexible linker containing a series of highly mobile residues connects the two domains.

In addition, the C-terminal subunit displays hemolytic and sphingomyelinase activities and primarily contributes to the toxin's lethal effect, even if it is completely devoid of toxic activity when used alone. Nevertheless, immunization with this domain affords full protection from disease in mouse models, thus indicating that the protective epitopes are located in this portion of the molecule (Titball et al., 1993; Nagahama et al., 2002).

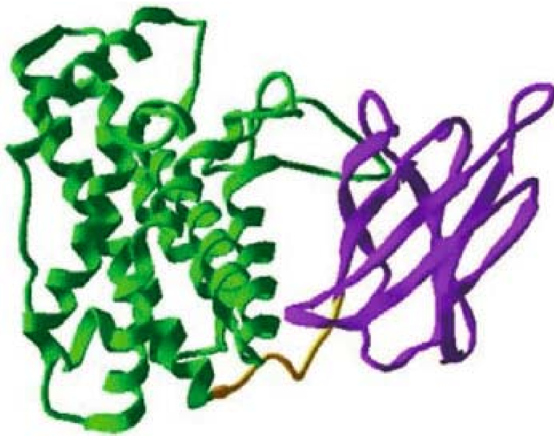


Fig. 36. Three-dimensional structure of *Clostridium perfringens* α-toxin. The N-terminal and C-terminal domains are green and violet, respectively, and the flexible linker is orange.

Recently, other bacterial PLCs, like those from *L. monocytogenes* and *Mycobacterium tuberculosis*, have been implicated in the pathogenesis of a number of diseases (Wadsworth et al., 1999; Raynaud et al., 2002).

Clostridium difficile Toxins A and B

Enterotoxin A (TcdA) and cytotoxin B (TcdB) of *Clostridium difficile* are the two virulence factors responsible for the induction of antibiotic-associated diarrhea. These toxins have molecular masses of 308 and 270 kDa, respectively, and belong to the class of large clostridial cytotoxins (Lyerly et al., 1986; Knoop et al., 1993).

The toxin genes *tcdA* and *tcdB* together with three accessory genes (*tcdC–E*) constitute the pathogenicity locus (PaLoc) of *C. difficile* (Cohen et al., 2000). Primary sequence homology between *tcdA* and *tcdB* gene products is higher than 60% identity (von Eichel-Streiber et al., 1994).

Upon binding to eukaryotic cells and translocation across membranes via receptor-mediated endocytosis, TcdA and TcdB monoglucosylate small GTP-binding proteins such as Rho, Rac and Cdc42 at a threonine residue (Just et al., 1995a, 1995b; Ciesla and Bobak, 1998). In most cells, *C. difficile* toxins induce depolymerization of the actin cytoskeleton, leading to a morphology similar to that induced by C3-like transferases. While toxin B has potent cytotoxic activity in vitro, the enterotoxic activity of *C. difficile* in animals has been mainly attributed to toxin A.

From the structural point of view, they are composed of two portions: the N-terminal nonrepetitive two thirds corresponding to the catalytic subunit, and the C-terminal third characterized by a highly repetitive domain called the "clostridial repetitive oligopeptide" (CROP), identified as the site of interaction with a carbohydrate structure as well as the ligand to which neutralizing antibodies bind (von Eichel-Streiber, 1994). A central hydrophobic region contains several predicted transmembrane segments and is believed to function as the translocation unit.

Bordetella pertussis Adenylate Cyclase

Adenylate cyclase (CyaA) is a toxin produced by *Bordetella pertussis*, *B. bronchiseptica* and *B. parapertussis* (Weiss and Hewlett, 1986). It is essential in the early stages of bacterial colonization of the respiratory tract and can induce apoptosis of lung alveolar macrophages (Goodwin and Weiss, 1990; Khelef et al., 1993).

Organized as a bifunctional protein, CyaA (177 kDa) is composed of an N-terminal cell-invasive and calmodulin-dependent adenylate cyclase domain (residues 1–400) fused to a pore-forming hemolysin (residues 401–1706; Glaser et al., 1988; Bejerano et al., 1999; see also the section Pore-Forming Toxins: RTX Hemolysins). Unlike most of the other members of the RTX family that are secreted into the supernatant, CyaA remains associated to the bacterial surface, through interactions with filamentous hemagglutinin (FHA). This toxin forms small cation-selective channels in lipid bilayer membranes and delivers into the cytosol of target cells the adenylate cyclase (AC) domain, which, upon binding to calmodulin, catalyzes an uncontrolled conversion of ATP to cAMP, thus causing intoxication and disruption of cellular functions (Ladant and Ullmann, 1999). Calcium has been shown to play a fundamental role in channel formation (Knapp et al., 2003). Furthermore, it was also demonstrated that the ability of the AC domain to form pores and translocate across the membrane is strictly linked to the correct folding of an amphipathic α-helix spanning residues 509–516. Substitution of Glu-509 with a helix-breaker proline residue, in fact, significantly reduced the capacity of the toxin to undergo translocation (Osickova et al., 1999).

A very similar function and mechanism of action is that of ExoY, an adenylate cyclase produced by *Pseudomonas aeruginosa* and injected into the cytoplasm of eukaryotic cells by the type III secretion apparatus (see Table 1, and the section Toxins Injected into Eukaryotic Cells in this Chapter). However, differently from CyaA, ExoY is not activated by calmodulin. In vivo, following infection with ExoY-expressing strains, CHO cells showed a rounded morphology, which correlated with increased cAMP levels (Yahr et al., 1998).

Anthrax Edema and Lethal Factors

Lethal factor (LF) and edema factor (EF) proteins, produced by *Bacillus anthracis*, combine with the protective antigen PA to give the lethal (PA+LF) and edema (PA+EF) toxins (Brossier et al., 2000; Collier and Young, 2003; Fig. 1, panel 17). In both complexes, the PA has the pore-forming, receptor-binding activity (see the section Pore-Forming Toxins in this Chapter), whereas EF and LF display, in turn, the toxic activities.

The EF and LF genes are located on a large plasmid (Mikesell et al., 1983) and encode precursors of approximately 800 residues. Cleavage of the N-terminal signal peptides yields mature EF and LF proteins with molecular masses of 88.8 kDa and 90.2 kDa, respectively. These virulence factors enter cells by binding to proteolytically activated, receptor-bound, oligomeric PA; following receptor-mediated endocytosis, the low pH causes a conformational change in PA, allowing the translocation of EF-LF across cell membrane (Collier, 1999). The EF-LF is then endocytosed and translocated from endosomes directly to the cytosol of cells, where both toxins perform their toxic activities (Fig. 37). The binding sites of EF and LF on PA have been recently mapped (Cunningham et al., 2002).

Once inside the cells, EF binds calmodulin and catalyzes an unregulated production of the second messenger cAMP, thereby perturbing the normal cell regulatory mechanisms (Goyard et al., 1989). Calcium influx is required for inducing cyclic AMP toxicity in target cells (Kumar et al., 2002).

Whereas the PA-binding domain displays a strong sequence homology to lethal factor LF, the catalytic domain is more similar to the other known adenylate cyclase CyaA toxin of *Bordetella pertussis* (Escuyer et al., 1988). On the other hand, LF cleaves the amino-terminus of the cellular mitogen-activated protein kinase kinases (MAPKK1 and MAPKK2), thus causing inhibition of the MAPK signal transduction pathway, which is key to cellular proliferation and signal transduction processes in the cell (Duesbery et al., 1998; Vitale et al., 1999).

Recently, the 3D structures of LF and EF have been solved (Fig. 38). LF comprises four domains: domain I binds the membrane-translocating component of anthrax toxin (PA); domain II resembles the ADP-ribosylating toxin from *Bacillus cereus*; domain III is inserted into domain II, and seems to have arisen from a repeated duplication of a structural element of domain II. Domain IV is distantly related to the zinc metalloprotease family, and contains the catalytic center (Pannifer et al., 2001). The catalytic portion of EF is made by three globular domains. The active site is located at the inter-

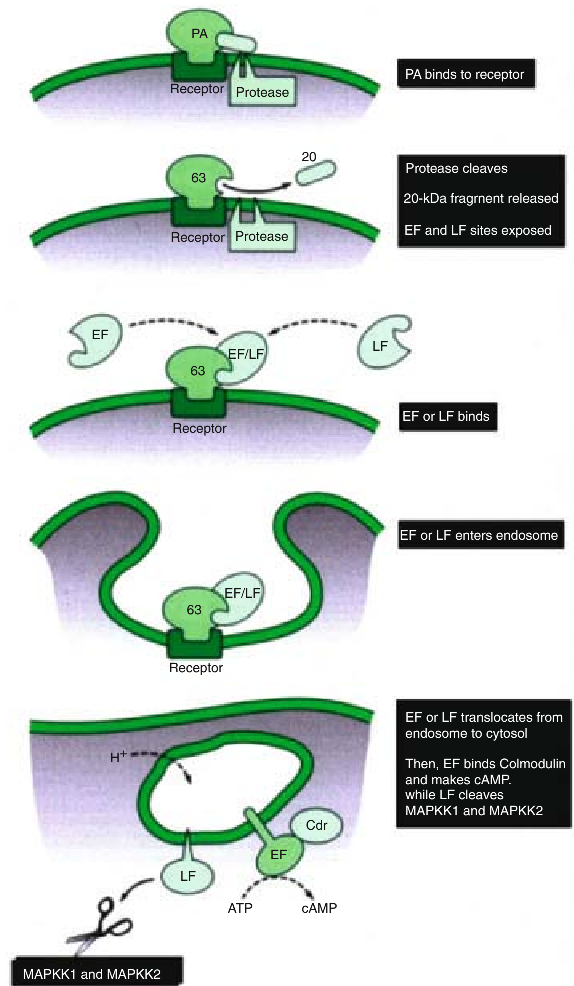


Fig. 37. Mechanism of PA-mediated entry and intoxication of anthrax LF and EF toxins.

face of two domains (C_A and C_B), which together form the catalytic core, containing the catalytic residue His351. EF has been crystallized both alone and in complex with calmodulin. The differences between the two forms are induced by calmodulin, which acts by stabilizing the conformation of the substrate-binding-site of EF (Drum et al., 2002). Interestingly, a remarkable level of primary sequence similarity can be detected between EF and the N-terminal, calmodulin-binding domain of *Bordetella* adenylate cyclase CyaA. In particular, His351 is conserved between the two proteins.

Once in the cytoplasm, LF acts as a zinc-metalloprotease disrupting normal homoeostatic functions. The macrophage is a uniquely sensitive cell type that seems to be a vital global mediator of toxin-induced pathologies. Removal of macrophages from mice renders them insensitive to LF challenge (Hanna, 1999).

In addition, LF, but not EF, is able to cause apoptosis in human endothelial cells. As a con-

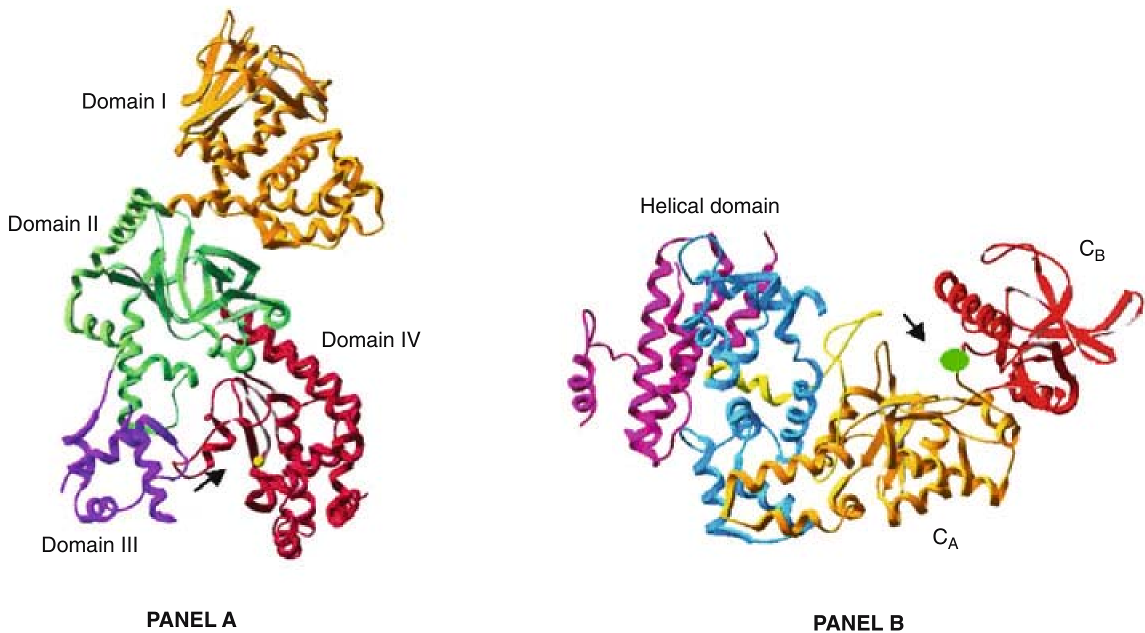


Fig. 38. Crystal structures of the catalytic portion of anthrax lethal factor (panel A) and edema factor in complex with calmodulin (panel B). Panel A. The four domains are in different colors. The zinc atom complexed by domain IV is indicated by an arrow.

sequence, the observed endothelial toxicity contributes to vascular pathology and hemorrhage during systemic anthrax (Kirby, 2004).

E. coli Cytotoxin Necrotizing Factors

Cytotoxin necrotizing factors (CNF1 [Fig. 1, panel 18] and CNF2), single-chain proteins of 115 kDa produced by a number of uropathogenic and neonatal meningitis-causing pathogenic *E. coli* strains (Caprioli et al., 1984; De Rycke et al., 1987), are immunologically related and share 85% identity. They also share some similarity with the dermonecrotic toxin of *Pasteurella multocida* and *Bordetella pertussis* (Schmidt et al., 1999). Both CNF1 and CNF2 toxins are encoded by a single structural gene with a low G+C content (35%). However, whereas *cnf1* is chromosomally encoded, cnf2 is carried on a large transmissible F-like plasmid called "Vir" (Oswald and De Rycke, 1990; Falbo et al., 1992).

These toxins induce ruffling, stress fiber formation, and cell spreading in cultured cells by activating the small GTP-binding proteins Rho, Rac and Cdc42, which control assembly of actin stress fibers (Oswald et al., 1994). CNF1 induces only a transient activation of Rho GTPase and a depletion of Rac by inducing the addition of an ubiquitin chain, which is known to drive to specific degradation by the proteasome. Reduction of Rac GTPase levels induces cell motility and cellular junction dynamics allowing efficient cell invasion by uropathogenic bacteria (Doye et al., 2002). The catalytic region of CNF1 has been crystallized (Buetow et al., 2001; Fig. 39). The active site contains a catalytic triad, which is positioned in a deep pocket, thus explaining the restricted access to unspecific substrates and therefore its specificity. Very likely, some type of conformational rearrangement is required also to accomodate Rho in this narrow cavity.

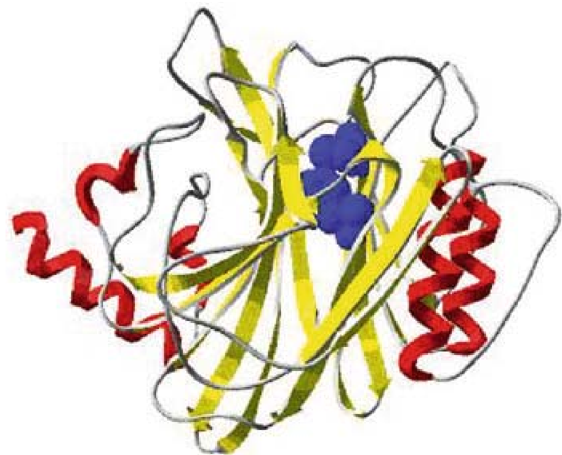


Fig. 39. Crystal structure of the active site of *E. coli* CNF1. The catalytic site composed by Cys866-His881 is colored in blue.

Recently, a CNF1-like toxin (CNFY) has been identified also in *Yersinia pseudotubercolosis* (Lockman et al., 2002). Differently from the *E. coli* CNFs, CNFY has been shown to selectively activate RhoA (Hoffman et al., 2004).

Bordetella Dermonecrotic Toxin

Dermonecrotic toxin (DNT) is produced by *Bordetella* species as a single-chain polypeptide chain of 1464 amino acids, which is composed of a C-terminal portion that contains the catalytic site, and of an N-terminal receptor-binding domain. DNT shares about 30% identical residues in the catalytic domain with *E. coli* CNF1, including the catalytic Cys and His residues. DNT is a transglutaminase, which catalyzes the deamidation or polyamination at Gln63 of Rho and of the corresponding residues of Rac and Cdc42 (Horiguchi, 2001). This activity causes alteration of cell morphology, reorganization of stress fibers, and focal adhesions on a variety of animal models. Recently, it has been demonstrated that the initial 54 amino acids of DNT are sufficient for cell surface recognition. However, the receptor is still unknown.

Cytolethal Distending Toxins

The cytolethal distending toxin (CDT) produced by *Haemophilus ducreyi* (HdCDT) is the prototype of a growing family of bacterial toxins that act by inducing cell enlargement followed by cell death (Cortes-Bratti et al., 1999; Frisan et al., 2003). HdCDT is a complex of three proteins (CdtA, CdtB and CdtC) encoded by three genes that are part of an operon. Members of this family have been identified in *E. coli*, *Shigella*, *Salmonella*, *Campylobacter*, *Actinobacillus* and *Helicobacter hepaticus* (Okuda et al., 1995; Lara-Tejero and Galan, 2001; Hoghjoo et al., 2004; Pickett et al., 2004; Shenker et al., 2004; Young et al., 2004). The overall sequence similarity varies among the different members of this family of toxins. HdCDT intoxicates eukaryotic cells by causing a three- to fivefold gradual distension and induces cell cycle arrest in the G_2 phase. It has also been shown to induce DNA double-strand breaks and formation of actin stress fibers via activation of the small GTPase RhoA. Recently it has been shown that CdtB is the active subunit of the CDT toxin and acts as a nuclease. All the amino acids predicted to be important for nuclease activity are conserved in the CdtB of different bacteria, suggesting that the mechanism of action is the same for all CDT toxins. On the other hand, CdtA and CdtC are able to bind to the surface of HeLa cells, therefore playing a role in the delivery of the active domain to target cells (Lee et al., 2003).

Toxins Acting on the Cytoskeleton Structure

The cytoskeleton is a cellular structure that consists of a fiber network composed of microfilaments, microtubules, and the intermediate filaments. It controls a number of essential functions in the eukaryotic cell and participates in all kinds of cellular movement and transport; furthermore, the cytoskeleton is involved in processes like exo- and endocytosis, vesicle transport, cell-cell contact, and mitosis (Kabsch and Vandekerckhove, 1992).

The group of cytoskeleton-affecting bacterial toxins comprises not only virulence factors that directly act on particular elements of the cytoskeleton, but also proteins that perform an indirect action by affecting regulatory components, which control its organization (Aktories, 1994; Richard et al., 1999). Most of them do it by modifying the regulatory, small G proteins, such as Ras, Rho, Rac and Cdc42, which control cell shape. These toxins, which have a dramatic but indirect effect on the cytoskeleton and are described in the section Toxins Acting on Signal Transduction, are *E. coli* CNF and *C. difficile* enterotoxins A and B. Other toxins acting on regulatory G proteins are exoenzyme S, C3 and YopE, which are described below as toxins that are directly injected into the eukaryotic cells. Other bacterial molecules that cannot be strictly considered toxins but that have a powerful ability to polymerize actin are ActA and IcsA of *Listeria* and *Shigella,* respectively. These are described elsewhere in this volume (see Listeria and Relatives in Volumn 4 and The Genus Shigella in Volumn 6). Another toxin acting indirectly on the cytoskeleton is the zonula occludens toxin (Zot) produced by *V. cholerae*, a toxin with an unknown mechanism of action that modifies the permeability of tight junctions (Zot is described in the paragraph Toxins with Unknown Mechanism of Action in this Chapter). In the following section we consider only toxins that have the cytoskeleton as a direct target. The only toxin shown to affect directly the cytoskeleton is the C2 toxin of *C. botulinum*, which ADP-ribosylates monomeric actin, making it unable to polymerize. A second protein that has recently been described as being able to bind actin and stabilize the fibers supporting the ruffles induced by the *Salmonella* type III secretion system is SipA (described in the section Toxins Injected into Eukaryotic Cells in this Chapter).

Representatives of both subgroups can be identified among the class of ADP-ribosylating factors that ultimately display their toxic effect on the cytoskeleton of eukaryotic cells. In fact, whereas the family of *Clostridium botulinum* toxin C2, clostridial toxin C3 (and related pro-

teins), and *Pseudomonas aeruginosa* exoenzyme S (Exo S) act on small GTP-binding proteins that regulate the correct functioning of the cytoskeleton, and thus have an indirect toxic effect (Coburn et al., 1999).

Clostridium botulinum Toxin C2 and Related Proteins

Clostridium botulinum toxin C2 is the main representative of a class of binary cytotoxins produced by clostridial species that predominantly act on polymerized actin microfilaments of 7–9 nm in diameter (Aktories et al., 1986; Aktories and Wegner, 1992). C2 ADP-ribosylate monomeric G-actin at an arginine residue (Aktories et al., 1986; see the section ADP-Ribosylating Toxins in this Chapter). Because this arginine (Arg-177) is a contact site between actin monomers, the binding of the ADP-ribose moiety prevents actin's polymerization.

Other members of this family are *C. perfringens* iota toxin (Stiles and Wilkins, 1986; Perelle et al., 1993) and the related *C. spiroforme* and *C. difficile* ADP-ribosylating toxins (Popoff and Boquet, 1988a; Just et al., 1994), which are generally classified as iota-like toxins. These binary toxins are constructed according to the A/B model architecture, but in this case the two domains reside in separate molecules that interact to cause the toxic effect. Therefore, these toxins have an enzymatically active and toxic domain (A) and a binding component (B), which is essential for the binding at the cell surface and for the translocation inside the cell.

Clostridium botulinum toxin C2 is an extremely toxic agent, which induces hypotension, increase in intestinal secretion, vascular permeability, and hemorrhaging in the lungs. In contrast to botulinum neurotoxins, C2 does not seem to display any neurotoxic effect. The two molecules that constitute its toxic moiety are classified as C2-II (for the binding component) and C2-I (for the enzymatic component). The C2-II is a 100-kDa protein that must be proteolytically cleaved to a 75-kDa fragment before it can bind to the surface receptor; upon this interaction, a binding site for the 50-kDa C2-I component is activated and the toxic domain is taken up by receptor-mediated endocytosis (Ohishi, 1987). Substrates of the C2-I toxin are β/γ-non-muscle actin and γ-smooth muscle actin, but not α-actin isoforms. Conversely, the related iota toxin of *Clostridium perfringens* has been found to ADP-ribosylate all actin isoforms (Mauss et al., 1990). The iota toxin is a binary toxin produced by *Clostridium perfringens* type E, which has been implicated in fatal calf, lamb and guinea pig enterotoxemias (Madden et al., 1970). Structurally, it has two independent domains: Ia, which is the ADP-ribosyltransferase, and Ib, which is involved in the binding and internalization of the toxin by the cell (Stiles and Wilkins, 1986). The crystallization of the C2-I component in complex with its substrate NADH has recently been achieved (Tsuge et al., 2003; Fig. 40), showing a close relationship of iota toxin with insecticidal protein VIP2 of *Bacillus cereus*.

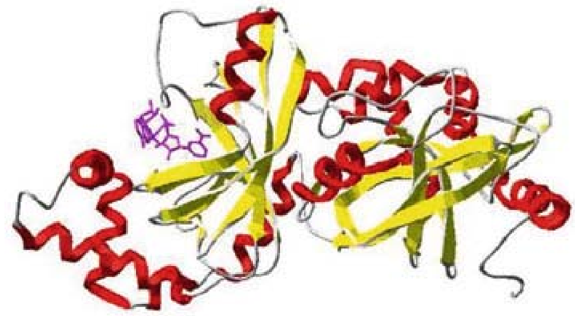


Fig. 40. Crystal structure of the catalytic domain C2I of *C. perfringens* C2 toxin (red and yellow) in complex with NADH (pink).

Clostridium difficile induces its pathogenic effects by secreting a number of potent cytotoxins; one, in particular, has been found to possess ADP-ribosyltransferase activity (CDT). CDT acts on the cytoskeleton structure by disaggregating actin filaments and thus provokes an increase of globular actin (G-actin; Popoff et al., 1988b; Gulke et al., 2001).

Another member of the group of iota-like toxins is the *Clostridium spiroforme* toxin, composed of a toxic subunit Sa and a binding subunit Sb (Popoff et al., 1989). The level of primary sequence homology detected among the enzymatic and binding components of this class of ADP-ribosylating toxin ranges from 32% to 80% identity, the binding domains being the better conserved. The C2 toxin is the one with the lower degree of sequence conservation, and this correlates with the fact that it does not appear to be crossreactive with the other iota-like toxins.

Experiments of site-directed mutagenesis have helped to define for these toxins an active site very similar to those described for the better studied members of the family of ADP-ribosyltransferases (Barth et al., 1998; see the section ADP-Ribosyltransferases: A Common Structure of the Catalytic Site in this Chapter).

Escherichia coli Lymphostatin

Lymphostatin is a very recently identified protein in enteropathogenic strains of *E. coli* (EPEC; Klapproth et al., 2000).

A leading cause of diarrhea among infants in developing countries, EPEC is also one of the few known bacterial causes of chronic diarrhea. These strains are characterized by their ability in host cells to induce cytoskeletal rearrangements that result in the formation of adhesion pedestals. This mechanism known as "the attaching and effacing effect" (Moon et al., 1983; Khoshoo et al., 1988) ultimately allows the bacterium to colonize the host for prolonged periods.

Lymphostatin also has been identified as one of the primary factors that selectively block the production of interleukin-2 (IL-2), IL-4, IL-5 and γ interferon by human peripheral cells and inhibit proliferation of these cells, thus interfering with the cellular immune response (Klapproth et al., 1995).

Lymphostatin, a very large toxin with a predicted molecular weight of 366 kDa, shares significant homology with the catalytic domain of the large clostridial cytotoxins, including toxins A and B of *Clostridium difficile*, lethal toxin of *C. sordelii*, and a toxin of *C. novyi*. Its corresponding gene, *lifA*, with 9669 bp, is the largest reported gene in *E. coli*. Some *lifA* mutants of EPEC have been constructed to verify the lymphocyte inhibitory factor (LIF) activity of its gene product; lysates of this mutant lacked the ability of wildtype EPEC lysates to inhibit expression of IL-2, IL-4 and γ interferon mRNA and protein in mitogen-stimulated lymphocytes, while the expression of IL-8 was unaffected (Klapproth et al., 2000). Experiments of colony hybridization performed using an internal fragment of the *lifA* gene identified a similar gene present in most of the EPEC and enterohemorrhagic *E. coli* (EHEC) strains able to produce the attaching and effacing lesions on host epithelial cells, but this gene was not found in other *E. coli* and related organisms (Klapproth et al., 2000).

Toxins Acting on Intracellular Trafficking

Vesicle structures are essential in the eukaryotic cell for a number of vital processes such as receptor-mediated endocytosis and exocytosis; these are used either to internalize portions of the plasma membrane and address them to the specialized compartment, or to transport to the cell surface molecules synthesized in the ER and modified in the Golgi apparatus.

One example of exocytic pathway is that involving the release of neurotransmitters that are contained within small synaptic vesicles packed at synaptic terminals; the majority of these vesicles are bound to the cytoskeleton and are not directly available for immediate release, but some of them are present at the cytosolic face of the presynaptic membrane and are ready to release their content. However, at low calcium concentrations, only an occasional vesicle fuses to the presynaptic membrane, giving rise to a depolarization event. This event leads to the opening of calcium channels and thus to an increase of calcium concentration, which finally triggers the fusion of the neurotransmitter vesicles with the plasma membrane.

Recently, this field was greatly advanced by the identification of the eukaryotic molecules responsible for vesicle docking and membrane fusion. Three of these proteins (namely vesicle-associated membrane protein [VAMP]/synaptobrevin, synaptosome-associated protein [SNAP-25], and syntaxin) are the specific targets of a number of neurotoxins produced by bacteria of the genus *Clostridium* (CNTs; Montecucco and Schiavo, 1994; Fig. 41).

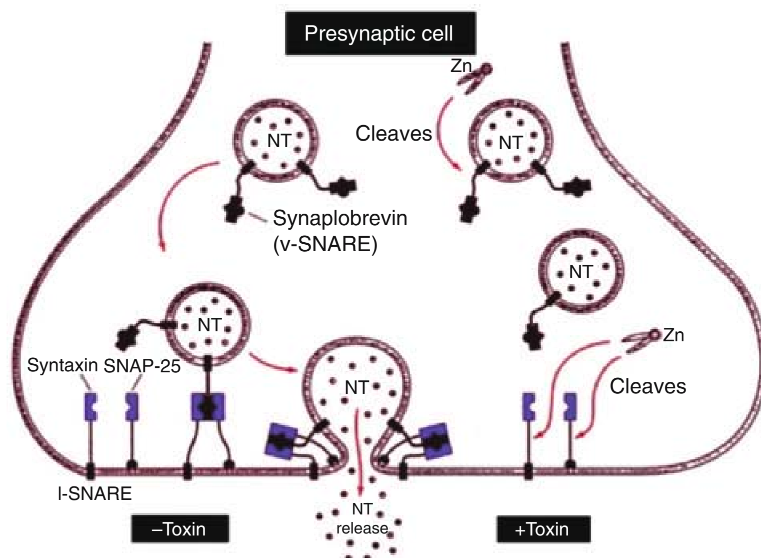


Fig. 41. Mechanism of action of clostridial neurotoxins.

The CNT family is composed of tetanus neurotoxin (TeNT) and seven serotypes of botulinum neurotoxins (BoNT/A–BoNT/G), which are specific zinc-dependent proteases whose action finally causes the block of neuroexocytosis (Schiavo et al., 1992; Pellizzari et al., 1999; Lalli et al., 2003). The degree of sequence homology detected among this group of toxins is high, ranging from 30% to more than 50% identity.

Clostridium tetanii Neurotoxin

Tetanus neurotoxin (TeNT; Fig. 1, panel 19) is the unique causal agent of the pathological condition of spastic paralysis known as tetanus. This is one of the most potent toxins known so far, with a 50% lethal dose (LD_{50}) in humans of 0.1–1.0 ng/kg.

The TeNT is produced by *Clostridium tetanii* as a single chain polypeptide of 150 kDa that, following proteolytic cleavage, is divided into fragments H (heavy) and L (light) held together by a disulfide bridge. Its overall structure is similar to that of A/B toxins, where the toxic subunit A is represented here by the light chain L, and subunit B is constituted by the H_C and H_N domains. The heavy chain is composed of fragments HC, which has recently been found to bind di- and trisialylgangliosides on neuronal cell membranes, and HN, which is involved in the transmembrane translocation of the L chain to the cytosol (Schiavo et al., 1990; Shapiro et al., 1997). The L chain is a 50-kDa fragment containing the –HExxH– motif typical of metalloproteases. It binds zinc and specifically cleaves VAMP/synaptobrevin, a eukaryotic factor essential for membrane fusion (Rossetto et al., 1995).

The first step of intoxication is the specific binding of domain H_C of TeNT to both high and low affinity receptors exposed on the presynaptic neuronal membrane at neuromuscular junctions (Montecucco, 1986); the second step is internalization of TeNT into the peripheral motoneuron and then retrograde axonal transport. The TeNT is released through the postsynaptic membrane into the synaptic space where it enters into the inhibitory interneurons of the central nervous system through receptor-mediated endocytosis (Halpern and Neale, 1995). At this point, while the H_C domain is in the vesicle, the translocation domain H_N helps the catalytic light chain L to cross the vesicle membrane and gain access to the cytosolic compartment where L performs its toxic activity on VAMP/synaptobrevin (Montal et al., 1992).

Interestingly, domain H_C retains the unique transport properties of the intact holotoxin and is capable of eliciting a protective immunological response against the full-length tetanus neurotoxin. A single zinc atom is bound to the L chain of TeNT and is essential for toxicity. This specific metallo-dependent proteolytic activity is common to the other clostridial toxins and to the lethal factor (LF) of *Bacillus anthracis*.

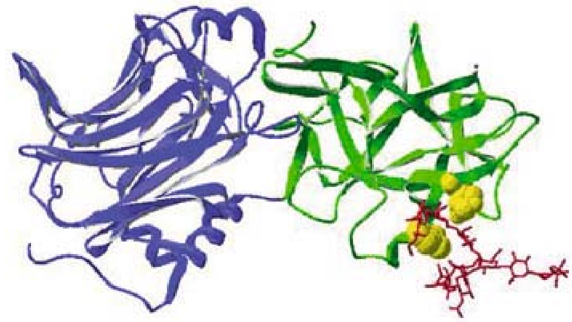


Fig. 42. Crystal structure of the receptor-binding domain HC of tetanus neurotoxin in complex with a ganglioside analogue (in red). The N-terminus and C-terminus are colored in blue and green, respectively. The residues probably involved in ganglioside binding are yellow (see Fig. 1, panel 18).

The crystal structure of the receptor-binding fragment HC of tetanus neurotoxin has been recently determined at 2.7 Å resolution (Umland et al., 1997; Fig. 42) revealing an N-terminal jellyroll domain and a C-terminal β-trefoil domain.

To determine which amino acids in tetanus toxin are involved in ganglioside binding, homology modeling was performed using recently resolved X-ray crystallographic structures of the tetanus toxin HC fragment. On the basis of these analyses, the amino acids tryptophan 1288, histidine 1270, and aspartate 1221 were found to be critical for binding of the HC fragment to ganglioside GT1b (Fotinou et al., 2001; Louch et al., 2002).

Although the overall sequence homology detected among clostridial neurotoxins is significant, this similarity weakens in the region encompassing the C-terminal domain (Murzin et al., 1992); the fact that each toxin possesses its own unique receptor and is immunologically distinct from the others has been attributed to sequence divergence of this domain which, therefore, could be responsible for receptor specificities (Lacy et al., 1999).

Clostridium botulinum Neurotoxins

These neurotoxins (BoNT/A-G; Fig. 1, panel 20) are the causative agents of the flaccid paralysis typical of clinical botulism intoxication (Hatheway, 1995). All of them are zinc-dependent proteases that show a strong tropism for the neuromuscular junction (Simpson, 1980; Rossetto et al., 1995), where they bind to still unidentified receptors in a strictly serotype-specific manner. This binding step is followed by the entry of the toxin into the cytoplasm of the motoneurons and

by specific proteolytic cleavage of intracellular targets belonging to the family of soluble *N*-ethylmaleimide-sensitive, fusion factor attachment protein receptors (SNARE). Four out of the seven botulinum neurotoxins (BoNT/B, D, F and G) cleave VAMP/synaptobrevin, another two act specifically on SNAP/25, whereas the last one, BoNT/C, cleaves both syntaxin and SNAP/25 substrates. In all cases, the ultimate effect is the total block of acetylcholine release (Montecucco and Schiavo, 1995).

These toxins are generally produced as large complexes of 300–900 kDa containing additional proteins such as hemagglutinin (300 kDa) and nontoxic peptides, which are believed to act as stabilizing agents of the neurotoxins in the gut environment (Sakaguchi, 1983).

The BoNTs are synthesized as inactive polypeptide chains of 150 kDa, which (following proteolytic cleavage) divide into two chains of 50 and 100 kDa that remain linked by a disulfide bridge. The catalytic function is carried by the 50-kDa fragment, the light chain L (residues 1–437), whereas the 100-kDa subunit (heavy chain, H) contains both the translocation (residues 448–872) and the receptor-binding domains (residues 873–1295; Krieglstein et al., 1994). The crystal structure determined for the full-length polypeptide of BoNT serotype A (Lacy et al., 1998; Fig. 43) reveals a number of remarkable features, particularly related to the peculiar structure of the translocation domain. This contains, in fact, a central pair of α-helices 105 Å long and a 50-residue loop that wraps around the catalytic domain in a belt-like fashion, partially occluding the active-site pocket. This unusual loop bears the site of the proteolytic cleavage, which is required for activation of the toxin; the fact that in the protoxin, the translocation domain shields the active site explains why the catalytic activity in test tube experiments is greatly enhanced by reduction of the disulfide bond. The fold of the translocation domain suggests a mechanism of pore formation different from that displayed by other pore-forming toxins. The helices are antiparallel and amphipathic and twist around each other in a coiled-coil-like structure. In addition, the domain has two strand-like segments that lie parallel to the helical axis and are predicted to be directly involved in membrane spanning. Very recently, the X-ray structure obtained for the recombinant form of chain L of BoNT-A has shed light on a possible novel mode of substrate binding and catalytic mechanism (Segelke et al., 2004).

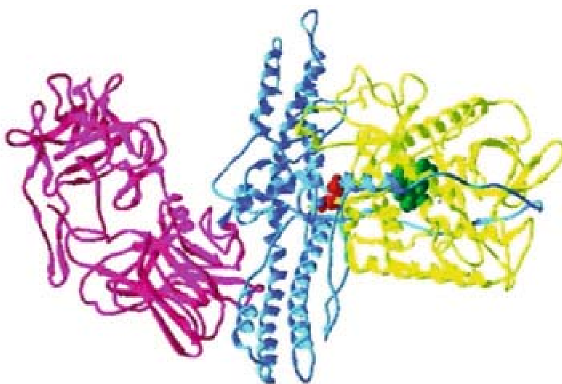


Fig. 43. X-ray structure of *Clostridium botulinum* neurotoxin serotype A. The 50-kDa catalytic domain (L) is colored in yellow, with the zinc-binding domain in green. The N-terminal portion of the 100-kDa subunit involved in translocation is blue, whereas the C-terminal receptor-binding moiety is in magenta. The disulfide bond linking the two 50- and 100-kDa fragments is colored in red (see Fig. 1, panel 20).

The highest degree of homology detected among this family of clostridial neurotoxins is concentrated in the light chain L (30–60% identity; particularly its N-terminus), probably involved in substrate recognition, and in the central portion that contains the catalytic zinc-binding motif –HexxH– characteristic of zinc endopeptidases. The zinc atom coordinated by this pocket is required for the in vivo toxicity of BoNTs.

Years ago, medical experiments demonstrated that injection of BoNT/A is very effective in strabismus; since then, the therapeutic applications of these neurotoxins have been extended to a variety of diseases which benefit from a functional paralysis of the neuromuscular junction, and all the BoNTs are under clinical testing (Jankovich and Hallett, 1994).

Helicobacter pylori Vacuolating Cytotoxin Vac A

Highly pathogenic strains of *Helicobacter pylori*, the etiological agent of peptic ulcer and gastritis (Cover and Blaser, 1992), produce vacuolating cytotoxin A (VacA; Papini et al., 1994; Fig. 1, panel 21). This toxin is responsible for massive growth of vacuoles within epithelial cells and, when administered to mice, VacA causes loss of gastric gland architecture, cell necrosis, and gastric ulceration (Telford et al., 1994). Synthesized as a 140-kDa precursor, VacA is secreted from the bacterium through its 45-kDa carboxy-terminal domain, using a mechanism similar to that of neisserial IgA proteases (Schmitt and Haas, 1994; Fiocca et al., 1999). When purified from the culture supernatant of Type I *H. pylori* strains, the protein has a molecular weight of approximately 600–700 kDa, suggesting the idea of a multimeric complex; electron microscopy studies have in fact demonstrated the flower-shaped structure of the toxin (Lupetti et al., 1996; Fig. 44) resulting from the aggregation of either six or seven monomers, each

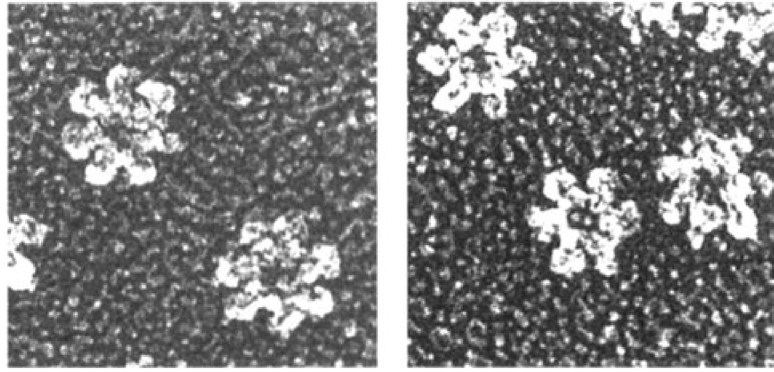


Fig. 44. Vacuolating cytotoxin structure: heptameric and hexameric forms of VacA as observed in electron micrographs of quick-freeze, deep-etched preparations. The oligomers are approximately 30 nm in diameter with a 10–12 nm central cavity.

comprising the 95-kDa amino-terminal region of the VacA precursor. Recently, a model has been proposed to show how VacA can insert into membranes forming hexameric, anion-selective pores (Kim et al., 2004).

Each monomer can be cleaved at a protease-sensitive site into two fragments of 37 kDa and 58 kDa (p37 and p58 moieties) that may represent the A and B moieties of AB-like bacterial toxins. The 37-kDa, amino-terminal portion is highly conserved at the sequence level and is able to induce vacuoles when the *vacA* gene is placed under the control of a strong eukaryotic promoter and transfected into epithelial cells. This evidence suggests that the active site could be located in this region of the molecule, whereas the carboxy-terminal portion is likely to be devoted to receptor recognition and binding. Although VacA is exported over the outer membrane and is released from the bacteria, recent data have been presented to show that a portion of the toxin remains associated with the bacterial surface. Surface-associated toxin is biologically active and organized into distinct toxin-rich domains on the bacterial surface. Upon bacterial contact with host cells, toxin clusters are transferred to the host cell surface via a contact-dependent mechanism, followed by uptake and intoxication (Ilver et al., 2004).

The mechanism of toxicity exploited by this virulence factor has not yet been completely elucidated. What is known is that VacA causes an alteration of the endocytic pathway, which results in the selective swelling of late endosomes or prelysosomal structures. The small GTP-binding protein Rab7 is necessary for vacuole formation (Papini et al., 1994, 1997). Even though it is unknown, the target of VacA action is strongly believed to be a fundamental effector in membrane trafficking.

Streptococcus pyogenes NAD$^+$ Glycohydrolase

NAD$^+$ glycohydrolase is an important virulence factor produced by group A streptococci (GAS), which is thought to enhance pathogenicity by facilitating the spread of the microorganism through host tissues. This enzyme catalyzes the hydrolysis of the nicotinamide-ribose bond of NAD to yield nicotinamide and ADP-ribose. Differently from ADP-ribosylating toxins, NAD$^+$ glycohydrolases possess a much higher rate of NADase activity and do not require an ADP-ribose acceptor. Interestingly this GAS virulence factor is functionally linked to streptolysin O (SLO), a pore-forming toxin, which has been shown to be required for efficient translocation of NAD$^+$ glycohydrolase into epithelial cells. In contrast to the wildtype GAS, isogenic mutants deficient in the expression of SLO, NAD$^+$ glycohydrolase, or both proteins resulted in reduced cytotoxicity and keratinocyte apoptosis. These results suggest that NAD$^+$ glycohydrolase modulates host cell signaling pathways and contributes to the enhancement of streptolysin O cytotoxicity (Bricker et al., 2002).

Toxins Injected into Eukaryotic Cells

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

In the classical view, toxins were believed to be molecules that cause intoxication when released by bacteria into the body fluids of multicellular organisms. This definition failed to explain the pathogenicity of many virulent bacteria such as *Salmonella*, *Shigella* and *Yersinia*, which did not release toxic proteins into the culture supernatant. Today we know that these bacteria also intoxicate their hosts by using proteinaceous weapons. These bacteria intoxicate individual eukaryotic cells by using a contact-dependent secretion system to inject or deliver toxic proteins into the cytoplasm of eukaryotic cells (Fig. 2, panel 3). This is done by using specialized secretion systems that in Gram-negative bacteria are called "type III" or "type IV," depending on whether they use a transmem-

brane structure similar to flagella or conjugative pili, respectively.

Mediators of Apoptosis

Pathogens use different mechanisms to induce or prevent apoptosis in host cells. Virulence factors produced by the pathogen can interact directly with effector molecules of apoptosis or interfere with factors involved in cell survival (Weinrauch and Zychlinsky, 1999).

They include: pore-forming toxins which induce cell death by altering host cell permeability, bacterial toxins (such as DT, PAETA, Shiga and Shiga-like toxins) which induce cell death by inhibition of host protein synthesis, and type III secreted proteins of *Shigella*, *Salmonella* and *Yersinia* which are directly delivered into host cell compartment and trigger apoptosis by altering the signal transduction pathway. This latter class of toxins will be described here in more detail.

IpaB

Shigella, the causative agent of bacillary dysentery produces IpaB. *Shigella* invades the epithelial cells by causing the cell cytoskeleton to reorganize during bacterial entry. The bacteria are phagocytosed by macrophages and rapidly escape from phagosomal compartment to the cytosol where they induce apoptosis of the macrophages. Invasion and cytotoxicity require *Shigella* invasion plasmid antigen (Ipa) proteins, which are secreted by a type III secretion apparatus. Invasion and escape from the phagosome are dependent upon the expression and secretion of the IpaB, IpaC and IpaD. Only IpaB is required to initiate cell death by interaction with the interleukin-1β converting enzyme, or caspase I, which is one of the effector molecules of apoptosis. The IpaB-induced apoptosis results in an inflammation that has the effect not only of clearing and possibly localizing the infection but also promoting bacterial spread in the intestinal epithelium (Hilbi et al., 1998). Protein domains directly involved in pathogenicity have recently been mapped (Guichon et al., 2001).

SipB

An analog of *Shigella* invasin IpaB, *Salmonella* invasion protein (SipB) is produced by *Salmonella* and is delivered to the host cells by a type III secretion system. In contrast to *Shigella*, *Salmonella* does not escape from the phagosome, but it survives and multiplies within the macrophages. *Salmonella* virulence genes responsible for invasion and killing of macrophages are encoded by a chromosomal operon named *sip* containing five genes (sipEBCDA; Hermant et al., 1995). The sip genes show high sequence homology with the *ipa* operon of *Shigella*, and the Sip proteins show functional similarities with Ipa proteins. Both proteins have a predominant alpha-helical structure and contain two helical transmembrane domains, which insert deeply into the bilayer (Hume et al., 2003). Similarly to IpaB, SipB also induces apoptosis by binding interleukin-1β-converting enzyme.

Necessary for *Salmonella*-induced macrophage apoptosis, SipB acts through a caspase-I-activating mechanism similar to that used by IpaB (Hersh et al., 1999). Also, SipB can complement IpaB mutants, enabling them to invade cells and escape macrophage phagosomes.

YopP, YopJ and Related Proteins

Yersinia enterocolitica and *Yersinia pestis* produce YopP and YopJ, respectively (Straley et al., 1986; Mills et al., 1997). Following contact with the host cell, *Yersiniae* deliver into the cytoplasm of eukaryotic cells, through a type-III secretion system, plasmid-encoded proteins named "*Yersinia*-outer-membrane proteins" (Yop). These proteins are able to induce alteration of cytoskeleton (YopE and YopT), inhibition of phagocytosis (YopH), and in the case of YopP and YopJ, induction of apoptosis.

The mechanism by which *Yersinia* induces apoptosis is probably different from that described for *Shigella*, inasmuch as *Yersinia* induces apoptosis from the outside of host cells. The binding of YopJ directly to the superfamily of MAPKKs blocks both their phosphorylation and subsequent activation. These activities of YopJ are responsible for the inhibition of extracellular signal-regulated kinase, downregulation of TNF-α and suppression of the nuclear factor kappa B (NF-κB) signaling pathways, preventing cytokine synthesis and promoting apoptosis (Orth et al., 1999). The YopJ-related proteins that are found in a number of bacterial pathogens of animals and plants, such as AvrRxv from *Xanthomonas campestris* (Whalen et al., 1993), AvrA from *Salmonella* (Hardt et al., 1997), and y410 from *Rhizobium* (Freiberg et al., 1997) may function to block MAPKKs so that host signaling responses can be modulated upon infection. Whereas no function is known for AvrA and y410, AvrRxv is a plant pathogen virulence protein involved in the programmed cell death pathway.

Toxins Interfering with Inositol Phosphate Metabolism: SopB and IpgD

The SopB protein, secreted by *Salmonella dublin*, is a virulence factor essential for

	Motif 1	Motif 2
SopB	VVTFNFGVNELALKM	AWNCKSGKDRTGMMSDE
IpgD	VAAFNVGVNELALKL	CWNCKSGKDRTGMQDAE
PTPaseI	PVLFNVGINEQQTLA	FTSCKSAKDRTAMSVTL
PTPaseII	PVLFNVGINEQQTLA	FTCCKSAKDRTSMSVTL

Fig. 45. Alignment of conserved motifs.

enteropathogenicity. The toxin hydrolyzes phosphatidylinositol triphosphate (PIP_3), which is a messenger molecule that inhibits chloride secretion, thus favoring fluid accumulation and diarrhea (Norris et al., 1998). Furthermore, SopB, mediates actin cytoskeleton rearrangements and bacterial entry in a Rac-1 and Cdc42-dependent manner. Consistent with an important role for inositol phosphate metabolism in *Salmonella*-induced cellular responses, a catalytically defective mutant of SopB failed to stimulate actin cytoskeleton rearrangements and bacterial entry (Zhou et al., 2001).

SopB is homologous to the *Shigella flexneri* virulence factor IpgD, suggesting that a similar mechanism of virulence is also present in *Shigella*. Both proteins contain two regions of sequence similarities (motifs 1 and 2, Fig. 45) with human inositol polyphosphatases types I and II. Motif 2 contains a consensus sequence (Cys-X5-Arg) characteristic of Mg^{+2}-independent phosphatases in which the cysteine is the residue essential for catalysis. Recent studies have shown that IpgD acts as a potent inositol 4-phosphatase and is responsible for dramatic morphological changes of the host cell, ultimately leading to consistent actin filament remodeling (Niebuhr et al., 2002).

Toxins Acting on the Cytoskeleton

PSEUDOMONAS AERUGINOSA EXOENZYME S. This toxin is one of several products of *Pseudomonas aeruginosa* that contributes to its pathogenicity (Woods et al., 1989; Kulich et al., 1993; Fig. 1, panel 22). It belongs to the group of ADP-ribosylating factors that lack both the receptor-binding and translocation domains, and are directly injected by bacteria into the cytoplasm of eukaryotic cells. In this case, bacteria intoxicate individual eukaryotic cells by means of a contact-dependent type III secretion system (Yahr et al., 1996).

The 49-kDa ExoS protein ADP-ribosylates the small GTP-binding protein Ras at multiple sites but preferably at Arg-41 (Ganesan et al., 1998; see the section ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity in this Chapter). To become enzymatically active, ExoS requires the interaction with a cytoplasmic activator named "FAS" or "14.3.3" (Fu et al., 1993). When cells are transfected with the *exos* gene under the control of a eukaryotic cell promoter, a collapse of the cytoskeleton and a change of the morphology of the cells can be observed as primary consequences.

Pseudomonas aeruginosa ExoS is a bifunctional cytotoxin where the ADP-ribosyltransferase domain is located within its C-terminus portion. Recent studies showed, in fact, that when transfected or microinjected into eukaryotic cells, the N-terminus part of ExoS (amino acid residues 1–234) stimulates cell rounding. The N-terminus of ExoS (1–234) does not influence nucleotide exchange of Rho, Rac and Cdc42 but increases GTP hydrolysis. It has also been shown that Arg-146 of ExoS is essential for the stimulation of GTPase activity of Rho proteins (Goehring et al., 1999). The GTPase activating domain (GAP) of ExoS has been crystallized (Wurtele et al., 2001). In addition to these toxic effects performed on the cytoskeleton, other activities have been demonstrated for Exo S, such as the adhesive property on buccal cells (Baker et al., 1991) and the induction of human T lymphocyte proliferation (Mody et al., 1995). From sequence analysis, it has been possible to identify the regions of Exo S, which could be involved in NAD binding and thus constitute the common structure of the catalytic site.

CLOSTRIDIUM BOTULINUM EXOENZYME C3 AND RELATED PROTEINS. Produced by certain strains of *Clostridium botulinum* types C and D, exoenzyme C3 is a 251-amino acid protein that specifically ADP-ribosylates *rho* and *rac* gene products in eukaryotic cells (Moriishi et al., 1993; Fig. 1, panel 23). These substrates belong to the group of small GTP-binding proteins and seem to have a fundamental role in cell physiology and cell growth. The ADP-ribosylation process occurs at asparagine residues (Asn-41) located in the putative effector binding domains of *rho* and *rac* and thus alter their functions (Sekine et al., 1989). The enzymatic activity is identical to that of all ADP-ribosylating enzymes; however, the recently solved 3D structure has shown that the C3 exoenzyme structure can be distinguished by the absence of the elongated α-helix, which generally constitutes the ceiling of the active site cleft in the ADP-ribosylating toxins crystallized so far. Seemingly, this feature does not impair the ability of C3 either to accommodate the NAD substrate or to carry out the enzymatic reaction (Han et al., 2001; Fig. 46).

This exoenzyme is the prototype of the group of A-only toxins because it apparently lacks the receptor-binding B domain and thus is unable to enter the cells; for this reason, C3 cannot be considered a real virulence factor, and still unknown is whether C3 alone is able to intoxicate the cells.

Nevertheless, when microinjected into cells, it causes complete disruption of actin-stress fibers, rounding of the cell body, and formation of arborescent extensions.

Other members of this family of C3-related exoenzymes have been isolated from Gram-positive bacteria, such as certain strains of *Staphylococcus aureus* (Sugai et al., 1992), *Clostridium limosum* (Just et al., 1992) and *Bacillus cereus* (Just et al., 1995c). Whereas *C. botulinum* C3 and *C. limosum* exoenzyme are about 70% homologous and immunologically related, the epidermal cell differentiation inhibitor (EDIN) produced by *S. aureus* is only 35% homologous with C3 and shows no immunological crossreactivity (Fig. 47). However, crystal data recently obtained for *S. aureus* C3 exotoxin (EDIN-B) have disclosed a very similar structure (Evans et al., 2003). *Bacillus cereus* exoenzyme exhibits the same substrate specificity as the other C3-like transferases (it was found to act specifically on rho proteins). Nevertheless some differences can be observed for this toxin, such as the higher molecular weight (28 kDa) and, more importantly, the lack of immunological relationship to any other member of this family (Just et al., 1995a).

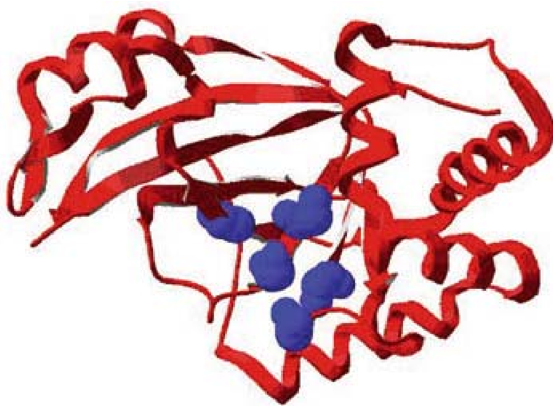


Fig.46. Crystal structure of exoenzyme C3 of *C. botulinum*. The residues which constitute the catalytic site are in blue.

SALMONELLA SOPE AND SIPA. *Salmonella typhimurium* achieves entry into cells by delivering effector proteins into the cytosol through a type III secretion system. These effectors stimulate signal pathways leading to reorganization of the cell's actin cytoskeleton, membrane ruffling and stimulation of nuclear response to promote efficient bacterial internalization. One of the proteins that stimulate the cellular response is SopE, which is able to activate signaling pathways through Rho GTPases by stimulating GTP/GDP nucleotide exchange on proteins such as Cdc42 and Rac (Hardt et al., 1998).

These signaling events lead to the recruitment of cellular proteins such as actin and T-plastin (an actin-binding protein that bundles actin), which finally induce actin cytoskeleton rearrangement and membrane ruffling. In addition, SopE stimulates nuclear responses that induce the synthesis of proinflammatory cytokines that contribute to the induction of diarrhea.

These cytoskeletal rearrangements are further modulated by SipA, which binds directly to actin, stabilizes actin filaments inhibiting depolymerization, and forms a complex with T-plastin thus increasing its actin-bundling activity (Zhou et al., 1999a, 1999b). SipA activities result in localized actin cytoskeleton reorganization and more pronounced extension of membrane ruffles, which facilitate bacterial uptake. The actin-cytoskeleton reorganization induced by *Salmonella* is reversible and infected cells are able to recover their normal architecture after bacterial internalization.

Crystal structures are available for SipA (Lilic et al., 2003) and for the catalytic fragment of SopE in complex with its host cellular target

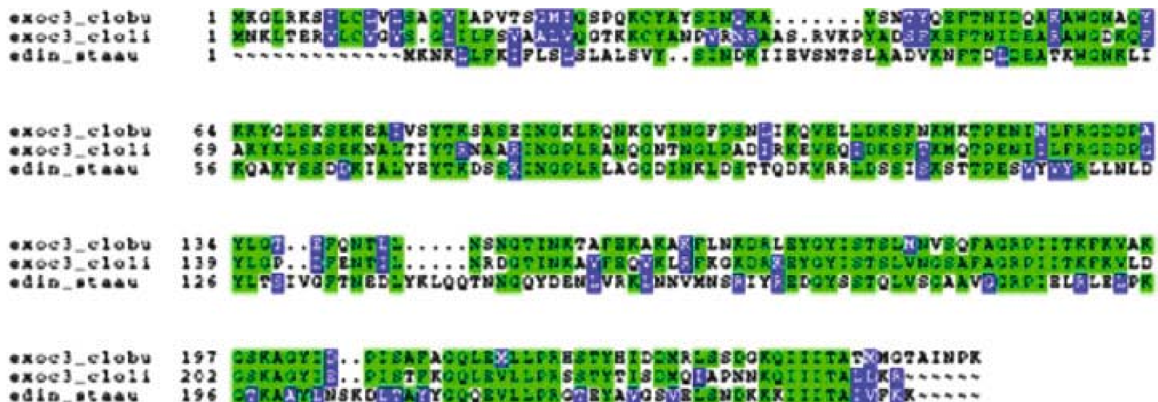
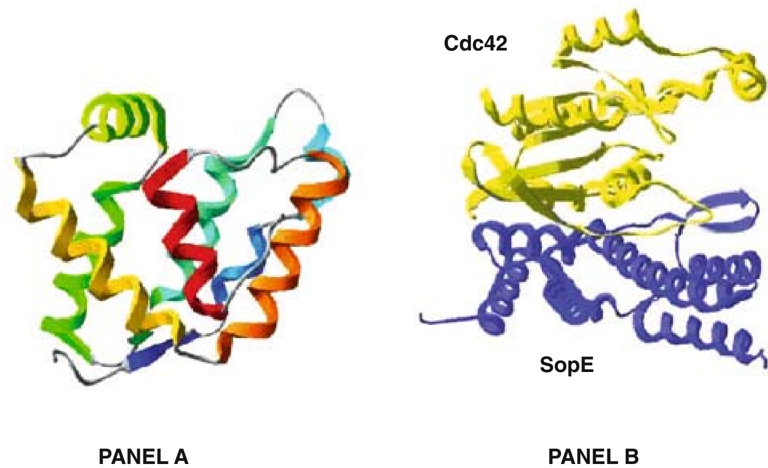


Fig. 47. Multiple sequence alignment of protein toxins belonging to the group of exoenzyme C3-like ADP-ribosyltransferases.

Fig. 48. Crystal structures of SipA (panel A) and of SopE in complex with Cdc42 (panel B).



Cdc42 (Buchwald et al., 2002; Figs. 1 [panels 24 and 25] and 48).

SHIGELLA IPAA. The entry of *Shigella* into epithelial cells requires the Ipa proteins, which are secreted upon cell contact by the type III apparatus and act in concert. The IpaB and IpaC proteins form a complex that binds B1 integrin and CD44 receptors and induces actin polymerization at the site of bacterium-cell contact, allowing the formation of membrane extension that probably requires also the action of Cdc42, Rac and Rho GTPases (Nhieu and Sansonetti, 1999).

The translocation of IpaA into the cell cytosol probably favors *Shigella* entry. The IpaA protein binds with high affinity to the N-terminal residues 1–265 of vinculin, a protein involved in linking actin filaments to the plasma membrane. The vinculin-IpaA complex interacts with F-actin inducing subsequent depolymerization of actin filaments. Presumably, these interactions further modulate the formation on the membrane of adhesion-like structures required for efficient invasion.

Shigella internalization still occurs at low levels in the absence of IpaA, suggesting that IpaA acts in concert with other bacterial effectors to promote cell entry. Binding of the *Shigella* protein IpaA to vinculin induces F-actin depolymerization (Bourdet-Sicard et al., 1999). The IpaA and vinculin rapidly associate during bacterial invasion. Although defective for cell entry, an *ipaA* mutant is still able to induce foci of actin polymerization but differs from wildtype *Shigella* in its ability to recruit vinculin and α-actinin. It has been postulated that IpaA-vinculin interaction initiates the formation of focal adhesion-like structures required for efficient invasion (Tran Van Nhieu et al., 1997).

YERSINIA YOPE. A protein secreted by *Yersinia* through a type III secretion system, YopE contributes to the ability of *Yersinia* to resist phagocytosis (Rosqvist et al., 1990). Following infection of epithelial cells with *Yersinia*, the microfilament structure of the cells changes leading to a complete disruption of the actin microfilaments, which finally results in cell rounding and detachment from the extracellular matrix (Rosqvist et al., 1991). The effector YopE was recently shown to possess GAP activity towards the Rho GTPases RhoA, Rac and CDC42 in vitro (Aili et al., 2003; Fig. 1, panel 26). Further experimentation has shown that in vivo YopE is able to inhibit Rac- but not Rho- or Cdc42-regulated actin structures. Furthermore, the structure of this toxin has recently been solved, showing a close relationship with the analogous ExoS Gap domain (Evdokimov et al., 2002).

YERSINIA YOPT. YopT is the prototype of a new family of 19 cysteine proteases with potent

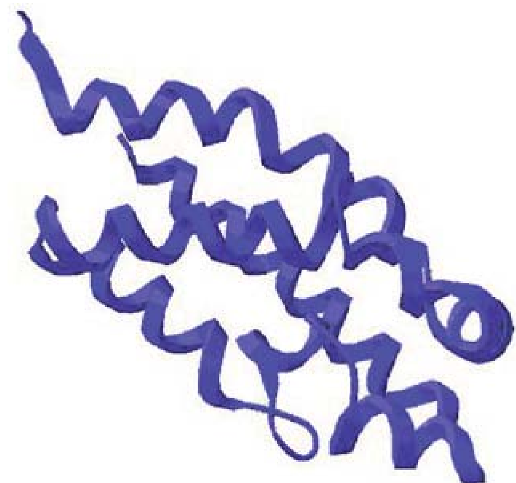


Fig. 49. Crystal structure of YopE catalytic domain.

effects on host cells. These include the AVr protein of the plant pathogen *Pseudomonas* and possibly Yop-J of *Yersinia*. YopT cleaves the posttranslationally modified cysteine located at the C-terminal end of Rho GTPases (DKG-CASS), causing the loss of the prenyl group from RhoA, Rac and cdc42, and releasing them from the membrane (Shao et al., 2003). The inability of Rho to be located to the membrane causes disruption of the cytoskeleton. While the C terminus of YopT is crucial for activity, the N terminus of YopT is crucial for substrate binding (Sorg et al., 2003).

SHIGELLA VIRA. The invasiveness of *Shigella* is an essential pathogenic step and a prerequisite of bacillary dysentery. VirA is a *Shigella* effector protein, which is delivered into the host cell by a specialized type III secretion system. This protein can interact with tubulin to promote microtubule destabilization and membrane ruffling (Yoshida et al., 2002). With this mechanism, *Shigella* is able to remodel the cell surface and thus promote its entry into the host. Recent data have shown that *VirA* deletion mutants displayed decreased invasiveness and were unable to stimulate Rac1.

Toxins Acting on Signal Transduction

YERSINIA YPKA AND YOPH. Phosphorylation is central to many regulatory functions associated with the growth and proliferation of eukaryotic cells. Bacteria have learned to interfere with these key functions in several ways. The best-known system is that of *Yersinia*, where a protein kinase (YpkA; Barz et al., 2000) and a protein tyrosine phosphatase (YopH; Zhang, 1995; Fig. 1, panel 27) are injected into the cytoplasm of eukaryotic cells by a type III secretion system to paralyze the macrophages before they can kill the bacterium.

YpkA is a Ser/Thr protein kinase that also displays autophosphorylating activity in vitro. In vivo experiments have shown that this protein is essential for virulence: in fact, challenge with a *YpkA* knockout mutant causes a nonlethal infection, whereas all mice challenged with wildtype *Y. pseudotuberculosis* die. Recently, natural eukaryotic substrates of YpkA have been identified by using a two-hybrid assay. These belong to the class of small GTPases and comprise RhoA and Rac-1, but not Cdc42.

YopH is a modular protein where the tyrosine phosphatase domain shows a structure and catalytic mechanism very similar to those of eukaryotic enzymes. YopH acts by dephosphorylating cytoskeletal proteins thus disrupting phosphotyrosine-dependent signaling pathways necessary for phagocytosis. Host protein targets include Crk-associated substrate, paxillin, and focal adhesion kinase. In vivo, YopH inhibits phagocytosis by polymorphonuclear leukocytes (PMNs) and macrophages (Fallman et al., 1995; Ruckdeschel et al., 1996). The protein has a molecular weight of 51 kDa and is composed of an N-terminal domain important for translocation and secretion (Sory et al., 1995) and a C-terminal domain homologous to eukaryotic PTPases (Guan and Dixon, 1990; Bliska, 1995).

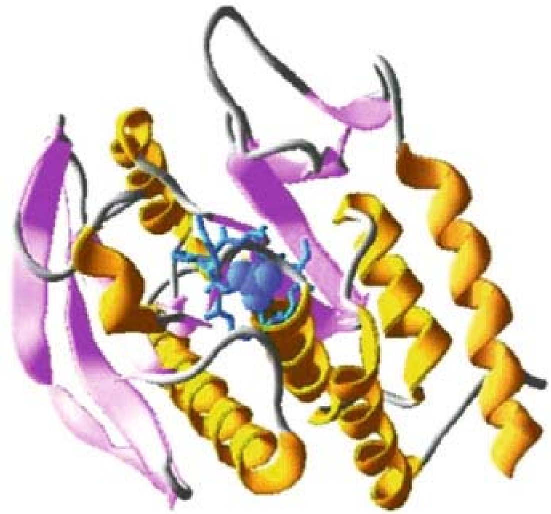


Fig. 50. X-ray structure of YopH. Colors have been assigned on the basis of secondary structure (yellow for helix and pink for β-sheet). The PTPase phosphate-binding loop and Cys-403 are in blue.

The three-dimensional structure of YopH has been solved (Stuckey et al., 1994; Su et al., 1994) revealing the presence of a catalytic domain which, despite its low level of sequence identity to the human PTP1B, still contains all of the invariant residues present in eukaryotic PTPases. Its tertiary fold is a highly twisted α/β structure with an eight-stranded β-sheet flanked by seven α-helices. Residues 403–410 form the PTPase phosphate-binding loop with the invariant Cys-403 thiol centered within the loop (Fig. 50).

EPEC TIR. A 78-kDa protein produced by enteropathogenic *E. coli* (EPEC) strains, Tir mediates the attachment of bacteria to eukaryotic cells and is essential for EPEC virulence. The Tir protein is tyrosine phosphorylated upon injection into eukaryotic cells by a type III secretion system. While in the host cell, it becomes an integral part of the eukaryotic cell membrane and functions as receptor for intimin, the major EPEC adhesin (Kenny et al., 1997). It is believed that, once in the host, Tir adopts a hairpin-like structure using its two putative transmembrane domains (TMDs) to span the host cell membrane. The region between the two TMDs constitutes the extracellular loop that functions as the intimin-binding domain. Following tyrosine

phosphorylation, the protein mediates actin nucleation, resulting in pedestal formation and triggering tyrosine phosphorylation of additional host proteins, including phospholipase C-γ. Tir is essential for EPEC virulence and was the first bacterial protein described to be tyrosine phosphorylated by host cells (Crawford and Kaper, 2002).

HELICOBACTER PYLORI CAGA. Cytotoxin-associated gene A (CagA) is an immunodominant protein produced by most virulent strains of *Helicobacter pylori*, with a size that can vary from 128 kDa to 146 kDa and which is commonly expressed in peptic ulcer disease (Covacci et al., 1993b). CagA is characterized by a central region containing an EPIYA motif, which can be repeated up to six times increasing the molecular weight of the protein. The gene is encoded within a pathogenicity island, which also encodes the type IV secretion system necessary to inject the protein into eukaryotic cells. Once injected into the host cell, the protein is tyrosine phosphorylated at the EPIYA motif by the kinase C-Src and Lyn. The signal is proportional to the number of EPIYA motives present (Stein et al., 2000). The tyrosine phosphorylated CagA (CagA-P) activates SHP-2, inactivates C-Src leading to cortactin dephosphorylation triggering a signal transduction cascade (which results in cellular scattering proliferation, a phenotype indistinguishable from that induced by the hepatocyte growth factor [HGF]).

The long-term chronic infection and the continuous stimulation increase the risk of cancer of people infected by CagA+ *H. pylori*. CagA is the first bacterial protein linked to cancer in humans and the *cagA* gene can be considered the first bacterial oncogene.

YERSINIA PESTIS YOPM. YopM is an effector protein delivered to the cytoplasm of infected cells by the type III secretion mechanism of *Yersinia pestis.* YopM is a highly acidic protein, which is essential for virulence, but whose mechanism of action is still elusive. Differently from other effectors, this toxin has been shown to accumulate not only in the cytoplasm but also in the nucleus of mammalian cells. Recently, McDonald and colleagues have found that YopM interacts with two kinases, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1). These two kinases associate only when YopM is present, and expression of YopM in cells stimulates the activity of both kinases. These results indicate that PRK2 and RSK1 are the first intracellular targets of YopM (McDonald et al., 2003).

The X-ray structure determined for YopM has shown a modular architecture constituted by leucine-rich repeats, mainly organized in an extended β-sheet structure (Evdokimov et al., 2001; Figs. 1 [panel 28] and 51). This organization is very similar to that found for other important proteins, such as rab geranylgeranyltransferase and internalin B produced by *Listeria*.

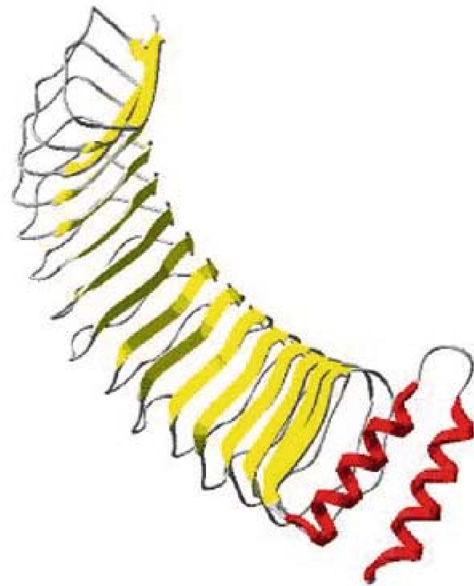


Fig. 51. Crystal structure of YopM effector protein of *Yersinia pestis*.

SALMONELLA SPTP. *Salmonella* protein tyrosine phosphatase (SptP) is an effector protein secreted by the type III secretion apparatus of *Salmonella enterica*. SptP is a modular protein composed of two functional domains, a C-terminal region with sequence similarity to *Yersinia* tyrosine phosphatase YopH, and an N-terminal domain showing homology to bacterial cytotoxins such as *Yersinia* YopE and *Pseudomonas* ExoS (Murli et al., 2001). Recently, it was demonstrated that this domain possesses strong GTPase activating domain protein (GAP) activity for Cdc42 and Rac1. The crystal structure of SptP-Rac1 complex has shown that SptP is strongly stabilized by this interaction (Stebbins and Galan, 2000; Fig. 52).

PSEUDOMONAS AERUGINOSA EXOU. Several extracellular products secreted by the *P. aeruginosa* type III secretion system are responsible for virulence. Among these, the 70-kDa protein, ExoU, is responsible for causing acute cytotoxicity in vitro and epithelial lung injury. Recent studies demonstrated that ExoU has lipase activity, and that the cytotoxicity of ExoU is dependent on its patatin-like phospholipase domain. The results suggest that ExoU requires the presence of a catalytically active site Ser(142) and that a yet unknown eukaryotic cell factor(s) is necessary for its activation (Tamura et al., 2004).

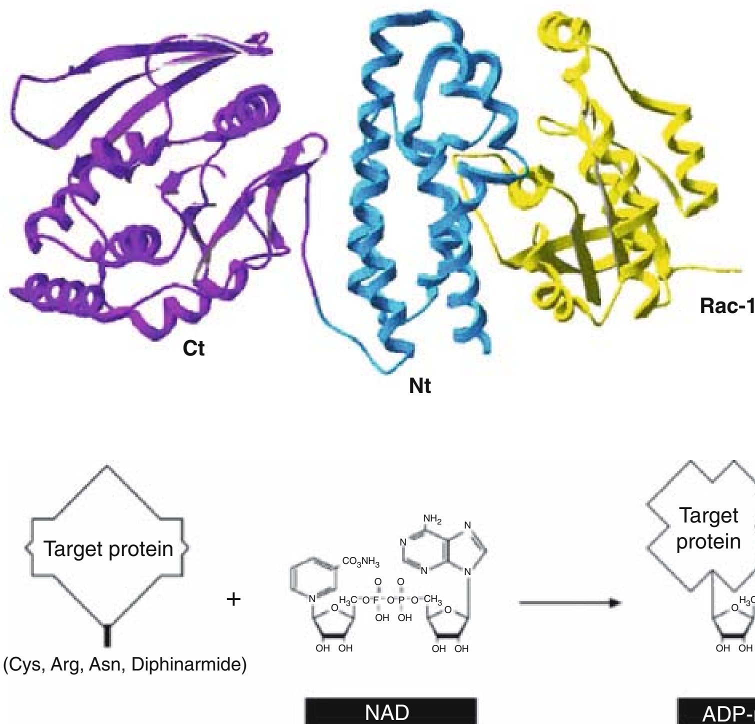


Fig. 52. Crystal structure of SptP in complex with Rac-1 (yellow). The N-term and C-term domains of SptP are colored in cyano and purple, respectively.

Fig. 53. Mechanism of ADP-ribosylation reaction catalyzed by ADP-ribosyltransferases.

ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity

ADP-Ribosylating Toxins: Main Features

The ADP-ribosylating toxins are a class of bacterial proteins that characterized by an enzymatic domain with ADP-ribosyltransferase activity (Ueda and Hayaishi, 1985; Althaus and Richter, 1987). During ADP-ribosylation (Fig. 53), these toxins bind NAD and transfer the ADP-ribose moiety to a specific substrate molecule, which is thus forced to undergo a dramatic functional modification. The toxic effect is totally dependent upon the enzymatic activity.

On the basis of their overall structure, ADP-ribosyltransferases can be separated into A/B toxins, binary toxins, and A-only toxins, where A is the subunit with the enzymatic activity, and B is the carrier domain involved in the recognition of the specific surface receptor and in the translocation of the toxic moiety into the eukaryotic cell. Most of the best characterized ADP-ribosylating toxins belong to the class with an A/B architecture: pertussis toxin (PT; Locht et al., 1986; Nicosia et al., 1986), cholera toxin (CT; Mekalanos et al., 1983), and *E. coli* heat-labile enterotoxin (LT; Spicer and Noble, 1982; Yamamoto et al., 1984) are typical examples of this family where the A domain (called "S1" in PT) bears the enzymatic core and the B domain is an oligomer that helps the translocation across the cell membrane; the two subunits are linked together by noncovalent bonds. The genes coding for CT and LT are highly homologous (Dallas and Falkow, 1980) and are organized into operons located on the chromosome of *Vibrio cholerae* and on a plasmid of *E. coli* (So et al., 1978).

Diphtheria toxin (DT; Pappenheimer, 1977; Collier et al., 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; Gray et al., 1984b; Wick et al., 1990) are A/B toxins with a three-domain structure: the catalytic domain C, contained in fragment A, and the transmembrane domain T and receptor-binding domain R, both within the B subunit.

The binary (as opposed to the A/B) toxins have a fairly similar organization, but in this case the A and B domains are separately secreted in the culture supernatant where the B domain initially binds the receptor on the surface of the target cell and only then is able to bind the A subunit and help its translocation into the cytosol. Examples of this family of ADP-ribosyltransferases are the C2 toxin of *Clostridium botulinum* (Aktories et al., 1986), the iota toxin of *C. perfringens* (Perelle et al, 1995), the toxin of *C. spiroforme* (Popoff and Boquet, 1988a), the mosquitocidal toxin (MTX) of *Bacillus sphaeri-*

cus (Thanabalu et al., 1993), and the *C. difficile* transferase (Just et al., 1994).

Finally, the "A-only" toxins include Exo S of *Pseudomonas aeruginosa* (Kulich et al., 1994) and other toxins such as C3 of *Clostridium botulinum* (Nemoto et al., 1991), EDIN of *Staphylococcus aureus* (Sugai et al., 1990), and the toxins of *Bacillus cereus* (Just et al., 1995b) and of *Clostridium limosum* (Just et al., 1992). All the A-only toxins possess a still unknown mechanism of cell entry, with the notable exception of Exo S, which has been shown to be directly injected into eukaryotic cells by a specialized secretion system (Yahr et al., 1996).

With the exception of actin, all the eukaryotic proteins that are ADP-ribosylated by these toxins are GTP-binding proteins (G-proteins); these proteins are molecular switches involved in a number of essential cell functions including protein synthesis and translocation, signal transduction, cell proliferation, and vesicular trafficking (Hamm and Gilchrist, 1996).

ADP-Ribosylating Toxins: A Common Structure of the Catalytic Site

Bacterial enzymes with ADP-ribosyltransferase activity include a variety of toxins with different structural organizations; the better-represented class is that comprising proteins with an A/B structure (PAETA, DT, CT, LT and PT), where subunit A is responsible for enzymatic activity and subunit B is involved in receptor binding.

Other toxins, termed "binary toxins" (*Clostridium botulinum* toxin C2 and related proteins) are still composed of the two functional domains A and B. However, they reside on different molecules and need to interact to acquire activity. Finally, there is a group of ADP-ribosylating toxins that do not possess the receptor-binding domain B at all and are thus named "A-only toxins." This group includes *Clostridium botulinum* exoenzyme C3 and related proteins, which are unable to invade the cells, and toxins which are directly injected into eukaryotic cells (ExoS) by means of a specialized secretion apparatus.

From primary sequence analysis, it is possible to identify two main groups of homology (Fig. 54): the DT-like group, mainly composed of DT and PAETA, and the CT-like group comprising the remaining ADP-ribosyltransferases.

Although some homology is present among the members of the CT group, no overall significant and extended sequence similarity can be detected to justify the observed common mech-

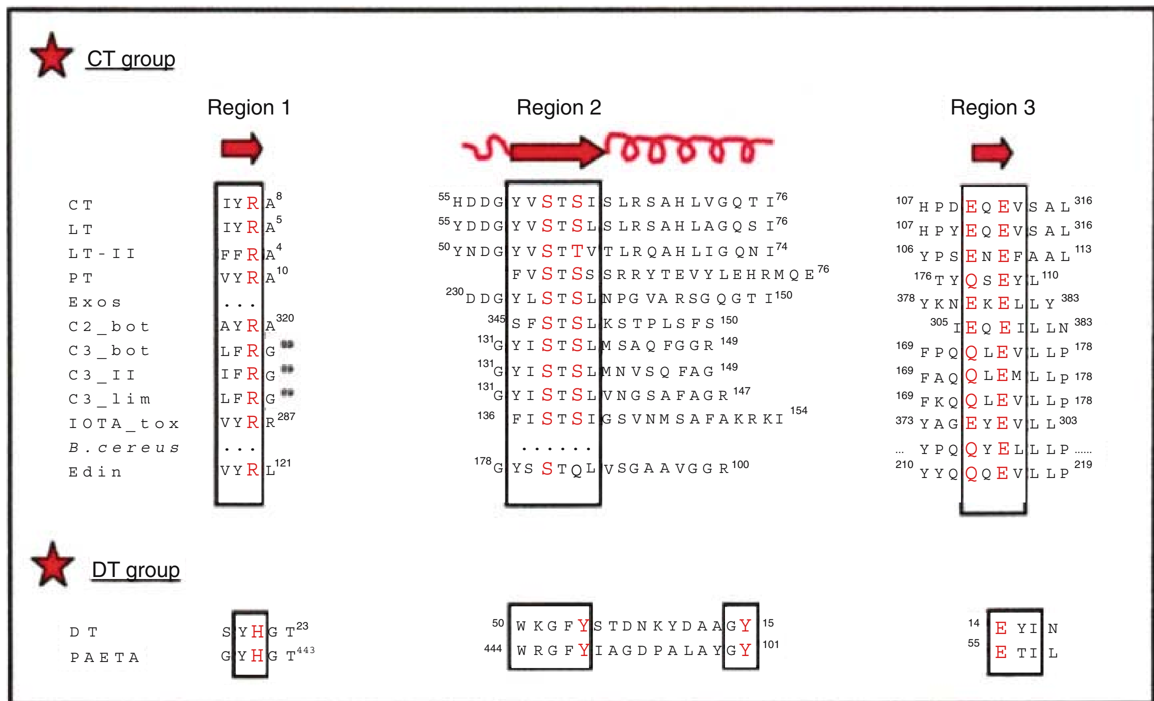


Fig. 54. Sequence alignment of protein segments containing Regions 1, 2 and 3 of bacterial ADP-ribosylating enzymes. The two groups of homology (DT-like and CT-like groups) are distinguished. Catalytic residues of Regions 1 and 3, and most relevant and conserved residues of Region 2 are colored in red; extended consensus sequences detected in the three regions are boxed, whereas other partially conserved residues are in boldface. Predicted and observed secondary structure folding is indicated for each region: Regions 1 and 3 are β-strands (arrows), while Region 2 is characterized by a short coil (solid line), followed by a β-strand and by an α-helix.

anism of catalysis; nevertheless, biochemical experiments of photoaffinity labeling and studies of site-directed mutagenesis had previously demonstrated for most of the toxins that the presence of a glutamic acid is so important for catalytic activity, even a conservative substitution with an aspartate could not be tolerated without loss or drastic decrease of toxicity (Douglas and Collier, 1987; Wilson et al., 1990; Lobet et al., 1991; Antoine et al., 1993).

On the basis of these experimental data and on the crystallographic structures which are now available for LT (Sixma et al., 1991), CT (Zhang et al., 1995), PT (Stein et al., 1994), DT (Choe et al., 1992) and PAETA (Allured et al., 1986), a common catalytic site could be identified which, despite the low level of sequence homology, is almost perfectly superimposable for all them (Domenighini et al., 1994).

In terms of tertiary structure, the active site is a cleft formed by a β-strand followed by a slanted α-helix that has a different length in the various toxins (spanning from 12 residues for DT, PAETA and LT, to 21 in the case of PT). The β-strand and the α-helix represent, respectively, the lower and upper face of the cavity in which the nicotinamide ring of NAD is anchored during the enzymatic reaction (Region 2 of Fig. 54).

Although all the toxins share this similar folding in the region of the active site, at the amino acid level, the only residue which is well conserved among all the representatives of the CT- and DT-groups is a glutamic acid (Glu-148 of DT, Glu-553 of PAETA, Glu-112 of CT and LT, and Glu-129 of PT), which corresponds to the core of Region 3 (Fig. 54). These residues retain an equivalent spatial position and orientation residing in a short β-strand flanking the external side of the cavity (Fig. 55). With the exception of the conserved glutamate, the consensus sequence generated for Region 3 differs between the two groups of toxins. In the DT family, in fact, it is composed of the catalytic Glu followed by an aromatic and a hydrophobic residue, whereas in the CT-group, the consensus can be extended to a few neighboring residues (Fig. 45). On the basis of alignment of C2-I with iota toxin and with the other ADP-ribosyltransferases, the catalytic glutamate was identified (Glu-389 of C2) and its function experimentally confirmed by site-directed mutagenesis (Barth et al., 1998). In the case of *Pseudomonas aeruginosa* Exo S, the equivalent Glu has been mapped at position 381 (Liu et al., 1996).

Another well-conserved residue is His-21 of DT that can be aligned to His-440 of PAETA, and with the conserved Arg-7 of CT and LT, and Arg-9 of PT (Burnette et al., 1988, 1991; Papini et al., 1990; Lobet et al., 1991; Han and Galloway, 1995). The segment comprising this residue is termed “Region 1” (Fig. 54). These amino acids are once again located in essentially identical positions within the active site, lying opposite to the glutamic acid on an antiparallel β-strand close to the internal face of the catalytic cleft.

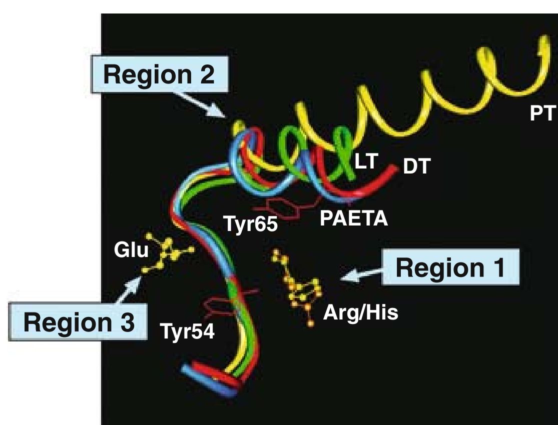


Fig. 55. Superimposition of the three-dimensional structures of the NAD-binding cavities (Region 2) of the bacterial toxins LT (green), PT (yellow), DT (red) and PAETA (blue). The catalytic residues carried by Region 1 (Arg/His) and by Region 3 (Glu) and common to the two regions of homology are shown. In addition, the two essential tyrosines of the DT group are colored in red.

Although several models have been proposed to explain the possible function of the conserved histidine/arginine of Region 1, that this residue does not play a direct role in catalysis seems now widely accepted; very likely it may have a function in maintaining the integrity of the active-site pocket upon formation of structurally stabilizing hydrogen bonds (Johnson and Nicholls, 1994). Nevertheless, mutations at the His-440 position of PAETA, though affecting the enzymatic activity, have little or no effect on NAD-binding (Han and Galloway, 1995); this suggests that His-440 may not be exactly homologous to His-21 of DT or to the arginines of the CT-group. In the case of the C2-I component of clostridial toxin C2, site-directed mutagenesis of Arg-299 induced a dramatic reduction of transferase activity, thus suggesting an equivalent role for this residue in the conformation of the active site (Perelle et al., 1995).

Region 2 includes a number of amino acids that, while maintaining the same secondary structure in both DT- and CT-families (Fig. 55), result in a major sequence difference (Fig. 54). This is mainly a structural region corresponding to the core of the active site cleft, which is devoted to the docking of NAD. The consensus sequence generated for the DT group is characterized by two conserved tyrosines spaced by ten amino acids, and located on the middle portion

of the β-strand and on the internal face of the α-helix, respectively. Tyr-54 and Tyr-65 of DT, and Tyr-470 and Tyr-481 of PAETA have been shown to play an important role in catalysis inasmuch as they anchor the nicotinamide ring during the reaction by creating a p pile of three aromatic rings which strengthen the overall binding of NAD and stabilize the complex (Carroll and Collier, 1984; Li et al., 1995). This consensus motif can be extended to four other residues which precede the first Tyr, and to a glycine residue which is located upstream of the second Tyr.

In PT, a similar role is likely to be played by Tyr-59 and Tyr-63, which have a similar spatial orientation and distance from each other. This observation is supported by the fact that in CT and LT, where the stacking interactions produced by the two tyrosines are lacking, the affinity for NAD is 1000-fold lower (Galloway and van Heyningen, 1987).

In the case of the CT-group, Region 2 is centered on a consensus core domain characterized by the motif Ser-Thr-Ser that is observed and predicted to fold in a β-strand representing the floor of the cavity. Experiments of site-directed mutagenesis have confirmed the importance of these residues in maintaining the shape of the cavity. Substitutions of Ser-61 and Ser-63 of LT with Phe and Lys, respectively, have been shown to produce nontoxic mutants (Harford et al., 1989; Fontana et al., 1995). The core sequence of Region 2 can be extended to give the more general consensus aromatic-hydrophobic-Ser-Thr-Ser-hydrophobic.

Another amino acid that has been proposed as being important in catalysis is His-35 of PT (Xu et al., 1994) located near the beginning of the β-strand which forms the floor of the cavity, in a position equivalent to that of His-44 of LT and CT (Yamashita et al., 1991); a functional homologue, His is also present in the mosquitocidal toxin SSII-1 from *Bacillus sphaericus* (Thanabalu et al., 1991) but is absent in DT and PAETA. In the 3D structure, this residue appears to be sufficiently close to the oxygen atom of the ribose ring of NAD to interact with it and increase the electrophilicity of the adjacent anomeric carbon atom. The absence of an equivalent residue in DT and PAETA again supports the idea that the two groups of toxins perform the same enzymatic activity in a slightly different fashion.

An additional feature that is common to all ADP-ribosylating toxins is the need for a conformational rearrangement to achieve enzymatic activity.

In the native structure, in fact, the NAD-binding site of LT and CT is obstructed by a loop (amino acids 47–56) that needs to be displaced to obtain a functional NAD-binding cavity. A functionally homologous region is also present in PT where the loop comprises residues 199–207. In the case of DT, where the crystallographic data of the complex are available, the observation that the active-site loop consisting of amino acids 39–46 changes structure upon NAD-binding, suggests that these residues may be important for the recognition of the ADP-ribose acceptor substrate, EF-2 (Weiss et al., 1995; Bell and Eisenberg, 1996).

The recent publication of the crystallographic data of the DT-NAD complex, and the presence of common features within all ADP-ribosylating toxins, permits speculation on a possible common mechanism of catalysis (Fig. 56). The best hypothesis is that NAD enters the cavity, which is then made available for the recognition of the

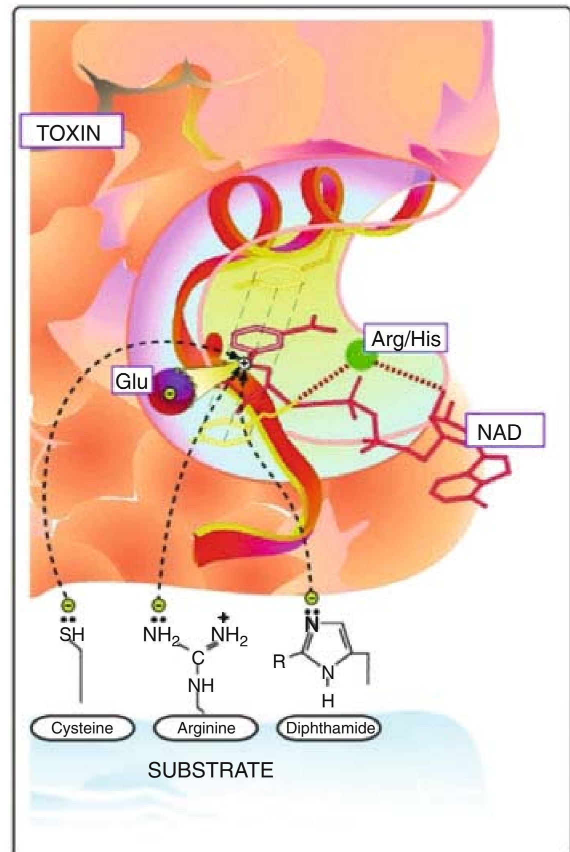


Fig. 56. Schematic representation of a possible common mechanism of catalysis: the nicotinamide adenine dinucleotide (NAD) molecule (red) is docked inside the cavity by means of stacking interactions provided by the two aromatic rings (yellow) that protrude from the scaffold of Region 2. The catalytic glutamic acid (purple) and its possible interactions with the acceptor residues of the various substrates are also reported. The Arg/His residue (green) provides stabilizing interactions with the backbone of the cavity and seems to be also responsible for the correct positioning of NAD inside the pocket.

substrate, upon displacement of the mobile loop. Then, NAD docks at the bottom of the pocket where a small residue (the conserved serine in Region 2 of the CT-group, the threonine-56 of DT, and the alanine-472 of PAETA) is required to allow good positioning. The nicotinamide moiety of NAD is then blocked in a suitable position by means of stacking interactions provided by a couple of aromatic rings (Tyr-54 and Tyr-65 of DT, Tyr-470 and Tyr-481 of PAETA, and possibly, Tyr-59 and Tyr-63 of PT). In this context the conserved arginine/histidine might display its key role in maintaining the correct shape of the active site pocket via hydrogen bonds formed with the backbone of the structure and possibly one with the ribose moiety. The enzymatic reaction is then catalyzed by the essential glutamic acid, which is likely to stabilize a positively charged oxocarbonium intermediate of NAD, to favor its subsequent interaction with the nucleophilic residue of the incoming substrate (diphthamide in the case of DT and PAETA, arginine in the case of LT and CT, and cysteine in the case of PT).

Novel ADP-Ribosylating Toxins Detected by Genome-Mining

With the advent of the Genomic Era, identification of bacterial factors possibly involved in virulence is an easier challenge. In fact, given the vast amount of information that we now possess on toxins—including sequence data—and thanks to the growing number of sequenced bacterial genomes, it is possible to proceed by homology criteria to predict novel members of important classes of bacterial toxins.

Several examples exist where computer-based methodologies have been instrumental to the identification of novel potential bacterial toxins in sequenced genomes. Among them, we will mention here the case of mono ADP-ribosyltransferases.

Mono-ADP-ribosyltransferases (mADPRTs) constitute a class of potent toxins in bacteria, which generally play an important role in the pathogenesis of related microorganisms. Despite the poor overall conservation at the primary structure level, the catalytic subunits of these toxins show a remarkable similarity within the enzymatic cavity, so that these portions of the proteins are quite well conserved.

For these reasons, and encouraged by the availability of a growing number of sequenced bacterial genomes, a series of studies have been directed towards the computer-based identification of novel members of this family of enzymes by means of sequence-homology criteria in finished and unfinished genome sequences. As a result, more than twenty novel putative ADP-ribosyltransferases have been identified both in Gram-positive and Gram-negative organisms, including five from *Pseudomonas syringae*, five from *Burkholderia cepacea*, two from *Enterococcus faecalis*, and one each from *Salmonella typhi*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, *Streptomyces coelicolor*, *Bacillus halodurans* and *Vibrio parahaemolyticus* (Pallen et al., 2001). With the exception of the protein detected in *Salmonella*, which is adjacent to an ORF protein similar to the S2 subunit of pertussis toxin, all the other genome-derived putative ADPRTs lack a predicted translocation domain. So far, none of these bacterial proteins has been tested either for their ADP-ribosyltransferase activity or for the capability of entering eukaryotic cells; however, sequence data indicate a possible role of these proteins in the pathogenesis of the corresponding microorganisms. Very recently, a new protein has been added to the list of ADP-ribosyltransferases detected by computer analysis (Masignani et al., 2003). This novel factor has been identified by means of primary and secondary structure analysis in the genomic sequence of a virulent isolate of *Neisseria meningitidis* and has been named "NarE" (*Neisseria* ADP-ribosylating enzyme). As predicted by "in silico" studies, biochemical analysis has demonstrated that NarE is capable of transferring an ADP-ribose moiety to a synthetic substrate.

Toxins with Unknown Mechanism of Action

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

The zonula occludens toxin (Zot) is produced by bacteriophages present in toxinogenic strains of *Vibrio cholerae*. Zot is a single polypeptide chain of 44.8 kDa, which localizes in the outer membranes. After internal cleavage, a carboxy-terminal fragment of 12 kDa is excreted and this is probably responsible for the biologic effect. Zot has the ability to reversibly alter the tight junctions of intestinal epithelium, thus facilitating the passage of macromolecules through mucosal barriers (Di Pierro et al., 2001). Zot has also been shown to act as mucosal adjuvant and to induce protective immune response in the animal model (Marinaro et al., 2003).

Hemolysin BL (HBL) is an enterotoxin produced by *B. cereus*, which is composed of three proteins (B, L1 and L2), each with a molecular mass of 40 kDa, and whose corresponding genes are located on the same operon. HBL has hemolytic as well as dermonecrotic and vascular permeability activities and is able to cause fluid accumulation in ligated rabbit ileal loops (Beecher et al., 1997; Beecher and Wong, 2000).

The bile-salt hydrolase (BSH) is a protein elaborated by *Listeria monocytogenes*, which is absent from the genome of the nonpathogenic *L. innocua*. The *bsh* gene encodes an intracellular enzyme and is positively regulated by PrfA, the transcriptional activator of known *L. monocytogenes* virulence genes (Dussurget et al., 2002). Furthermore, *bsh* deletion mutants show reduced virulence and liver colonization, thus demonstrating that BSH is a toxin specifically involved in the intestinal and hepatic phases of listeriosis.

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Prokaryotes (2006) 2:956–968
DOI: 10.1007/0-387-30742-7_29

CHAPTER 1.29

The Metabolic Pathways of Biodegradation

LAWRENCE P. WACKETT

Introduction

History

The decay (biodegradation) of organic matter has been a part of life throughout human history. When the organic matter was a person's food, clothing or dwelling, biodegradation was no doubt very undesirable. In this context, humans have, through most of their history, sought to prevent or, more practically, slow down biodegradation. Animal hides were treated with tannins to crosslink proteins and prevent their degradation. Food was dried, salted or pickled to prevent microbial growth, and hence, spoilage.

Though solutions to biodegradation were found, the underlying causes of the phenomenon were less clear. But no doubt, correlations were made by direct observation of macroscopic microorganisms, typically fungi, on rotting material. People could see wood rot fungi on decaying wood or hyphal masses on bread. And, in fact, some of the foundations for the science of microbiology were established with macroscopic fungi. Micheli showed that fungi now known as *Mucor*, *Botrytis* and *Aspergillis* could be cultivated on the surfaces of fresh-cut melon, quince and pear (Bull and Slater, 1982). Micheli serially transferred the fungi, thus initiating the practice of isolating, maintaining and characterizing specific genera of microorganisms. He also observed that different fungal genera showed preferences for certain fruits used as the cultivating medium, thus establishing the idea of selective culture.

In fact, these observations became entwined with the controversy over spontaneous generation because macroscopic fungal growth derived from microscopic fungal spores. Many interpreted proliferation of microscopic life, biodegrading different organic material, as the spontaneous generation of life from non-life. Louis Pasteur is generally credited with demonstrating most convincingly that the elimination of all bacterial and fungal contamination would prevent spoilage (Clarke, 1985). It was recognized from the work of Pasteur, Tyndall and others that bacteria are ubiquitous and difficult to remove from any given environment.

In a broad sense, virtually all prokaryotes participate in biodegradation. Prokaryotes decompose (biodegrade) organic molecules as part of their need to derive chemical energy to make ATP or to produce metabolic intermediates. In the early twentieth century, Beijerinck (1901) and Winogradsky (1890) contributed to the current idea that prokaryotes are important in the recycling of carbon, nitrogen, sulfur and other elements on a global scale. For example, we now know that 10^{15} grams of methane gas are produced annually by anaerobic Archaea known as "methanogens" and most of the biogenic methane is oxidized by aerobic methanotrophic bacteria (Lipscomb, 1994). This constitutes one small part of the global carbon cycle. If one considers that over ten million organic compounds are known, many of which are theoretically biodegradable, the magnitude of these cycles is enormous.

Scope of Biodegradation in the Modern World

Although naturally occurring compounds biodegrade on a massive scale, the biodegradation of synthetic compounds attracts more interest. Over the last century, some synthetic, industrial chemicals have been shown to exert toxic or carcinogenic effects on humans. For example, factory workers in aniline dye (Bulbulyan et al., 1995) and vinyl chloride polymer industries (Langard et al., 2000) were developing certain cancers at relatively high rates and the epidemiological studies were confirmed in animals. From these observations has emerged the awareness that, with increasing world population, more effort must be expended to maintain clean water, air and soil.

Problems of human exposure to potentially harmful organic compounds can be handled in different ways. For example, certain chemicals may be banned, manufacturing processes can be made cleaner, or wastes can be treated at the

source or in the open environment. In fact, all of those are occurring. And prokaryotes are increasingly being exploited for treating wastes, either in the manufacturing facility or for remediation of chemical spills or releases. Some high profile applications have been published (Harkness et al., 1993; Roberts et al., 1993; Strong et al., 2000; Wagner-Dobler et al., 2000) but in fact, most of these applications are quietly in use at manufacturing facilities all around the globe.

The University of Minnesota Biocatalysis/Biodegradation Database

To facilitate the use of microbial catalysis, either for developing cleaner manufacturing or treating wastes, we have developed the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD) (umbbd.ahc.umn.edu).

The UM-BBD provides information on microbial biocatalytic non-intermediary metabolism (i.e., reactions generally associated with biodegradation of synthetic industrial chemicals; Ellis et al., 2000). This metabolism is considered non-intermediary because it is restricted to only a few prokaryotes. It is these non-intermediary metabolic reactions that biotransform compounds and funnel them into the central metabolism of most prokaryotes. For example, only a limited number of prokaryotes can catabolize nitrobenzene, as shown, but many organisms can metabolize the open chain carboxylic acid products of those initial catabolic, or biodegradative, reactions. Intermediary metabolism databases such as KEGG, the Kyoto Encyclopedia of Genes and Genomes, which includes the LIGAND database of enzymes and reactions (Goto et al., 2000), depict this process. For example, one of the metabolic pathways for nitrobenzene is shown on the UM-BBD to yield 2-aminomuconate semialdehyde and the metabolic fate of this latter compound is shown on KEGG (http://www.genome.ad.jp).

It is important to study the pathways of biodegradation to insure that more highly toxic compounds are not generated as the result of microbial metabolism. This concern was raised by the recent observation that the widely used industrial solvent trichloroethylene undergoes reductive dechlorination, generating vinyl chloride as an intermediate (Vogel and McCarty, 1985). See umbbd.ahc.umn.edu/tce2/tce2_map.html for more information about this pathway. Because it is a strong human carcinogen, vinyl chloride is a greater environmental problem than trichloroethylene (Maltoni and Cotti, 1988). So it is not enough to know that a pollutant is disappearing from a given environment; regulatory agencies increasingly need to account for its complete environmental fate.

Methodological Advances Relevant to Biodegradation

Enrichment Culture

In the late 1800s, microbiologists largely focused on the bacteria that cause disease in humans. Isolating the disease-causing bacteria directly from an infected tissue was relatively easy because the infection was largely a monoculture. Thus, plating onto a rich medium might well yield a single organism that could be studied for its disease-causing properties.

This contrasted with the situation in a natural soil or water where thousands of different bacteria might well be present in a gram of material. In this case, culturing on a nonselective laboratory medium would yield a complex mixture, difficult to analyze for one particular metabolic trait. Thus, one needed to enrich the mixture to obtain one or a few different types of bacteria. This would simplify the system so that it could be studied productively.

As pioneered by the Dutch microbiologist Beijerinck (1901), the enrichment culture technique allowed selective cultivation of one or more bacterial strains obtained from complex environmental mixtures. Assume that one wanted to study the ability of microorganisms in a particular soil to metabolize a given compound. The compound would then be added as the sole carbon, nitrogen or sulfur source to a liquid laboratory medium lacking one of those major elements but containing the others and trace nutrients. The medium would then be inoculated with soil or water, perhaps adding as many as 10^{11} bacteria. If only a few of the bacteria are able to metabolize the compound to meet their nutritional needs, they will reproduce, or be enriched, selectively. The numbers of these specific bacteria will increase markedly in comparison to what is present in the native soil or water where alternative carbon, nitrogen or sulfur sources are present. With repeated transfer of the enriched microbial mixture into fresh growth medium, the numbers of the preferred bacterium will sometimes increase to the extent that it can be readily isolated.

The preferred bacterium is one that can metabolize the given compound, utilize the trace nutrients provided, grow at the temperature used in the laboratory and reproduce quickly. In this context, one may not necessarily obtain the bacterium most prevalent in the original sampled environment. For this reason, some people have

criticized the practice of obtaining and characterizing prokaryotes in pure culture as unsuited to yielding insights into what occurs in nature. But think of the difficulties inherent in trying to learn the details of biodegradation in a complex milieu such as soil. The metabolism of a particular compound might be inferred if it is disappearing from soil, but one has to rule out abiotic reactions in soil and soil can be difficult to sterilize. If the compound is available in a radiolabelled form, an accumulating intermediate may be obtained if it is stable in soil. This may or may not yield insights into metabolic pathways. But one would be hard-pressed to learn about the other metabolites, the enzymes, genes and specific microorganisms involved. In short, without obtaining pure cultures, one could learn whether a compound is metabolized but little about the molecular details.

The use of prokaryote pure cultures, many of which have been obtained by enrichment culture, has been instrumental in the identification of the many novel enzymes catalyzing metabolic transformations that drive the carbon, nitrogen and sulfur cycles of Earth. In turn, the corresponding genes have been identified; at first these were identified singly, and now wholesale as the result of genome sequencing efforts, which focused initially on prokaryotes because of their relatively small genome size. Without the development of enrichment culture, we would know far less about the Earth's biological cycles, the catalytic diversity of the planet and microbial phylogenetic diversity.

Anaerobic Culturing Methods and Biodegradation

Most of our early knowledge on biodegradation derived from studies on aerobic or facultative bacteria. This reflected the comparative ease of studying aerobic versus anaerobic bacteria. Anaerobic conditions were fairly easy to maintain with mixed-cultures because facultative organisms would consume oxygen and thus allow strict anaerobes to survive. Obtaining strict anaerobes in pure culture, and elucidating the novel biochemical reactions they catalyze, required the development of specialized techniques (Barker, 1940; Hungate, 1985).

Several decades ago, microbiologists used such techniques as roll-tubes to cultivate strictly anaerobic prokaryotes such as methanogenic bacteria. More recently, people routinely began using crimp-sealed, septum-plugged bottles for liquid cultures and putting Petri plates into anaerobic chambers containing an inert gas such as helium or argon. The latter can routinely be maintained at oxygen levels of around one part per million when coupled with oxygen-scrubbers for the gas mixtures and catalyst cartridges inside the anaerobic chamber.

Anaerobic biodegradation is also difficult to study in another context. Anaerobic enrichment cultures may initially show very long lag phases, perhaps six months or one year, before significant biodegradation occurs. Upon repeated transfer, the lag phase often shortens continually. Still, many years may be required to achieve significantly rapid rates of biodegradation and those may never approach the rates of comparable aerobic biodegradation. In most cases, a definitive explanation for the lag phase phenomenon is lacking. It is these kinds of impediments which have skewed the focus of laboratory studies in biodegradation toward the fast-growing aerobic prokaryotes such as *Pseudomonas* species, which can be grown overnight with simple equipment and typically yield high cell densities.

Despite this, anaerobes offer rewards to those who persevere by providing for the discovery of the most novel biochemical reactions on Earth. Some of these reactions have recently been elucidated. For example, bacteria are now known to catabolize toluene anaerobically. They initiate attack on the benzylic carbon via a radical mechanism that generates a new carbon-carbon bond to form benzylsuccinate as the first metabolite (Leuthner et al., 1998). Others, such as the anaerobic formation of methane from long-chain alkanes (Zengler et al., 1999), remain obscure biochemically.

Analytical Chemistry

Chemical methods for analyzing organic compounds have improved enormously since the late 1800s when use of enrichment culture methods began. Thus, obtaining pure cultures has gone hand in hand with new methods for analyzing the intermediates and products of their metabolism. Thus, one might anticipate a biodegradative metabolic pathway based on chemical logic, obtain authentic chemical standards, and screen for the presence of such compounds in growth cultures of the microbial isolate. But how does one screen for the compound(s)?

Chromatography, coupled to the use of authentically synthesized standard compounds, has been a powerful method for studying biodegradation over the last century, and it remains so today. There have been big advances in the science of chromatographic separations. A century ago, thin-layer chromatography (TLC) was state of the art. Later, gas chromatography (GC) provided better resolution and most recently, high pressure liquid chromatography (HPLC) gives

excellent resolution and the ability to capture and further analyze compounds.

Identification of compounds in complex mixtures is being aided by new developments in mass spectrometry (MS), which may be coupled with isotopic labelling for additional power. Similarly, with high-field nuclear magnetic resonance (NMR) spectroscopy, and the use of specifically labelled ^{13}C-compounds becoming increasingly available, it is now feasible to monitor metabolism in situ (Sauer et al., 1999). This, in turn, may lead to a revolution in environmental microbiology, in which systems more resembling natural systems can be analyzed with respect to biodegradation.

Whole Genome Sequencing and Analysis

Biodegradative genes have been identified, usually by transferring the DNA containing a specific gene(s) from a pure culture environmental isolate into *Escherichia coli* for sequencing and functional expression. With a substantial set of genes available, it is becoming routine to screen soils for the presence of homologous genes that might be involved in identical or similar biodegradative reactions. This gives insights into the environmental prevalence of certain biodegradative genes.

In the last several years, DNA sequencing techniques have advanced to the point that we can readily sequence entire prokaryote genomes (Nelson et al., 2000). With appropriate annotation techniques, this can provide insight into the metabolic pathways encoded by the genes. Theoretically, an organism's entire network of metabolism can be deduced. In practice, deducing metabolism is an imperfect task. Consider that the complete genomic DNA sequence of *Escherichia coli*, the most intensely studied biochemical entity on Earth, yielded 38% of the coding regions having unknown function (Blattner et al., 1997). If there are gaps in the metabolic map of *E. coli*, there will be many more as we proceed to sequence the genomic DNA of soil isolates important in biodegradation. Despite this caveat, it is exciting to contemplate the explosive increase in obtaining complete genome sequences for an expanding array of prokaryotes. This will spur a resurgence of interest in comparative biochemistry, with an attendant interest in new "exotic" genes. I predict that a significant number of the newly discovered gene functions in soil Eubacteria will be involved in the biodegradation of organic compounds. This will enhance interest in biodegradation and microbial biocatalysis, in general, as the era of functional genomics comes into full swing.

The Prokaryotes of Biodegradation

Our current perspective on the microorganisms of biodegradation derives largely from enrichment culture methods, isolating pure cultures and studying the individual reactions of biodegradation. Table 1 shows the list of prokaryotes and compounds they degrade; the biodegradative pathways each of them initiate are depicted in the UM-BBD. An analogous microorganism index can be found at umbbd.ahc.umn.edu (UM-BBD).

The UM-BBD Microorganism Index has links for the entries containing both genus and species names to the corresponding entries on websites maintained by the American Type Culture Collection (ATCC) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Another excellent web resource that deals with microorganisms which are important in biodegradation is the Biodegradative Strain Database (BSD) bsd.cme.msu.edu maintained at Michigan State University by John Urbance, Jim Cole and Jim Tiedje.

The BSD microorganism listings, in turn, link to http://www.cme.msu.edu (Ribosomal RNA Database) and to biodegradative pathways maintained on the UM-BBD.

Several trends are apparent from perusing the data in Table 1. First, biodegradative capabilities are widespread phylogenically within the *Proteobacteria*, high G+C Gram-positive bacteria, and *Flavobacterium* in the *Cytophageles*-green sulfur bacteria. As discussed in the section above, this reflects the facile transfer of genes, especially those that might be contained on plasmids or flanked by transposable elements.

Second, there are several genera of bacteria, which have emerged repeatedly as having diverse catabolism, particularly with starting compounds we think of as metabolically unusual, such as synthetic industrially relevant organic compounds. The latter include herbicides, insecticides, industrial solvents and synthetic intermediates. As illustrated in Table 1, the following genera are particularly well represented: *Arthrobacter*, *Burkholderia*, *Pseudomonas* and *Rhodococcus*. The caveat to these observations is that we have largely depicted biodegradation pathways catalyzed by prokaryotes, which have been obtained in pure culture via enrichment culture. Thus, we have selectively depicted microorganisms that grow well under conditions typically used for enrichment culture and the maintenance of pure culture isolates in the laboratory. These microbial strains may only reflect some fraction, perhaps a small fraction, of the prokaryotes that actively carry out biodegradation in the soils and waters of the Earth. The complete genome sequencing of both pure culture bacteria

Table 1. Prokaryote genera identified in biodegradation and the compounds they metabolize.[a]

Prokaryote genus	Compound undergoing biodegradation
Acetobacterium	Triethanolamine
	Carbon tetrachloride
Achromobacter	2,4-Dichlorobenzoate
Acinetobacter	Cyclohexanol
	2-Chloro-*N*-isopropylacetanilide
Actinomycetes	2,4,6-Trinitrotoluene (TNT)
Aeromonas	Phenanthrene
Agrobacterium	Glyphosate
	1,2,3-Tribromopropane
	Atrazine
Alcaligenes	2,4-Dichlorobenzoate
	2,4-Dichlorophenoxyacetic acid (2,4-D)
	2,4-Dichlorobenzoate
	2-Aminobenzenesulfonate
	Toluene-4-sulfonate
	Atrazine
Ancylobacter	1,2-Dichloroethane
	2,4-Dichlorophenoxyacetic acid
	4-Nitrophenol
	1,3-Dichloro-2-propanol
	Tyrosine
	2,4-Dichlorobenzoate
	Glyphosate
	Parathion
Arthrobacter	2,4-Dichlorophenoxyacetic acid (2,4-D)
	4-Nitrophenol
	Octamethylcyclotetrasiloxane
	Iprodione
	1,3-Dichloro-2-propanol
	Fluorene
	Tyrosine
	2-4-Dichlorobenzoate
	Glyphosate
	Methyl *tert*-butyl ether
	Nicotine
	2-Aminobenzoate
	Phenanthrene
	Parathion
Azoarcus	Benzoate
	Toluene
Azotobacter	2,4-Dichlorophenoxyacetic acid (2,4-D)
	Thiocyanate
Bacillus	2,4,6-Trinitrotoluene
	2-Phenylacetaldoxime
Beijerinckia	Xylene
Brevibacterium	Dibenzofuran
	Nitrobenzene
Brevundimonas	Parathion
Burkholderia	2,4-Dichlorophenoxyacetic acid (2,4-D)
	1,2,4-Trichlorobenzene
	Phthalates
	Benzoate
	Pentachlorophenol
	2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)
	3-Chloroacrylic acid
	Toluene
	Trichloroethylene
	o-Xylene
Clavibacter	2,4-Dichlorobenzoate
	Atrazine
Clostridium	2,4,6-Trirtotoluene (TNT)
	Phenol
Chelatobacter	Nitrilotriacetate

Table 1. *Continued*

Prokaryote genus	Compound undergoing biodegradation
Comamonas	Nirtobenzene
	3-Methylquinoline
	Phthalates
	Toluene-4-sulfonate
Corynebacterium	1,3-Dichloro-2-propanol
	2,4-Dichlorobenzoate
Dehalobacter	Tetrachloroethene
Dehalococcoides	Tetrachloroethene
Dehalospirillum	Tetrachloroethene
Desulfitobacterium	Tetrachloroethene
Desulfobacterium	Carbon tetrachloride
Desulfovibrio	2,4,6-Trinitrotoluene (TNT)
Enterobacter	Glyphosate
	1,1,1-Trichloro-2,2-*bis*-(4-chlorophenyl)ethane (DDT)
	Pentaerythritol tetranitrate
Escherichia	3-Phenylpropionate
	Arsonoacetate
	Phenylmercuric chloride
Eubacterium	Gallate
Exophiala	Styrene
Fiavobacterium	Bromoxynil
	2,4-Dichlorophenoxyacetic acid (2,4-D)
	Glyphosate
	Parathion
	Pentachlorophenol
Hydrogenophaga	4-Carboxy-4′-sulfoazobenzene
Hyphomicrobium	Dichloromethane
	Dimethyl sulfoxide
Klebsiella	Benzonitrile
	Bromoxynil
	Acetylene
	1,1,1-Trichloro-2,2-*bis*-(4′-chlorophenyl)ethane (DDT)
Lactobacillus	2,4,6-Trinitrotoluene (TNT)
Methanobacterium	Carbon tetrachloride
Methanosarcina	Tetrachloroethene
	Carbon tetrachloride
Methylobacterium	Dichloromethane
	Methyl *tert*-butyl ether
Methylococcus	Thiocyanate
Methylophilus	Dichloromethane
Methylosinus	Trichloroethylene
Methylosulfonomonas	Methanesulfonic Acid
Moorella	Carbon tetrachloride
Moraxella	4-Nitrophenol
	2-Chloro-*N*-isopropylacetanilide
	Naphthalenesulfonates
Mycobacterium	Methyl *tert*-butyl ether
Myrothecium	Cyanamide
Neurospora	2-Nitropropane
Nirtosomonas	Methyl fluoride
	Dimethyl ether
Nocaradia	Methyl *tert*-butyl ether
	Parathion
	Methyl ethyl ketone
Pelobacter	Acetylene
Proteus	1,1,1-Trichloro-2,2-*bis*-(4-chlorophenyl)ethane (DDT)
Pseudomonas	Acrylonitrile
	2-Aminobenzoate
	1,3-Dichloropropene
	Dichloromethane
	Dimethyl sulfoxide
	Carbazole

(continued)

Table 1. *Continued*

Prokaryote genus	Compound undergoing biodegradation
	Benzoate
	p-Xylene
	p-Cymene
	Carbon tetrachloride
	Fluorene
	Adamantanone
	3-Chloroacrylic Acid
	2-Chloro-*N*-isopropylacetanilide
	1,4-Dichlorobenzene
	Parathion
	Nitroglycerin
	Toluene
	Octane
	Nitrobenzene
	4-Chlorobiphenyl
	Dibenzothiophene
	Orcinol
	Xylene
	Ethylbenzene
	Mandelate
	Styrene
	Trichloroethylene
	Toluene-4-sulfonate
	m-Xylene
	Atrazine
	Naphthalenesulfonates
	2,4-Dichlorobenzoate
	Chlorobenzene
	2-Aminobenzoic Acid
	4-Chlorobiphenyl
	Ethylbenzene
	Naphthalene
	Chlorobenzene
	1-Aminocyclopropane-1-carboxylate
	Biphenyl
	Caprolactam
	Phenanthrene
	1,1,1-Trichloro-2,2-*bis*-(4-chlorophenyl)ethane (DDT)
	2,4,6-Trinitrotoluene
	m-Cresol
	Thiocyanate
	Phenylmercuric chloride
	n-Octane
	Dodecyl Sulfate
	Bromoxynil
	Dibenzothiophene
	2,4-Dichlorobenzoate
	Mandelate
	Methyl *tert*-butyl ether
	(+)-Camphor
	2,4-Dichlorophenoxyacetic Acid
Ralstorsa	Atrazine
	1,1,1-Trichloro-2,2-*bis*(4′-chlorophenyl)ethane (DDT)
	Dimetyl sulfoxide
Rhodobacter	Acetylene
Rhodococcus	Atrazine
	Acrylonitrile
	Methyl *tert*-butyl ether
	Cyclohexanol
	Bromoxynil
	Styrene
	Tetrahydrofuran
	Benzonitrile

Table 1. *Continued*

Prokaryote genus	Compound undergoing biodegradation
	Dibenzothiophene
	Benzoate
Rhodopseudomonas	2,4,6-Trinitrotoluene (TNT)
Salmonella	*n*-Octane
	Dibenzofuran
Sphingomonas	Carbazole
	γ-1,2,3,4,5,6-Hexachlorocyclohexane
	Dibenzo-*p*-dioxin
	Xylenes
	Tetrachloroethene
Sporomusa	Dibenzofuran
Staphylococcus	2,4,6-Trinitrotoluene
	Arsonoacetate
	Fluorene
	Atrazine
Streptomyces	Phenanthrene
	1,1,1-Trichloso-2,2-*bis*-(4-chlorophenyl)ethane (DDT)
Synechococcus	Phenanthrene
	Dibenzofuran
Terrabacter	
	Toluene
Thauera	Benzoate
	Phenol
	Thiocyanate
Thiobacillus	1,2-Dichloroethane
Xanthobacter	1,4-Dichlorobenzene
	2-Chloro-*N*-isopropylacetanilide
	2-Nitropropane
	Propylene

[a]A similar list with links to the metabolic pathways can be obtained on the UM-BBD at http://umbbd.ahc.umn.edu/search/micro.html

and genomic DNA from soil, the so-called "soil metagenome" (Rondon et al., 2000), may help address this question by helping unveil what percentage of the total genome of a given organism functions in non-intermediary catabolic metabolism. In another example, culture-independent molecular methods were used to analyze microbial communities in an aquifer contaminated with hydrocarbons and chlorinated solvents in which active biodegradation was occurring (Dojka et al., 1998). In that study, 16S rRNA sequences were determined for 21 bacterial members of the consortium, belonging to four recently described divisions of bacteria for which there are no cultivated representatives. Moreover, two particularly abundant 16S rRNA sequence types were implicated in the overall hydrocarbon metabolism. They were members of the genera *Syntrophus* and *Methanosaeta*, both of which were proposed to participate in aceticlastic methanogenesis at the end of the catabolic food chain.

In parallel with molecular non-culture methods, the well-established methods of enrichment culture are more frequently being applied under anaerobic and other nonstandard conditions in an effort to obtain novel microbial types. This approach also suggests that biodegradative capabilities are more widespread in the microbial world than has been appreciated by some. For example, halophiles have been identified which metabolize nitroarenes, and members of the *Heliobacterium* group are known that catabolize polychlorinated biphenyls (PCBs) and chlorophenols (Table 2). These and other recent observations are expanding the taxonomic range of bacteria that catabolize environmental pollutants. Further experiments are likely to expand this further.

Themes in Biodegradation Pathways

Occurrence of Similar Pathways in Divergent Prokaryotes

Gene transfer amongst prokaryotes is quite facile, and our appreciation of this seems to be increasing all the time. The genes most prone to transfer are those conferring survival advantage only under specialized conditions, the so-called "dispensible genes." Principal among those

Table 2. Microbes recently identified as organic pollutant biodegraders, but falling outside of the prokaryotic groupings typically isolated for studies on biodegradation.

Prokaryote[a]	Taxonomic group	Substrate	Reference
Haloanaerobium praevalens	*Haloanaerobiales*	Nitrobenzene *o*-Nitrophenol *m*-Nitrophenol *p*-Nitrophenol Nitroanilines 2,4-Dinitrophenol 2,4-Dinitroaniline	Oren et al., 1991
Sporohalobacter marismortui	*Haloanaerobiales*	Nitrobenzene *o*-Nitrophenol *m*-Nitrophenol *p*-Nitrophenol Nitroanilines 2,4-Dinitrophenol 2,4-Dinitroaniline	Oren et al., 1991
Borrelia burgdorferi *Borrelia hermsii*	Spirochaetales	Benzamides	Dettori et al., 1995
Bacteroides fragilis	*Cytophagales*	Alkylhydroperoxides	Rocha et al., 1999
Desulfitobacterium dehalogenans	*Heliobacterium*	Polychlorinated-biphenyls	Wiegel et al., 1999
Desulfitobacterium hafniense	*Heliobacterium*	3-Chloro-4-hydroxy-phenylacetate	Christiansen et al., 1998
Desulfitobacterium dehalogenans	*Heliobacterium*	Chorophenols	van de Pas et al., 1999

[a]These bacteria do not belong to the following groups: Proteobacteria, and high and low G+C Gram-positive bacteria.

genes are ones conferring antibiotic resistance, heavy metal resistance or new catabolic activities. These genes are commonly found on plasmids. Many catabolic plasmids have been shown to have a broad host-range and transfer by conjugation in the absence of helper plasmids. Thus, the genes, and the metabolic functions they encode, can show up in diverse prokaryotes. An example will best serve to illustrate this point.

In 1995, a *Pseudomonas* species, denoted strain ADP, was isolated from an enrichment culture in which the herbicide atrazine was supplied as the sole source of nitrogen (Mandelbaum et al., 1995). Subsequent studies over the ensuing three years elucidated the atrazine catabolic pathway and yielded the DNA sequences of the genes encoding the first three metabolic steps (Fig. 1). During the same period, other laboratories isolated atrazine-catabolizing prokaryotes using different enrichment and isolation conditions (Bouquard et al., 1997; de Souza et al., 1998a; de Souza et al., 1998b; Radosevich et al., 1995; Struthers et al., 1998). The bacteria were subjected to taxonomic determination and found to be members of the following genera, respectively: *Rhizobium*, *Agrobacterium*, *Ralstonia* and *Clavibacteria*. In our laboratory, DNA from each of the distinct atrazine-catabolizing bacteria was prepared (de Souza et al., 1998b). They were each found to contain genes with more than 99% sequence identity to the atrazine genes from the original *Pseudomonas* sp. ADP isolate. This occurred despite the fact that the organisms were isolated independently in different regions of Earth, by different groups and under different conditions. These observations are consistent with a facile transfer of the atrazine-catabolic genes amongst soil prokaryotes.

In another example, illustrated by perusing the UM-BBD, the same organic compound is metabolized by different genera of bacteria via

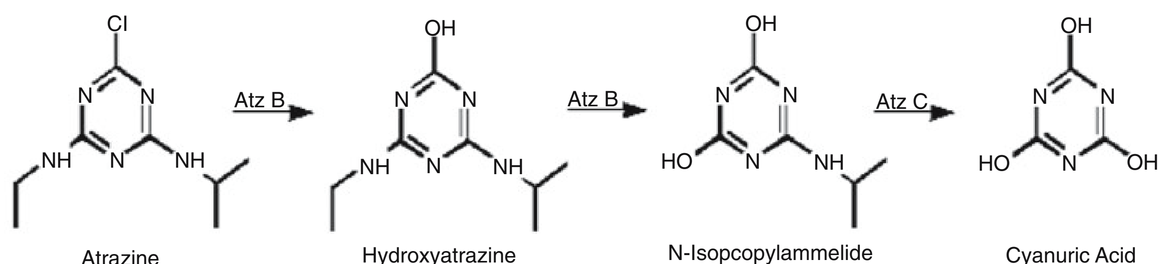

Fig. 1. Catabolic pathway for the catabolism of atrazine in *Pseudomonas* sp. ADP.

different intermediates. Examples of starting compounds are:

{atrazine
{fluorene
{glyphosate
{nitrobenzene
{4-nitrophenol
{parathion
{phenanthrene
{styrene
{toluene
{2,4,6-trinitrotoluene

Common Themes in Catabolic Pathways

Although some catabolic pathways are widespread, there are some correlations between certain types of metabolism and the prokaryotic group catalyzing those reactions. This can be attributed to the compatibility between the catabolic reactions and the core metabolic pathways that the catabolic intermediates feed into. This is particularly well illustrated for chemical compounds which are composed of a single carbon atom or which are readily metabolized to C-1 fragments.

Figure 2 shows the C-1 meta-pathway, which is also depicted in the [{umbbd.ahc.umn.edu/c1/c1_map.html}{UM-BBD}]. At the core of the map are the oxidative and reductive parts of the C-1 metabolic cycle, which is important on a global scale as discussed previously. Anaerobic Archaea (known as methanogens) catalyze the reductive reactions that transform carbon dioxide to methane. These organisms are important members of certain anaerobic consortia involved in the biodegradation of complex organic matter such as cellulosic wastes. Methanogens occupy the end of the anaerobic food chain in the overall biodegradative process.

A class of prokaryotes called “methanotrophs” (Fig. 2) carry out the oxidative reactions leading from methane, the most reduced C-1 compound, to carbon dioxide, the most oxidized. Some C-1 oxidizing organisms (known as “methylotrophs”) cannot oxidize methane to methanol, but can carry out the next three oxidative reactions. Methane is a common natural product; it is the main constituent of natural gas. Moreover, data suggests that a majority of the methane generated in lake sediments is oxidized in higher, aerobic levels of the lake by methanotrophs, and thus methane never enters the atmosphere.

Methanotrophic and methylotrophic metabolism may be expanded to include a set of oxidative, hydrolytic or thiolytic reactions whereby simple organic structures can be transformed to the methanotrophic intermediates methanol, formaldehyde or formate (Fig. 2). Some of these

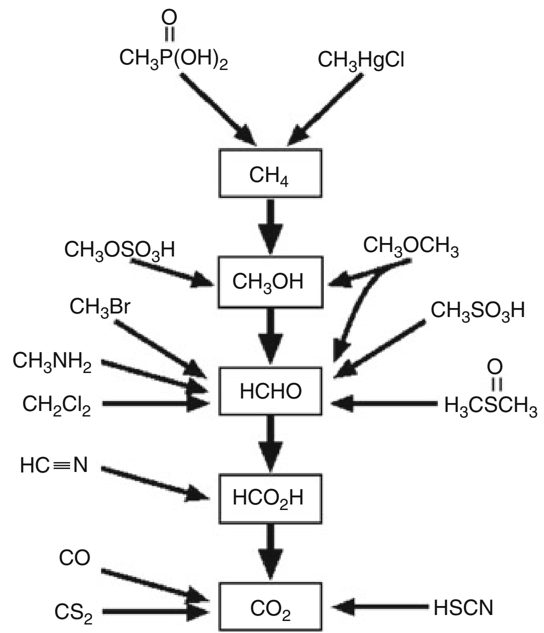

Fig. 2. Catabolism of organic compounds containing one carbon atom that funnel into the central intermediates of methanotrophy: methane, methanol, formaldehyde and formate.

compounds are natural products; for example dimethyl sulfide, methylamine and methyl chloride. Others are predominantly the products of organic synthesis: dichloromethane, dimethylether and methyl fluoride. Regardless of their origin, these compounds are readily transformable to methanotrophic metabolic intermediates and thus some methanotrophs will grow on them as their sole source of carbon and energy. This catabolic metabolism is not universal, however. Only some small subset of the total set of methanotrophs and methylotrophs will grow on a given compound shown at the periphery of Fig. 2. But methanotrophs and methylotrophs are common in nature and thus dichloromethane, dimethylether and methyl fluoride are generally thought of as being fairly biodegradable.

Another common theme is seen in the transformation of the commercially important BTEX compounds (i.e., benzene, toluene, ethylbenzene and xylenes). They are clustered because of their co-occurrence in environmental contamination stemming from spillage of petroleum materials. Because BTEX compounds are structurally analogous to each other, there are commonalities in their metabolism by prokaryotes. Anaerobic metabolism of BTEX compounds has been studied only more recently, and the biochemical basis of the biodegradation reactions is now being revealed. The aerobic metabolism of BTEX compounds is much better studied. For example, see Fig. 3 and (umbbd.ahc.umn.edu/BTEX/

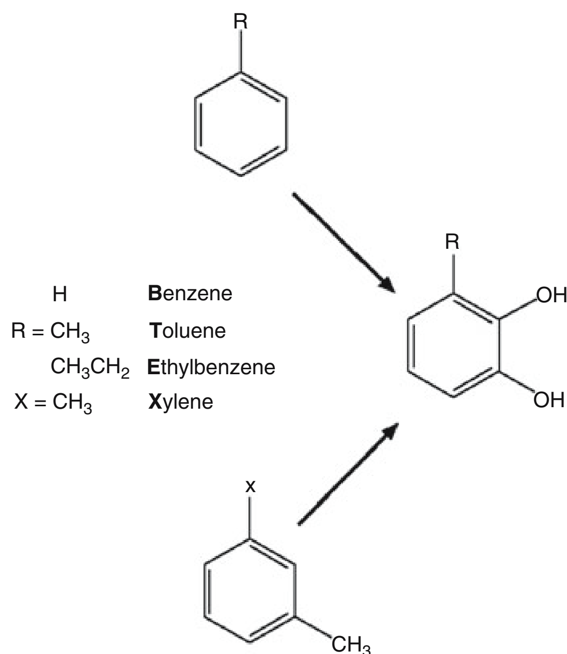

Fig. 3. Convergence of aerobic pathways for BTEX compounds leads to a catechol intermediate.

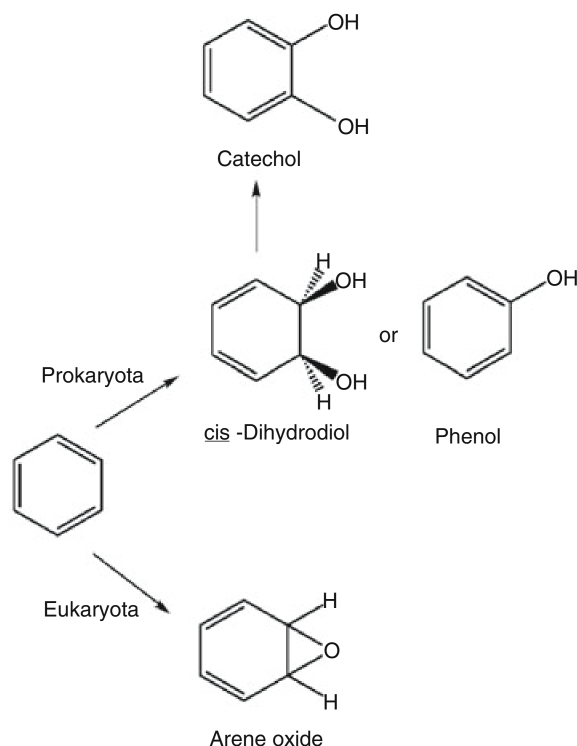

Fig. 4. Divergence in the catabolism of aromatic compounds by Prokaryota and Eukaryota.

BTEX_map.html#aerobic). Almost invariably, oxygenase enzymes initiate the metabolism to produce ring cis-dihydrodiols, phenols, benzyl alcohols and ultimately catechols, which undergo ring cleavage. These alcohol products are all more activated than their aromatic hydrocarbon starting compounds. There are multiple pathways possible but all of them produce catechol intermediates. To follow all known aerobic prokaryotic metabolic pathways for each of the BTEX compounds, follow the links:

{Benzene
{Toluene
{Ethylbenzene
{*o*-Xylene
{*m*-Xylene
{*p*-Xylene

The metabolic strategy for BTEX compounds used by aerobic prokaryotes differs from that used by aerobic eukaryotic organisms such as fungi (Cerniglia et al., 1978) and mammals (Jerina et al., 1968). The latter group also uses oxygenase enzymes to attack resonance-stabilized aromatic hydrocarbons. However, the initial products of the oxygenase-catalyzed reactions are often arene oxides (Fig. 4). Aromatic alcohols are detected but are shown to largely arise from spontaneous isomerization of the arene oxides and are not direct enzyme products. This contrasts with the prokaryote aromatic ring monooxygenation reactions in which the phenol product is detected directly. For example, with toluene catabolism by *Burkholderia cepacia* G4, the initial reaction product is 2-hydroxytoluene, or o-cresol, exclusively. There was no evidence for the intermediate formation of toluene 2,3-epoxide, which would have isomerized to a mixture of o-cresol and m-cresol. The data do not rule out that an epoxide is an enzyme-bound intermediate that undergoes a controlled isomerization on the enzyme surface. This would be advantageous to the organism, as epoxides are reactive electrophiles and can alkylate proteins and other molecules in the cell. So a high-flux metabolic pathway that produces such a reactive species might well be selected against during evolution. In contrast, the mammalian metabolism of BTEX compounds is largely low flux metabolism, to scavenge stray hydrocarbons that may enter the body. Other enzymes further metabolize the epoxide products to make intermediates that are excreted from the animal. A nonspecific detoxification metabolism such as this may work best when it proceeds through an initial arene oxide intermediate.

Acknowledgments. I thank the many talented colleagues with whom I have collaborated on metabolic pathways of biodegradation. Special thanks go to Professor Lynda Ellis for her meticulous work on the University of Minnesota Bio-

catalysis/Biodegradation Database, making it a resource that has been worth accessing millions of times by researchers around the world.

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Prokaryotes (2006) 2:969–984
DOI: 10.1007/0-387-30742-7_30

CHAPTER 1.30

Haloalkaliphilic Sulfur-Oxidizing Bacteria

DIMITRY YU. SOROKIN, HORIA BANCIU, LESLEY A. ROBERTSON AND J. GIJS KUENEN

Introduction

Chemolithoautotrophic sulfur-oxidizing bacteria (SOB) play an important role in the element cycling in natural and man-made environments because of their extremely high capacity to transform various sulfur compounds and their contribution to secondary production of organic matter. They are widely distributed in various habitats, associating primarily with sulfide-oxygen interface layers, where they successfully compete with chemical sulfide oxidation by oxygen. Energetically, the reaction of complete oxidation of sulfide or thiosulfate to sulfate (8 electrons) is among the most attractive for chemosynthesis, and not surprisingly, sulfur oxidizers can be found in many different groups of prokaryotes. Currently, lithoautotrophic sulfur bacteria are mostly found among the proteobacteria (alpha, beta, gamma and epsilon subdivision; The Colorless Sulfur Bacteria in the second edition; Kuenen and Robertson, 1992; Kelly and Wood, 2000). The currently known exceptions outside the proteobacteria are among the Gram-positive bacteria (*Sulfobacillus*), Crenarchae (*Sulfolobus*), and deep lineages (*Aquificalis*; The Chemolithotrophic Prokaryotes in this Volume).

According to their response to pH, the known sulfur-oxidizing species include acidophiles (optimum pH <6) and neutrophiles (optimum pH 7–8). The former have received much attention because of their important role in acidic metal leaching processes (Pronk et al., 1990). The neutrophiles are more common in natural environments, dominating in fresh and marine waters, and in waste treatment plants (Robertson and Kuenen, 1992). However, until recently, very little was known about the possibility of sulfur oxidation at pH above 9. Several years ago, the authors of this chapter became interested in alkaliphilic sulfur oxidation, as this would offer new options for removing H_2S from industrial wastes. An analysis of the literature available on bacterial life at high pH clearly pointed to soda lakes as the best possible natural habitat for such bacteria.

Soda Lakes as a Unique Habitat

Soda lakes represent a specific type of salt lake that contains an alkaline sodium carbonate-bicarbonate fraction among its dominant salts. Like most of the other inland salt lakes, the soda lakes are located in areas with dry climate conditions that facilitate gradual salt accumulation in depressions. The main mechanism of their genesis is the leaching of salt from sodium-rich rocks by high CO_2-containing groundwaters low in Ca and Mg content (Eugster, 1970; Jones et al., 1977). The presence of sodium carbonate creates a uniquely stable natural alkaline habitat, although the total salt content and the sodium carbonate fraction in the soda lakes vary within a broad range, depending on the local conditions. Although a high pH can occur in several other habitats, such as low-salt alkaline springs or microzones of ammonification in soils, the alkaline conditions in these habitats are not stable. For this a high buffering capacity (alkalinity) is necessary. In soda lakes, the presence of sodium carbonate ensures that the high-to-extremely high pH (usually around 9.5–10.5) is maintained. Thus, the soda lakes seem to be the only natural environment with appropriate conditions for the stable development of obligately alkaliphilic microorganisms, which usually grow optimally at a pH around 10. Indeed, as will be described later in the text, the alkaliphilic sulfur bacteria cannot be isolated from the neutral saline lakes or from alkaline, but not sufficiently buffered, freshwater springs.

The well-known hypersaline soda lakes are located in the East African Rift Valley in Kenya and Tanzania (Bogoria, Magadi, Natron), the Libyan Desert in Egypt (Wadi Natrun), California, and Nevada. These lakes were extensively studied by geologists because of their deposits of soda minerals. Another area where saline alkaline lakes are numerous is Central Asia, where the Transbaikal dry steppe stretches from southeast Siberia to northeast China. Here much smaller, shallow, and usually less saline lakes predominate, with a relatively unstable water regi-

Fig. 1. Typical small soda lake (Kulunda steppe, Altai, Russia).

men and a freezing winter period causing frequent and substantial fluctuations of the water salinity and temperature. A typical example of such a lake is presented in Fig. 1.

Recent microbiological analysis of the soda lakes by both traditional and culture-independent molecular techniques revealed the remarkable fact that, despite the doubly extreme conditions of salt and pH, fully structured and active microbial communities are present, even in saturated alkaline brines (Imhoff et al., 1979; Grant and Tindall, 1986; Jones et al., 1998; Zavarzin et al., 1999; Zavarzin and Zhilina, 2000; Humayoun et al., 2003; Sorokin et al., 2004a). Among them, the main functional groups of anaerobes, such as fermentative, acetogenic, methanogenic and sulfate-reducing bacteria, represented by unique haloalkaliphilic species, have recently been isolated and identified (Zavarzin et al., 1999). The microbial sulfur cycle seems to be one of the most active cycles observed in the soda lakes, with anaerobic phototrophic purple sulfur bacteria and sulfate-reducing alkaliphiles as the main participants (Isachenko, 1951; Imhoff et al., 1979; Zhilina et al., 1997; Gorlenko et al., 1999). However, no information is available regarding the presence of chemolithotrophic sulfur bacteria in soda lakes.

The Investigated Area and the Samples

Surface sediment samples from all the main areas of soda lakes, as described above, were obtained for the study of sulfur bacteria, either from colleagues or during our own expeditions. The main characteristics of the samples are given in Table 1. The sampled lakes can be divided into three categories: 1) low-saline, small and shallow steppe lakes of the Transbaikal area; 2) moderately saline soda lakes of the Kulunda steppe and Mono Lake in California; and 3) hypersaline alkaline brines of Africa and south Kulunda, in areas with significant deposits of the trona mineral $NaHCO_3{\cdot}Na_2CO_3{\cdot}2H_2O$). Most of the lakes belong to the carbonate-chloride-sulfate type. In a few, sodium chloride is the dominant salt, e.g., the hypersaline, alkaline Stamp Lake in the Kulunda steppe of Altai in Russia.

Enrichment, Isolation and Cultivation of Haloalkaliphilic Sulfur-Oxidizing Bacteria

Following the published examples (Tindall, 1988; Horikoshi, 1991), our first attempts to enrich for alkaliphilic SOB using soda lake sediments as the inoculum were made using medium containing 20 mM thiosulfate as energy source, 10 g liter^{-1} of sodium carbonate, 0–50 g liter^{-1} NaCl, 0.5 g liter^{-1} NH_4Cl, and 1 g liter^{-1} of K_2HPO_4. Sodium carbonate was added after sterilization and the pH was adjusted to 10 by HCl. Some growth was observed on this medium using inoculum from the Hadyn soda lake in Tuva (Table 1), but the resulting cultures were not stable and, in particular, a drop in pH below 9 was followed by heavy sulfur precipitation. An optimization study resulted in the formulation of a mineral base medium, containing 0.6–4.0 M Na^+ (Table 2). For the inocula from the hypersaline lakes where NaCl was the dominant salt (Wadi Natrun), the amount of sodium carbonate in the base medium was reduced by substituting NaCl. The medium contained a minimum amount of sodium carbonate and bicarbonate, equivalent to 0.5 M Na^+, and NaCl equivalent to 0.1–3.5 M Na^+. Such mineral bases provided sufficient alkalinity so that even when a dense sulfur-oxidizing culture producing high amounts of sulfuric acid from thiosulfate developed, the pH of the

Table 1. Soda lake samples used for enrichment and isolation of alkaliphilic SOB.

Area	Lakes	Salinity		pH	Total carbonate alkalinity (M)
		Total salts (g liter^{-1})	Conductivity (mS/cm)		
East African Rift Valley, Kenya, 1996, 1999[a]	Nakuru	20[b]	12	10.5	0.12–0.29
	Crater Lake (Sonachi)	40	23	10.0	0.14
	Elmenteita	50	26	9.5–10.0	0.13
	Bogoria	50–130	50–80	10–10.5	0.55–1.0
	Magadi	220	40–111	10.5–11.0	1.16
Wadi Natrun, Libyan Desert, Egypt, 2000	Hamra, Umm-Risha, Rusita, Fazda, Gaara, Khadra, Zugm, Beidah	200–380	nd	9.5–10.3	0.11–0.75
California, United States, 2000[c]	Mono Lake	90	nd	9.7	nd
Tuva, Russia, 1995[d]	Hadyn Lake	20	nd	10.0	nd
Kunkur steppe, Chita region, Transbaikal area, Russia (1997)	10 small shallow lakes	5–40	nd	9.5–10.2	0.02–0.11
Borzinskii region, Russia Transbaikal area, 2003[e]	Lake Borzinskoe	380	nd	9.4	0.50
Northeastern Mongolia, Transbaikal area, 1999	8 small shallow lakes	5–20	nd	9.2–10.5	0.02–0.12
	6 small shallow lakes	30–60		9.7–10.3	0.07–0.30
	Shar-Burdiin	220		10.1	1.20
	Hotontyn	360		10.0	0.82
Kulunda steppe, Altai, Russia, 2002, 2003					
North	5 small shallow lakes	20–50	20–50	9.4–9.6	0.02–0.24
Central	Cock Soda Lake	54–60	50–63	9.93–10.1	0.62–0.80
South: Salt Lake steppe	7 small shallow lakes	55–178	54–122	9.3–9.76	0.14–1.48
South: Tanatar system	Tanatar V–VI	30–55	37–55	9.9–10.1	0.43–0.58
	Tanatar III	82–90	76–85	9.9–10.1	1.02–1.15
	Tanatar II	262	236	9.5	3.50
	Tanatar I	350–475	425–600	10.0–10.65	5.0–5.2
South: hypersaline alkaline lake	Stamp Lake	380	500	9.02–9.2	0.5

Abbreviations: SOB, sulfur-oxidizing bacteria; and n.d., not determined.
[a]Sampled by B. Jones and W. Grant.
[b]Salinity and alkalinity data for the Kenyan lakes have been obtained from the literature.
[c]Sampled by V. Gorlenko.
[d]Sampled by T. N. Zhilina.
[e]Sampled by B. Namsaraev.

Table 2. Composition of the basic mineral media suitable for cultivation of alkaliphilic SOB (pH 10-10.1).

Salt (g liter^{-1})	0.6M Na^+	2M Na^+	4M Na^+
Na_2CO_3	23	95	185
$NaHCO_3$	7	15	35
NaCl	6	16	16
KNO_3	0.5	1	1
K_2HPO_4	1	1	1

Abbreviation: SOB, sulfur-oxidizing bacteria.

medium remained at 9.5–10.2. Potassium nitrate provided the source of nitrogen and potassium. In addition, in some cases, ammonium chloride was added after sterilization at low concentration (<3 mM) to ensure enrichment of species unable to utilize nitrate. The low concentration is dictated by the almost complete conversion of ammonium to highly toxic, free ammonia at pH 10. After sterilization, the alkaline mineral base medium was supplied with 20–80 mM of thiosulfate, 1 ml per liter of trace elements solution (Pfennig and Lippert, 1966) and 1 mM $MgCl_2 \cdot 6H_2O$. The latter formed a soluble basic magnesium carbonate complex [$Mg_2(OH)_2CO_3$]. The use of this medium provided a basis for the successful enrichment and isolation of many strains of obligately alkaliphilic sulfur bacteria with varying degrees of salt tolerance. The preparation of solid alkaline media is complicated by the chemical instability of agar at high pH at temperatures above 50°C and by the low solubility of sodium carbonate. Therefore, the preparation of solid medium involves mixing equal volumes of sterile 4% agar and mineral base

medium in double strength at 50°C. Obviously, the maximum soda concentration in the final solid medium is then limited to 2 M Na^+.

The isolation strategy was based on the following procedure. Positive enrichment cultures were subcultured several times with 1:100 inocula to obtain a stable active culture. This stable culture was serially diluted and the successive dilutions were plated. Sometimes, especially when the low-salt medium (0.6 M Na^+) was used, it was necessary to make serial dilutions immediately without preliminary enrichment because of the high grazing activity of protozoa. This approach was also useful in combining enrichment, enumeration and isolation in a single procedure. Moreover, it later appeared that the efficiency of colony formation of many sulfur bacteria isolated from soda lakes was very low in comparison with their growth in liquid media. Therefore, in some cases the extinction dilution approach was used to isolate the dominant species growing in liquid culture. Growth was monitored by measuringthiosulfate consumption (iodimetric titration in presence of acetic acid) and by microscopy. Some variations in the selective enrichment strategy, such as the use of sulfide and thiocyanate instead of thiosulfate as substrate or nitrogen oxides instead of oxygen as electron acceptors will be discussed below.

Distribution and Diversity of Haloalkaliphilic Chemolithoautotrophic Sulfur-Oxidizing Bacteria

Enrichment on low-salt alkaline medium under fully aerobic conditions using inocula from the Siberian low-salt soda lakes gave the first indication of the presence of aerobic sulfur bacteria capable of stable growth at pH 10 (Sorokin et al., 2000). Two pure cultures, strains AL 2 and AL 3, isolated from lake Hadyn in Tuva (Russia) became the reference type strains for two different groups of the haloalkaliphilic SOB most often isolated from soda lakes. Subsequent investigation of the samples from different geographic locations (Table 1) resulted in the isolation of more than 100 strains of obligately alkaliphilic sulfur bacteria. Positive enrichment cultures were only obtained from the lakes with pH >9 and not from the neutral salt lakes with pH 7–8.5. Combining the enrichment and serial dilution procedures yielded sulfur bacteria (10^3–10^8 cells/cm^3 of sediment) capable of growth at pH 10 and a salt concentration of 0.5–4 M total Na^+. The cultivation and maintenance of the haloalkaliphilic sulfur bacteria is not complicated by acid production because of the extremely high buffering capacity of the soda-based medium. For example, full oxidation of 80 mM thiosulfate (20 g/liter) resulted in a pH drop from 10 to 9.2 even at the lowest buffering capacity used (0.6 M total Na^+). The dense liquid cultures of haloalkaliphilic sulfur bacteria obtained after utilization of 40–80 mM thiosulfate remained viable during storage at 4°C for 3–6 months. Most of the strains also survived freeze-drying and deep-freezing storage in 10% glycerol.

The soda lake isolates grouped into two distinct clusters and created a basis for the description of two new genera of Gammaproteobacteria—*Thioalkalimicrobium* and *Thioalkalivibrio* (Sorokin et al., 2001c; Sorokin et al., 2002a). The former is closely affiliated with the neutrophilic sulfur bacteria *Thiomicrospira*, while the genus *Thioalkalivibrio* is affiliated with the purple sulfur bacteria of the family Ectothiorhodospiraceae (Fig. 2), which includes both halophilic and haloalkaliphilic species (Imhoff and Süling, 1996).

In general, the *Thioalkalimicrobium* group dominated in enrichment cultures from the low-saline Siberian soda lakes. *Thioalkalimicrobium* also could be enriched from the more saline soda lakes in Central Asia and Africa, but only from fresh samples. *Thioalkalivibrio* dominated in enrichments from highly mineralized lakes and from old samples and was always dominant when the enrichment medium contained >1.5 M total Na^+. Therefore the most important selective force favoring survival of alkaliphilic sulfur bacteria in soda lakes appeared to be the salt concentration (Table 3).

Ecophysiology of Aerobic Haloalkaliphilic Sulfur-Oxidizing Bacteria

The main properties of the two groups of haloalkaliphilic sulfur bacteria are presented in Tables 4 and 5. The *Thioalkalimicrobium* group is represented by highly specialized, low-salt tolerant, fast-growing and low-yield strains with extremely high sulfide- and thiosulfate-oxidizing activity. In contrast, the *Thioalkalivibrio* group is more physiologically diverse and accommodates slowly growing organisms with more efficient substrate conversion. These are, in general, more salt-tolerant, with many strains able to grow in saturated soda brines. This group uniformly synthesized a membrane-bound yellow pigment not found in the low-salt tolerant *Thioalkalivibrio* strains. This pigment is a 23-carbon polyene compound with a structure unlike that of any known bacterial pigments (Takaichi et al., 2004). Although its complete formula and function is not yet completely understood, it is clearly

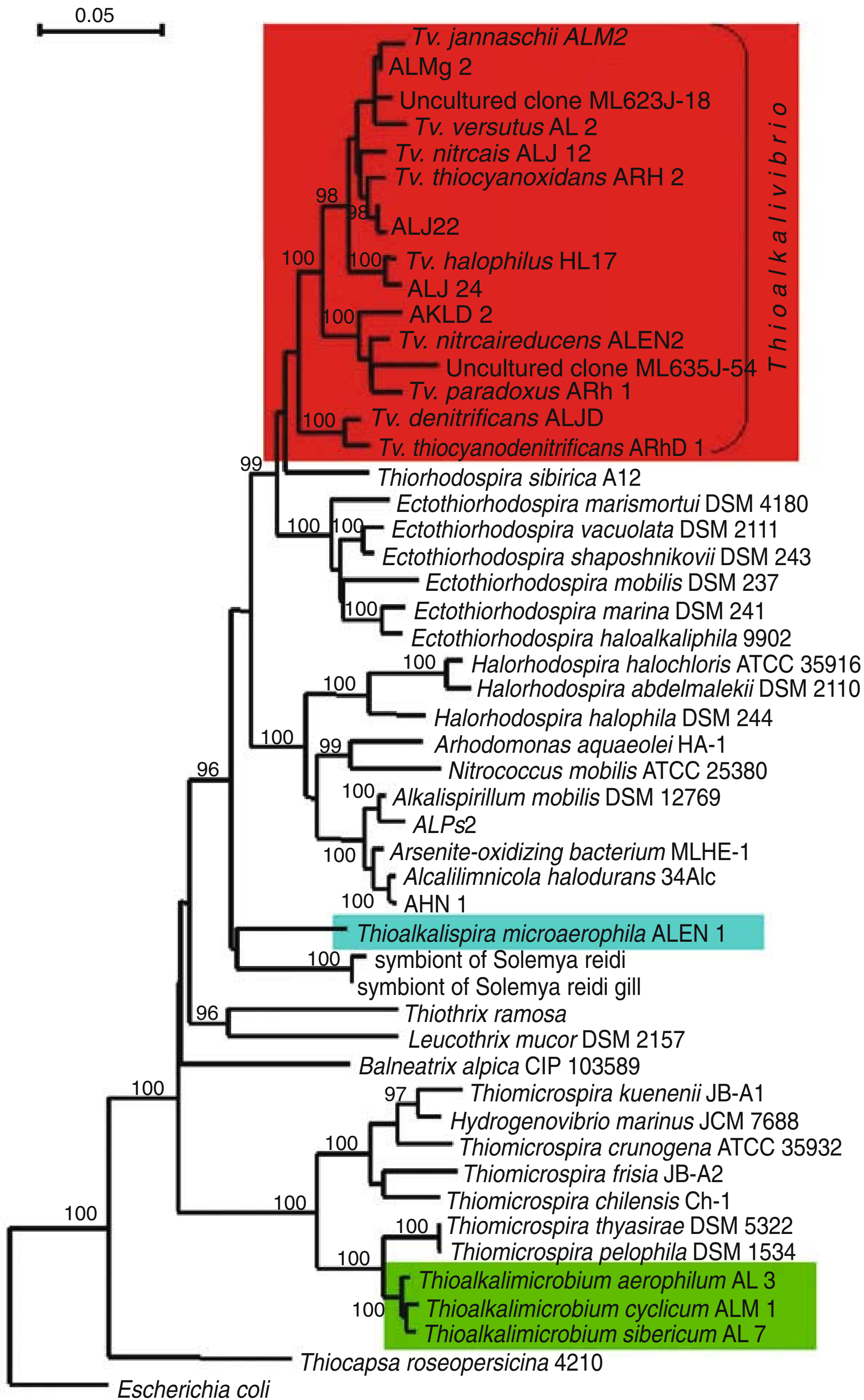


Fig. 2. Phylogenetic tree demonstrating position of the three new genera of haloalkaliphilic sulfur oxidizing bacteria isolated from the soda lakes. Numbers on the branches indicates bootstrap values (only the highest values are included). Unaffiliated strains among the genus *Thioalkalivibrio*: extremely salt tolerant strains from Mongolia (ALMg 2) and Kenya (ALJ 15, ALJ 22, and ALJ 24); AKLD 2 is a facultatively anaerobic nitrate-reducing strain from Kulunda. Bar, 5% sequence divergence.

Table 3. Results of enrichment and isolation of two different types of haloalkaliphilic SOB from soda lakes (pH 10).

	Low-salt medium (0.6M Na^+)			High-salt medium (2–4M Na^+)		
Location	MPN	Tm	Tv	MPN	Tm	Tv
Lake Hadyn (Tuva)	nd	1	1	nd	nd	nd
Kunkur Steppe (Siberia)	10^6	14	4	nd	nd	nd
Northeast Mongolia	10^6	20	0	10^5	0	20
Lake Borzinskoe (Siberia)	10^6	0	2	10^7	0	1
Kulunda Steppe (Siberia)	10^8	3	6	10^8	0	7
Kenya (Rift Valley)	10^6	3	20	10^6	0	5
Egypt (Wadi Natrun)	10^6	4	5	10^6	0	23
Mono Lake (California)	nd	1	0	nd	0	1

Abbreviations: MPN, maximum cell number/cm^3 of sediment; Tm, number of isolated *Thioalkalimicrobium* strains; Tv, number of isolated *Thioalkalivibrio* strains; and nd, not determined.

Table 4. Properties of haloalkaliphilic SOB from soda lakes.

Property	*Thioalkalivibrio* (43 strains)	*Thioalkalimicrobium* (43 strains)
DNA G + C mol%	61–66	47.3–51.2
Cell morphology	Mostly vibrios or short spirilla with a single polar flagellum; some strains are nonmotile, barrel-shaped or coccoid	From rods to spirilla with 1–3 polar flagella
Intracellular sulfur globules	+/–	–
Carboxysomes	+/–	+
Colony morphology	Compact, often with sulfur, often yellowish	Compact or spreading, pink, without sulfur
pH limits (optimum)	7.5–10.65 (10–10.2)	7.5–10.6 (10)
Upper temperature limit (°C)	50	39
Upper salt limit (M total Na^+)	4.3	1.5
Max.specific growth rate (pH 10)	$0.20 h^{-1}$	$0.33 h^{-1}$
Max.growth yield (g of protein/ mol of thiosulfate)	6.5	3.5
Survival during starvation	Long	Short
Rates of thiosulfate and sulfide oxidation	Low-moderate	Extremely high
Rates of sulfur oxidation	Moderate	Very low
Sulfur intermediates	Polysulfide, sulfur	Sulfite
Denitrification	+/–	–
Growth with thiocyanate	+/–	–
RuBisCo activity	+, type I	+, type I
Sulfite-dehydrogenase	+	–
Dominating cytochromes	*c* and *b*	*c*
Cytochrome oxidases	o, cbb_3, aa_3	cbb_3
N-sources for growth		
NH_3	+	+
NO_2^-, NO_3^-	+/–	+
SCN^-	+/–	–
Dominant ubiquinone	Q-8	Q-8
Compatible solutes[a]	Glycine betaine	Ectoine
Dominant fatty acids in membrane lipids[b]	C16:0, C18:1, and C19-cyclopropyl	C16:1, C18:1, and C16:0
Membrane-bound yellow pigment	+/–	–

Symbols and abbreviation: +, present; – absent; +/–, present in some strains; and RuBisCo, ribulose-1,5-bisphosphate carboxylase oxygenase.

[a]Data of E. Galinski.

[b]Data of J. Sinninghe Damste.

Table 5. Respiratory activity in haloalkaliphilic SOB grown with thiosulfate or thiocyanate at pH 10.

	Thioalkalimicrobium			Thioalkalivibrio		
Substrate	V	pH opt	N	V	pH opt	N
Thiosulfate ($S_2O_3^{-2}$)	2.5–5.2	9–10	40	0.15–1.1	9–10	60
Sulfide (HS^-)	2.3–5.2	9–10	40	0.15–1.5	9–10	60
Polysulfide (S_8^{-2})	1.1–3.0	10	38	0.2–0.9	10	55
Elemental sulfur (S_8)	0–0.2	10	40	0.08–0.6	10–10.5	60
Sulfite (SO_3^{-2})	0	n.d.	28	0–0.2	10	40
Trithionate ($S_3O_6^{-2}$)	0	n.d.	9	0–0.2	9	20
Tetrathionate ($S_4O_6^{-2}$)	0–1.1	9	40	0.05–0.5	9	60
Pentathionate ($S_5O_6^{-2}$)	0	n.d.	9	0.1–0.8	9	20
Thiocyanate (SCN^-)	No growth and respiration			0.09–0.4	10	9
CS_2 (carbon disulfide)				0.09	10	1

Abbreviations: V, respiration rate, μmol of O_2 (mg of protein min)$^{-1}$; N, number of tested strains; and n.d., not determined.

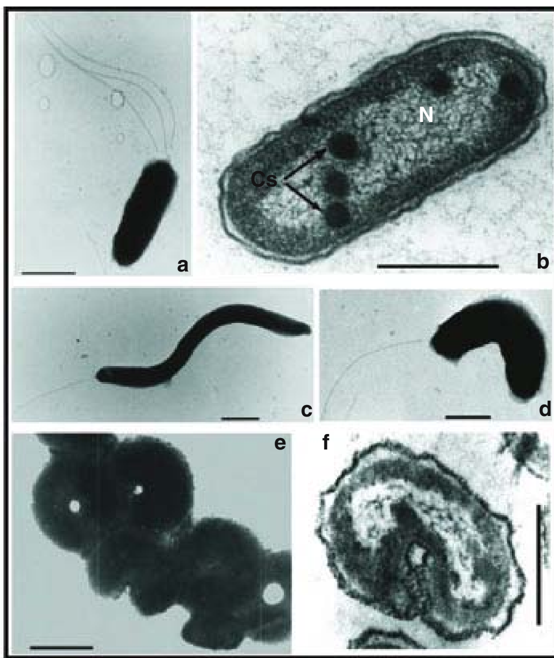


Fig. 3. Cell morphology of the genus *Thioalkalimicrobium*. (a, c–e) — total electron microphotographs; (b, f) — thin sections; (a–b) — *Tm.aerophilum* AL 3; (c) — str.ALJ 14 (Kenya); (d) — *Tm.sibiricum* AL 7; (e–f) — *Tm.cyclicum* ALM 2; Cs-carboxysomes; N-nucleoide. Bars: 0.5 µm.

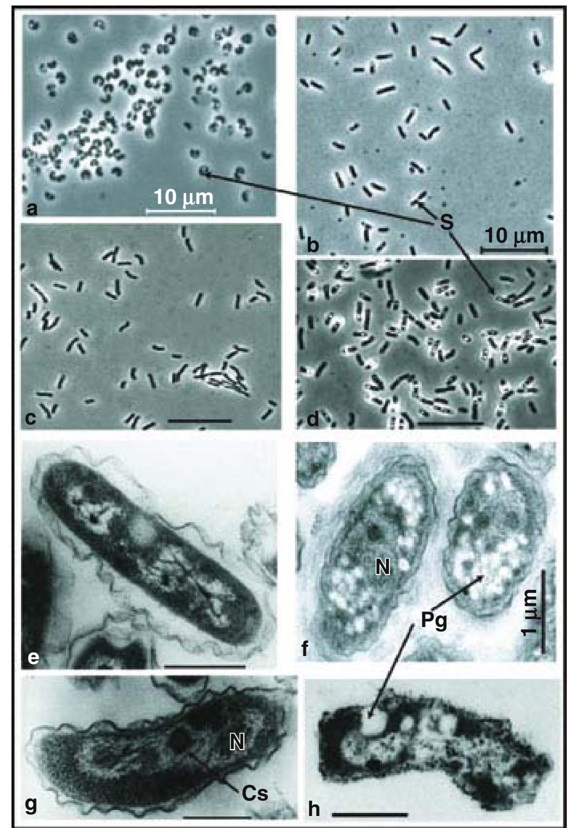


Fig. 4. Cell morphology of the genus *Thioalkalivibrio*. (a–d) — phase contrast of extremely salt tolerant isolates from Kulunda, Kenya and Egypt. (e–h) — thin sections of str.AL2, ALJ 15, ALJ 3, ALE 11. Bar (e,g,h) = 0.5 µm; Cs-carboxysome, N-nucleoide, Pg-polyglucose, S-intracellular sulfur.

essential for the functioning of these bacteria at extremely high salt and high pH conditions. The typical cell morphology of these bacteria is shown in Figs. 3 and 4.

Soda lake sulfur oxidizers differ from all other known sulfur bacteria because of their ability to grow and oxidize sulfur compounds at pH >9. The sodium carbonate-bicarbonate buffer appears to be the most appropriate mineral environment for such bacteria, providing both stable alkalinity and a source of carbon. The buffering capacity of the carbonate system is maximal at pH 9.5–10.2. This pH range was suitable for batch cultivation. However, to explore a realistic pattern of the pH-dependence of growth, continuous cultivation under pH-controlled conditions was necessary (Sorokin et al., 2003b). This investigation confirmed the obligately alkaliphilic

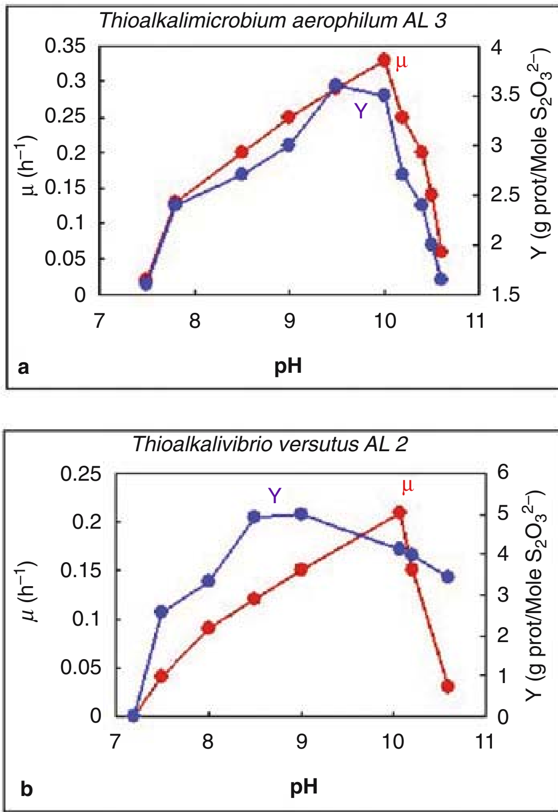


Fig. 5. pH profiles for growth rate (μ) and growth yield (Y) of alkaliphilic sulfur oxidizing bacteria measured in pH-controlled thiosulfate-limited continuous culture (0.6 M total Na^+).

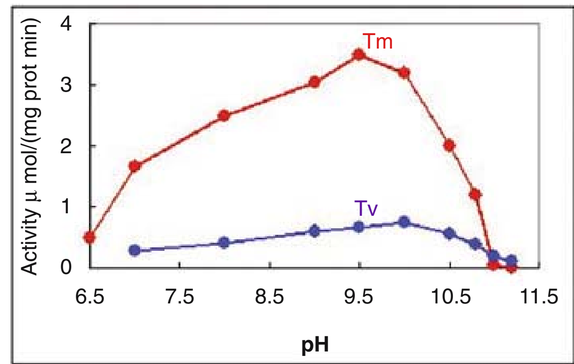


Fig. 6. pH profile of the activity of thiosulfate-dependent respiration for *Thioalkalimicrobium* (Tm) and *Thioalkalivibrio* (Tv).

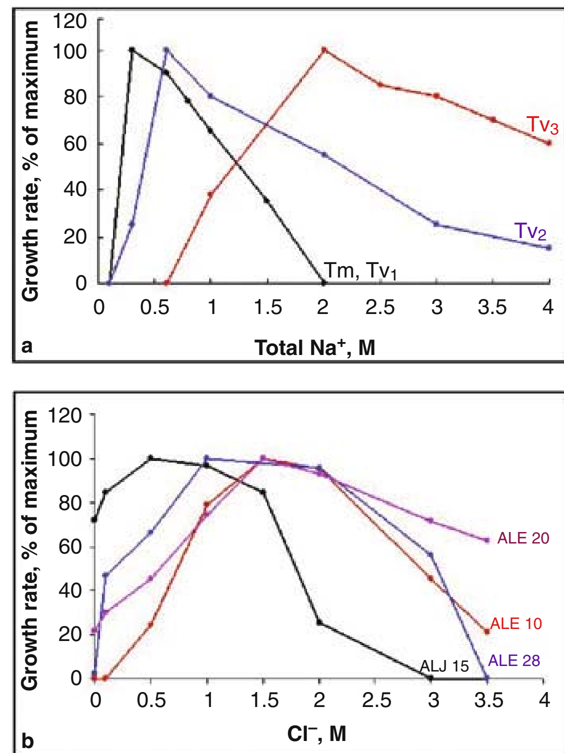


Fig. 7. Influence of sodium carbonate concentration (a) and Cl^- (b) on growth of different subgroups of extremely salt-tolerant alkaliphilic sulfur oxidizing bacteria at pH 10. The background Cl^- concentration in (a) was 0.1 M, the background carbonate concentration in (b) was 0.5 M of total Na^+. Tm, *Thioalkalimicrobium*; Tv_1, low-salt tolerant *Thioalkalivibrio*; Tv_2, extremely natronotolerant *Thioalkalivibrio*; Tv3, extremely natrono*philic Thioalkalivibrio*; ALJ 15, Kenyan natronophilic *Thioalkalivibrio* strain; and ALE 10, ALE 20 and ALE 28, haloalkaliphilic *Thioalkalivibrio* isolates from Wadi Natrun in Egypt.

nature of the representative strains of *Thioalkalimicrobium* and *Thioalkalivibrio* and, for the first time, demonstrated that chemolithoautotrophic bacteria are capable of stable growth at pH >10 (Fig. 5). Both the growth rate and the growth yield of the soda lake isolates were maximum at pH values around 10. The maximum pH for growth registered in chemostat cultures was 10.6. On the other hand, the pH for maximum respiratory activity was at least 11.0 (Fig. 6). The failure to grow at pH >10.6 might be explained by an anabolic constraint, most probably the unavailability of carbon in the form of CO_3^{-2}, as has been suggested previously for alkaliphilic cyanobacteria (Kaplan et al., 1982).

Another important environmental factor in the selection of a particular type of SOB is the total salt content. Although all strains isolated from the soda lakes belonged to the haloalkaliphiles, three different subgroups can be identified on the basis of their salt tolerance and requirement (Fig. 7a). 1) All *Thioalkalimicrobium* and some of the *Thioalkalivibrio* isolates

belong to a moderately salt tolerant type, being able to grow in up to 1.2–1.5 M total Na^+. They originated mostly from the hyposaline lakes and were isolated on medium containing a low salt concentration. 2) The biggest group of the *Thioalkalivibrio* isolates was extremely salt-tolerant and able to grow in saturated soda brines (4–4.5 M Na^+). However, most of them grew optimally at moderate salt concentrations (0.5–1 M Na^+). 3) The third type consisting of only a few isolates was the true extreme halophiles that cannot grow at salt concentrations below 1 M Na^+. All extremely salt tolerant *Thioalkalivibrio* strains were isolated from hypersaline soda lakes, mostly in Mongolia (Sorokin et al., 2004a) and Egypt (our unpublished results). The extreme halophiles were also the most thermotolerant, some being able to grow up to 50°C. Continuous culture experiments with one of these isolates, *Thioalkalivibrio versutus* ALJ 15, demonstrated its excellent adaptation to doubly extreme conditions (Banciu et al., 2004b). Its growth rate and growth yield in soda brine at pH 10 and 4 M Na^+ were only 3 and 2 times lower, respectively, than found at 0.6 M Na^+. Not only the total sodium concentration was important for optimal growth and activity, but also the anionic composition of the sodium salts. In particular, the ratio of carbonates to Cl^- was critical. Most of the extremely salt tolerant *Thioalkalivibrio* (isolates from Kenyan, Mongolian and Kulunda soda lakes) were able to grow in pure soda brines without Cl^-, but for maximum growth they required 0.1–0.5 M Cl^-. Higher concentrations of Cl^- inhibited growth, resulting in complete inhibition at >2 M Cl^-. In contrast, the strains isolated from the NaCl-dominated Wadi Natrun lakes, had an obligate requirement for 0.5 M Cl^-, grew optimally at 1–2 M Cl^- and still grew at 3–3.5 M NaCl in the presence of only 0.5 M Na^+ and carbonate to maintain an alkaline pH and provide the carbon source (Fig. 7b). The latter strains can be regarded as true haloalkaliphiles, while the dominant subgroup of the extremely salt-tolerant *Thioalkalivibrio* strains does not fit this term. We suggest calling such bacteria "natronophiles"—the soda-loving bacteria.

Under low-salt conditions, both *Thioalkalimicrobium* and *Thioalkalivibrio* representatives developed in some of the enrichment cultures. Competition experiments in thiosulfate-limited continuous culture at low salt and high pH conditions demonstrated that *Thioalkalivibrio* has a competitive advantage over *Thioalkalimicrobium* at extremely low dilution rates ($<0.02\ h^{-1}$), which in fact is close to starvation. This was confirmed in a direct starvation experiment, which proved that *Thioalkalivibrio* survives much better than *Thioalkalimicrobium* (Sorokin et al., 2003b). Furthermore, it was found that starvation induced the formation of thermotolerant cyst-like resting cells in both groups, but with different efficiency. *Thioalkalivibrio* formed several orders of magnitude more cyst-like cells as compared to *Thioalkalimicrobium* , but the cysts of the latter were of "higher quality," e.g., more resistant to stress conditions (Loiko et al., 2003).

Overall, the data suggest that the two groups of sulfur-oxidizing bacteria dominating in soda lakes exhibit different ecological strategies. The *Thioalkalimicrobium* group fits to the *r*-strategy—fast but inefficient growth, and advantage probably taken of short periods of substrate excess and wet weather. The *Thioalkalivibrio* group fits more the *K*-strategy—slow but efficient growth, relatively larger metabolic diversity, and broader adaptation to the extreme environment of saline soda lakes. Such organisms are usually more persistent in the environment. Recently, evidence from a culture-independent molecular ecology approach, including denaturing gradient gel electrophoresis and cloning, confirmed the ubiquitous presence of the *Thioalkalivibrio* group in various soda lakes (Baumgarte, 2003; Humayoun et al., 2003; Rees et al., 2004; G. Muyzer, unpublished results).

Sulfur Oxidation Mechanisms in Haloalkaliphilic Bacteria

Significant difference in the rates of oxidation of various sulfur compounds (Table 5) and production of different intermediates during thiosulfate oxidation suggest different pathways of sulfur metabolism in *Thioalkalimicrobium* and *Thioalkalivibrio*. The former never produced elemental sulfur during oxidation of thiosulfate, unless severe oxygen limitation was applied, while most of the *Thioalkalivibrio* strains formed extracellular or intracellular sulfur from thiosulfate during growth in liquid and solid culture media. Moreover, *Thioalkalimicrobium* was virtually unable to oxidize external elemental sulfur in contrast to *Thioalkalivibrio*, which converted it to sulfate. Another important difference between these two groups is sulfite metabolism. Sulfite is considered a key intermediate in sulfur oxidation pathways of many sulfur bacteria (Kappler and Dahl, 2001). The *Thioalkalimicrobium* strains released up to 3 mM sulfite into the medium during batch growth with thiosulfate (Sorokin et al., 2000), but it was never detected in the cultures of *Thioalkalivibrio*. Furthermore, neither of the *Thioalkalimicrobium* strains studied was able to oxidize external sulfite even at micromo-

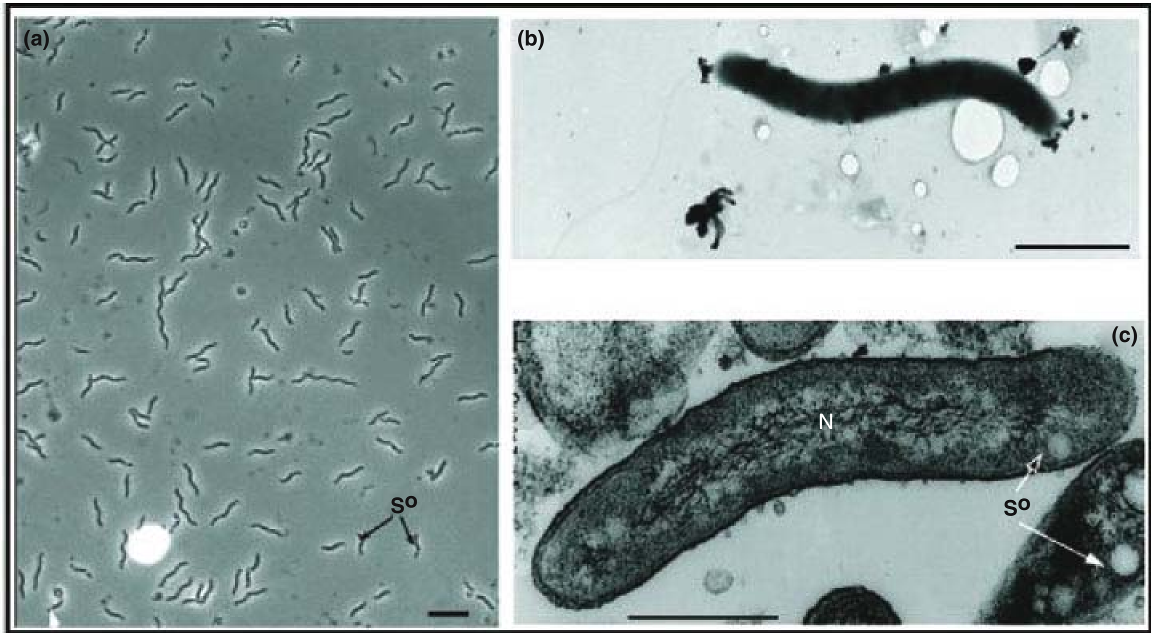


Fig. 8. Cell morphology of *Thioalkalispira microaerophila*. (a) – phase contrast, bar = 10 µm; (b) – total electron microphotograph, bar = 1 µm; (c) — thin section, bar = 0.5 µm; So — sulfur globules; N-nucleoide.

lar concentrations, while some of the *Thioalkalivibrio* isolates did. Sulfite oxidation correlated with the presence of sulfite dehydrogenase activity (AMP-independent type), which was uniformly detected in *Thioalkalivibrio* but not *Thioalkalimicrobium* (Sorokin et al., 2001c). Among the other activities, sulfide dehydrogenase (cytochrome *c*-dependent), SQR (sulfide-quinone reductase), and thiosulfate reductase activities have been detected in both groups. Cytochrome *c*-dependent sulfide dehydrogenase was purified from *Thioalkalimicrobium aerophilum* AL 3 (Sorokin et al., 1998). In contrast to the type of flavocytochrome *c*-containing enzymes found in chemotrophic sulfur bacteria (Visser et al., 1997), the enzyme from the alkaliphiles only contained cytochrome *c* as a cofactor. The presence of a second, quinone-dependent, sulfide dehydrogenase in alkaliphiles implies that more than one mechanism of sulfide oxidation in sulfur bacteria might exist in a single organism. Overall, the data so far suggest that in *Thioalkalimicrobium* the oxidation pathway of reduced sulfur is probably similar to the one proposed for facultatively autotrophic *Paracoccus* species, i.e., complete oxidation of the sulfane atom by a multienzyme complex without releasing free intermediates (Friedrich et al., 2001). In contrast, *Thioalkalivibrio* seems to employ different mechanism with formation and consumption of free sulfur intermediates, such as elemental sulfur and sulfite, more common for neutrophilic sulfur-oxidizing bacteria (Kelly et al., 1997).

Specific Physiological Subgroups of the Soda Lake Sulfur-Oxidizing Bacteria

Microaerophiles

Apart from the numerous aerobic alkaliphilic SOB discussed above, several different strains have been obtained from the soda lakes using specific enrichment conditions. Micro-oxic conditions with thiosulfate as substrate and a low salt content favored the enrichment and isolation of an obligately microaerophilic spirillum (Fig. 8). This has been described as a new genus and species *Thioalkalispira microaerophila* (Sorokin et al., 2002b)—another haloalkaliphilic gammaproteobacterium (Fig. 2). A micro-oxic enrichment with sulfide as substrate, using a gradient culture technique (Nelson et al., 1986) resulted in the isolation of five obligately microaerophilic *Thioalkalivibrio* strains, most of which had a relatively low DNA homology (<40%) with the aerobic strains. The common property of these species is the formation of multiple intracellular sulfur globules during growth with sulfide or thiosulfate.

Denitrification

Anoxic enrichments from the soda lake sediments, with thiosulfate or sulfide as the electron donor and nitrate as the electron acceptor, resulted in the domination of partial denitrifiers,

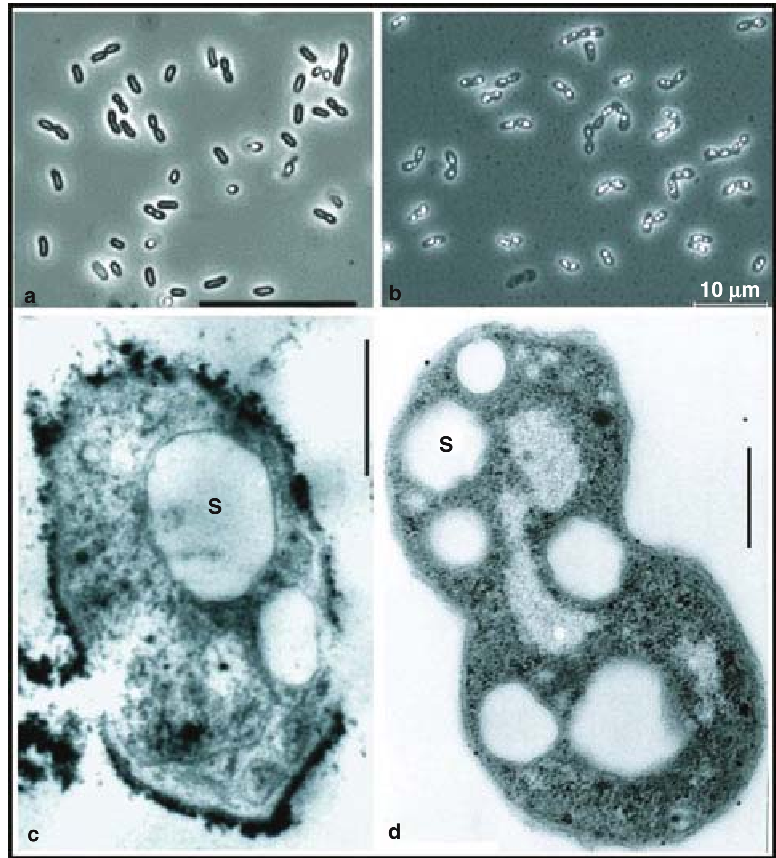


Fig. 9. Cell morphology of coccoid Thioalkalivibrio. (a,c) — *Tv. paradoxus*, (b,d) — *Tv. nitratireducens*; (a–b) — phase contrast, (c–d) — thin sections; S — intracellular sulfur globules; bars: a = 20 µm, b = 10 µm; c–d = 0.5 µm.

mostly reducing nitrate only to nitrite with copious sulfur formation. Only one enrichment culture from the hypersaline Lake Fazda in Wadi Natrun (Egypt) resulted in complete denitrification of nitrate, although with intermediate nitrite production (Sorokin et al., 2003a). Purification of this culture resulted in the selection of a stable coculture of two facultatively anaerobic alkaliphilic sulfur oxidizers. There was a numerically minor population of large nonmotile coccoid cells with intracellular sulfur globules. In pure culture, this bacterium reduced nitrate only to nitrite despite having a very active nitrite reductase (Antipov et al., 2003). Despite its obvious phenotypic difference from the known alkaliphilic SOB species (Fig. 9), the 16S rDNA sequenced-based phylogenetic analysis placed this unusual bacterium into the genus *Thioalkalivibrio*, and the strain ALEN 2 was described as a new species *Tv. nitratireducens* (Sorokin et al., 2003c). The second (numerically dominant) organism, strain ALED, was a thin motile rod, which reduced nitrite to dinitrogen gas, and could grow anaerobically with thiosulfate and nitrite or nitrous oxide (N_2O) but not nitrate. A similar organism, *Thioalkalivibrio denitrificans* ALJD, was isolated previously from a Kenyan soda lake using N_2O as electron acceptor (Sorokin et al., 2001a). One of the essential properties of this bacterium was its preference for N_2O as an electron acceptor. In pH controlled continuous culture, *Tv. denitrificans* was able to grow within the pH range 7.5–10.5 with an optimum at pH 9.0. Growth with N_2O as electron acceptor was more stable and faster than with O_2 at pH >10. Moreover, in the chemostat it grew well with N_2O and polysulfide—a form of autotrophic denitrification unique to alkaliphiles, since polysulfide is only chemically stable at highly alkaline pH.

Thiocyanate Oxidation

Thiocyanate ($N{\equiv}C\text{-}S^-$) represents a one-carbon reduced sulfur compound, which is produced both naturally and as an industrial waste product from cyanide. Thiocyanate is not an easy substrate to metabolize for bacteria. Only a few neutrophilic, autotrophic sulfur-oxidizing species can use it as a source of energy after first breaking it down to sulfide, ammonia and CO_2. Aerobic enrichment culturesin medium containing thiocyanate at pH 10 from various soda lake sediments resulted in the isolation of nine strains of haloalkaliphilic, obligately autotrophic SOB capable of growth on thiocyanate as energy and

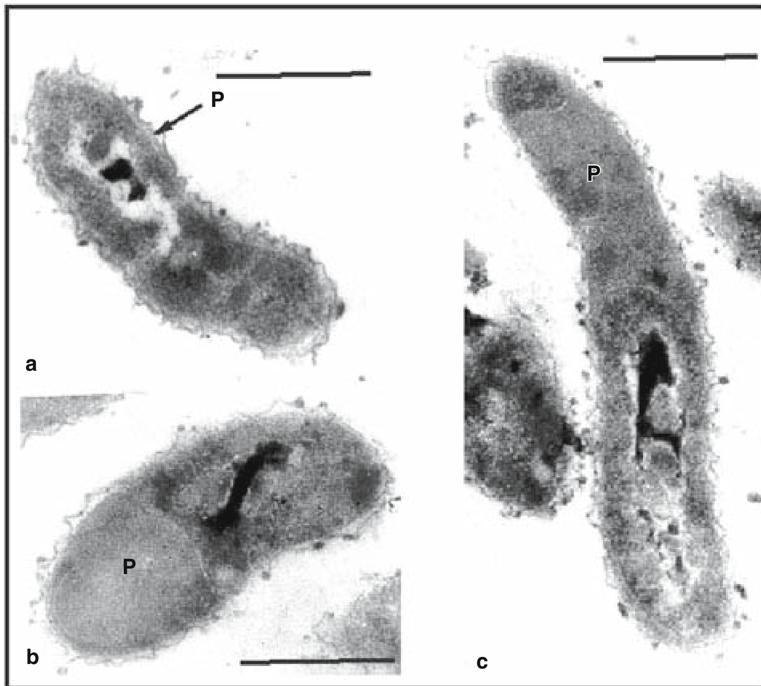
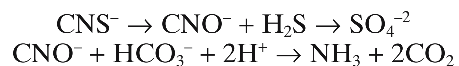


Fig. 10. Ultrastructure of the cells of *Thioalkalivibrio thiocyanoxidans* ARh 2 grown with thiosulfate (a) or thiocyanate (b, c) as electron donor at pH 10. P, periplasm. Bar = 0.5 μm.

nitrogen source (Sorokin et al., 2001b). The isolates fell into two distinct groups. The four motile, vibrio-shaped strains were genetically closely related to the previously described sulfur-oxidizing alkaliphiles from the genus *Thioalkalivibrio*. They were described as a new species *Tv. thiocyanoxidans* (Sorokin et al., 2002c; Fig. 2). These strains had unusually extended periplasmic compartments when grown with thiocyanate but not thiosulfate as energy source (Fig. 10). The other five isolates had nonmotile barrel-shaped cells, accumulating large amounts of intracellular sulfur, and were able to oxidize carbon disulfide (Fig. 9). Despite their obvious phenotypic difference, these strains were also *Thioalkalivibrio* on the basis of 16S-rDNA sequence-based phylogenetic analysis and were described as a new species *Tv. paradoxus* (Sorokin et al., 2002c) clustering with another coccoid nonmotile member of the *Thioalkalivibrio*—*Tv. nitratireducens* (Fig. 2). Successful enrichments with thiocyanate as substrate at pH 10 were also obtained from the soda lake sediments under denitrifying conditions with nitrate as electron acceptor—a mode of chemolithoautotrophic metabolism suggested previously by De Kruyff et al. (1957) for the neutrophilic *Thiobacillus denitrificans* but never confirmed. Two closely related haloalkaliphilic SOB strains, ARhD 1 and ARhD 2, isolated from these enrichments were identified as members of the genus *Thioalkalivibrio* with closest relationship to *Tv. denitrificans*. They are described as a new species *Tv. thiocyanodenitrificans* (Sorokin et al., 2004b; Fig. 2). They were able to grow anoxically on thiocyanate and thiosulfate with nitrate or nitrite as the electron acceptor, representing, therefore, the first known complete denitrifiers among the sulfur-oxidizing haloalkaliphiles (D. Sorokin, unpublished results). All thiocyanate-utilizing soda lake isolates accumulated cyanate (($N{=}C{=}O^-$) as an intermediate of thiocyanate degradation. This was the first direct confirmation of the involvement of the "cyanate pathway" in the primary thiocyanate degradation in pure bacterial cultures:



$$CNS^- \rightarrow CNO^- + H_2S \rightarrow SO_4^{-2}$$
$$CNO^- + HCO_3^- + 2H^+ \rightarrow NH_3 + 2CO_2$$

However, in contrast to the mechanism of primary anaerobic hydrolysis of thiocyanate, resulting in the formation of cyanate and sulfide, as suggested previously (Youatt, 1954), the alkaliphiles appear to employ a different mechanism: direct oxidation of the sulfane atom of thiocyanate, producing cyanate and elemental sulfur, has been observed in cell-free preparations. The enzyme responsible for this action was produced in large amounts by thiocyanate-grown alkaliphilic strains and has recently been purified from the *Tv. thiocyanoxidans* ARh 4. A soluble monomer with an approximate molecular mass of 60 kDa, the enzyme oxidizes the sulfane atom of thiocyanate in the presence of cytochrome *c* as an electron acceptor (D. Sorokin, unpublished result).

Table 6. Species of the soda lake SOB.

Species and type strains	G+C mol%	pH range (optimum)	Salt range, M Na^+	Cell morphology	Motility	Growth with SCN	Nitrate reduction to nitrite	Denitrification	Isolated from	Collection numbers	Accession number
Thioalkalimicrobium	47.3–51.2	7.5–10.6 (10)	0.3–1.5	Rods, vibrios, and short spirilla	+	–	–	–			
aerophilum AL 3	49.5								Siberia	CBS 100465 DSM 13739	AF126548
sibiricum AL 7	48.9								Siberia	NCCB 100000 DSM 13740	AF126549
cyclicum ALM 1	49.6		0.3–1.8	Open rings	–				Mono Lake	DSM 14477 JCM11371	AF329082
Thioalkalivibrio	61–66	6.5–10.6 (9–10)	0.3–4.3	Rods-spirilla	+/–	+/–	+/–	+/–			
versutus AL 2	n.d.	8–10.6 (10)	0.3–1.8	Rods	+	–	–	–	Tuva	CBS 100464 DSM 13738	AF126546
nitratis ALJ 12	62.1		0.3–1.8	Vibrios		–	+, no growth	–	Kenya	NCCB 100002 DSM 13741	AF126547
jannaschii ALM 2	63.7		0.3–4.3	Vibrios		–	–	–	Mono Lake	DSM 14478 JCM 11372	AF329083
thiocyanoridans ARh 2	n.d.		0.3–4.3	Vibrios		+	–	–	Kenya	DSM 13532 JCM 11368	AF302081
paradoxus ARh 1	n.d.		0.3–1.5	Barrel-shaped	–	+	–	–	Kenya	DSM 13531 JCM 11367	AF151432
nitratireducens ALEN 2	64.8		0.3–1.5	Coccoid		–	+, growth	–	Egypt	DSM 14787 UNIQEM 213	AY079010
denitrificans ALJD	62.9		0.3–1.8	Rods	+	–	–	+	Kenya	NCCB 100001 DSM 13742	AF126545
thiocyanodenitrificans ARhD 1	63.1		0.3–1.8	Rods		+	+, growth	+	Egypt	UNIQEM 226	AY360060
halophilus HL. 17	65.1	6.5–9.8 (8–9)	0.3–4.0	Rods		–	–	–	Altai	DSM 15791 UNIQEM 225	AY346464
Thioalkalispira microaerophila ALEN 1	58.9	8–10.5 (10)	0.3–1.5	Spirilla	+	–	+, no growth	– microaerophile	Egypt	DSM 14786 UNIQEM 212	AF481118

Symbols: +, present; and –, absent; and +/–, present in some strains. Abbreviations: SOB, sulfur-oxidizing bacteria; n.d., not determined; CBS, Centraalbureau voor Schimmelcultures, Utrecht. The Netherlands; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig. Germeny; NCCB, The Netherlands Culture Collection of Bacteria formerly LMD and Phabagen c/o Utrecht University; JCM, Japan Collection Of Microoganisms, The Institute of Physical and Chemical Research. Hirosawa, Wako-shi, Japan; and UNIQUEM, Unique and Extremophilic Microorganisms (Collection of Unique Microbial Cultures, Russian Academy of Sciences), Moscow, Russia.

Facultatively Alkaliphilic Sulfur-Oxidizing Bacteria

The numerous cultures enriched from the soda lakes using highly buffered sodium carbonate-based mineral medium invariably yielded obligately alkaliphilic isolates. On the other hand, nothing from the neutral salt lakes grew on such media. When NaCl-based medium was used with 0.1 M $NaHCO_3$ as a buffer and carbon source at pH 8.5, a halophilic strain was obtained from Stamp Lake (Table 1). It was able to grow both in neutral NaCl brines and at pH values above 9 in the presence of high sodium carbonate concentrations. Stamp Lake represents an intermediate lake type between soda and salt lakes, where NaCl dominates, but a small fraction of sodium carbonate provides the elevated pH. Strain HL 17 was identified as a member of the genus *Thioalkalivibrio* and will be described as a new species, *Tv. halophilus* (Fig. 2). It grew within the pH range 6.5–9.8, with a broad optimum range of 8.0–9.0. Being able to directly compare the biomass composition of this bacterium growing in NaCl- and sodium carbonate-based medium, it was possible to demonstrate that the specific content of its main compatible solute, glycine betaine, was twice as high in the NaCl-growing cells (Banciu et al., 2004a). This implies that the osmotic properties of the NaCl and sodium carbonate brines are substantially different. Direct measurements confirmed that the osmotic pressure of NaCl solutions was three times higher than that of the sodium carbonate media, which gives a certain energetic advantage to the natronophiles—the organisms preferring to live in the sodium carbonate brines.

Taxonomy of Haloalkaliphilic Sulfur-Oxidizing Bacteria

So far, the genus *Thioalkalivibrio* includes seven validly described species, and the genus *Thioalkalimicrobium* contains three species. Two more species in the genus *Thioalkalivibrio* are currently being described. The third genus, *Thioalkalispira*, contains a single species (Fig. 2). The most important properties of the type strains of these three genera are compared in Table 6. A large number of the isolates, especially those belonging to the high-salt tolerant *Thioalkalivibrio* subgroup from the Mongolian, Kulunda and Egyptian soda lakes, remain as yet undescribed. This is a common problem encountered when working with a wide range of genetically homologous strains. The more strains are isolated, the more it becomes clear that a bacterial group is represented by a continuous spectrum of organisms rather than a discrete number of distinct species. Despite their definite genetic difference (DNA-DNA hybridization), it is very difficult at this moment to find proper phenotypic descriptors sufficient to create an adequate taxonomy of all the soda lake isolates. As a rule, for a given geographic location, most of the isolates grouped together, with a DNA-DNA homology level typical for the single-species level (>60%). On the other hand,DNA homology between various "geographic species" was usually low (15–50%) despite their very similar phenotypes. At this moment, several molecular fingerprinting techniques are being employed to solve this problem.

Molecular techniques are now being used to improve detection of haloalkaliphilic sulfur bacteria in mixed populations in soda lake sediments and sulfide-removing bioreactors. So far, successful oligonucleotide probes have been designed forfluorescence in situ hybridization and polymerase chain reaction detection of the representatives of the *Thioalkalivibrio* (G. Muyzer and D. Sorokin, unpublished results).

Acknowledgments. We would like to thank our colleagues B.E. Jones and G.A. Zavarzin for making possible our work with their samples from the Kenyan soda lakes. We are also grateful to T.P. Tourova and A.M. Lysenko for help in genetic work, to M. Mityushina and K. Sjollema for electron microscopy and to E. Galinski and J. Sinninghe Damste for analyses of compatible solutes and fatty acids.

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Prokaryotes (2006) 2:985–1011
DOI: 10.1007/0-387-30742-7_31

CHAPTER 1.31

The Colorless Sulfur Bacteria

LESLEY A. ROBERTSON AND J. GIJS KUENEN

The name "the colorless sulfur bacteria" has been used since the time of Winogradsky to designate prokaryotes that are either able, or believed to be able, to use reduced sulfur compounds (e.g., sulfide, sulfur and organic sulfides) as sources of energy for growth. Today, it is known that this group comprises a very heterogeneous collection of bacteria, many of which have little or no taxonomic relationship to each other. The colorless sulfur bacteria play an essential role in the oxidative side of the sulfur cycle (Fig. 1). Like all of the element cycles, the sulfur cycle has an oxidative and a reductive side, which, in most ecosystems, are in balance. However, this balance does not always exist, and accumulations of intermediates such as sulfur, iron sulfides, and hydrogen sulfide are often found. On the reductive side, sulfate (and sometimes elemental sulfur) functions as an electron acceptor in the metabolic pathways used by a wide range of anaerobic bacteria, leading to the production of sulfide. Conversely, on the oxidative side of the cycle, reduced sulfur compounds serve as electron donors for anaerobic, phototrophic bacteria or provide growth energy for the extremely diverse group of (generally) respiratory colorless sulfur bacteria. Common oxidation products of sulfide are elemental sulfur and sulfate (Fig. 1). The adjective "colorless" is used because of the lack of photopigments in these bacteria, although it should be noted that colonies and dense cultures can actually be pink or brown because of their high cytochrome content. This chapter will concentrate on the colorless sulfur bacteria, while the sulfate reducers and phototrophs will be discussed in 13 and 24.

There is a wide range of different types of colorless sulfur bacteria with very diverse morphological, physiological and ecological properties and with equally diverse environmental requirements. Table 1 lists the genera that have traditionally been regarded as colorless sulfur bacteria (part A), as well as genera containing species originally not classified as such that have now been shown to be able to obtain energy from the oxidation of reduced sulphur compounds (part B). As will be discussed later, the apparent similarity of the metabolic pathways for sulfur oxidation disguises a high level of variation in these pathways indicating that the diversity among the colorless sulfur bacteria is probably due to convergent rather than divergent evolution. In addition to inorganic sulfur compounds, some species can also gain energy from the oxidation of other inorganic compounds such as hydrogen or ferrous iron. As well as differences in substrate range, there is also some variation in electron acceptor usage. Although most colorless sulfur-oxidizing bacteria require oxygen, a few are able to grow anaerobically using nitrogen oxides (e.g., nitrate) as their terminal electron acceptor during denitrification. One or two species (of the genus *Acidianus*) are capable of anaerobic metabolism by the reduction of sulfur (Segerer and Stetter, 1989), during which organic compounds or hydrogen serve as electron donors. *Thiobacillus ferrooxidans* is known to be able to reduce ferric iron under anaerobic conditions (Sugio et al., 1985). A somewhat exotic example of a sulfate reducer that might also be considered to be a colorless sulfur bacterium is *Desulfovibrio sulfodismutans*, which can grow anaerobically by the disproportionation of thiosulfate to sulfate and sulfide (Bak and Pfennig, 1987). Some of the reactions that generate energy from inorganic reduced sulfur compounds using oxygen and nitrate as electron acceptors are shown in Table 2.

In the following sections, we will first discuss the physiology of the colorless sulfur bacteria, since physiology forms the basis of their present taxonomy, and then treat the taxonomy in the following section. This will be followed by a discussion of the habitats of the colorless sulfur bacteria, including artificial habitats, and finally some applications of their use. The chapter concludes with a brief section on the role of the colorless sulfur bacteria in the natural sulfur cycle, together with a description of the tech-

This chapter was taken unchanged from the second edition.

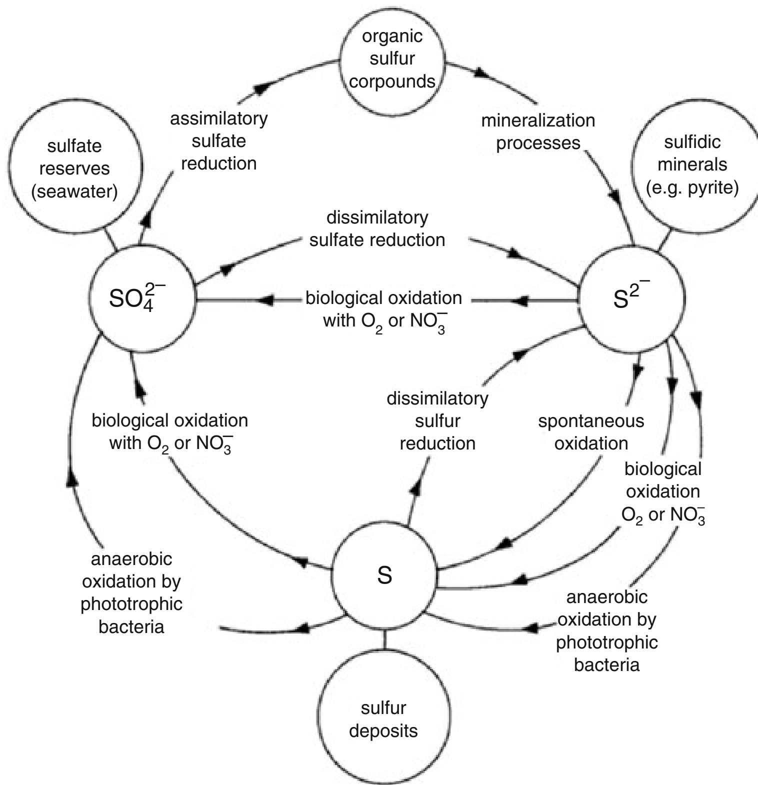


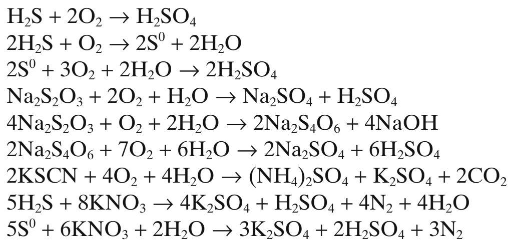
Fig. 1. The sulfur cycle. The colorless sulfur bacteria are involved primarily in those steps in which S^{2-} and S are oxidized with O_2 or NO_3^-. (Adapted from Bos and Kuenen, 1983).

Table 1. Genera of the colorless bacteria traditionally recognized as being capable of growth on reduced sulfur compounds and their environmental parameters.

	pH requirement		Thermal requirement		Anaerobic growth		
Genus	Neutrophilic	Acidophilic	Mesophilic	Thermophilic	Denitrifier	S^0/Fe^{3+} as electron acceptor	Symbiont
A. Traditional colorless sulfur bacteria							
Thiobacillus	+[a]	+	+	+	+	V	+
Thiomicrospira	+	–	+	–	+	–	?[b]
Thiosphaera	+	–	+	–	+	–	–
Sulfolobus	–	+	–	+[χ]	–	–	–
Acidianus	–	+	–	+[χ]	–	+	–
Thermothrix	+	–	–	+	+	–	–
Thiovulum[d]	+	–	+	–	–	–	–
Beggiatoa	+	–	+	–	–	+	–
Thiothrix	+	–	+	–	–	–	–
Thioploca[d]	+	–	+	–	–	–	–
Thiodendron[d]	+	–	+	–	–	–	–
Thiobacterium	+	–	+	–	–	+	–
Macromonas	+	–	+	–	–	+	–
Achromatium[d]	+	–	+	–	–	+	–
Thiospira[d]	+	–	+	–	–	–	–
B. Other bacteria capable of growth on reduced sulfur compounds							
Paracoccus	+	–	+	–	+	–	–
Hyphomicrobium	+	–	+	–	–	–	–
Alcaligenes	+	–	+	–	+	–	–
Pseudomonas	+	–	+	–	+	–	–
Hydrogenobacter	+	–	–	+	–	–	–

+, example known to exist; –, example unknown; V, variable.
16S rRNA analysis indicates a possible relationship.
Hyperthermophilic archaebacterium.
Axenic cultures are not available.

Table 2. Examples of the reactions used by the colorless sulfur bacteria to gain energy for growth.



$H_2S + 2O_2 \rightarrow H_2SO_4$
$2H_2S + O_2 \rightarrow 2S^0 + 2H_2O$
$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$
$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4$
$4Na_2S_2O_3 + O_2 + 2H_2O \rightarrow 2Na_2S_4O_6 + 4NaOH$
$2Na_2S_4O_6 + 7O_2 + 6H_2O \rightarrow 2Na_2SO_4 + 6H_2SO_4$
$2KSCN + 4O_2 + 4H_2O \rightarrow (NH_4)_2SO_4 + K_2SO_4 + 2CO_2$
$5H_2S + 8KNO_3 \rightarrow 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O$
$5S^0 + 6KNO_3 + 2H_2O \rightarrow 3K_2SO_4 + 2H_2SO_4 + 3N_2$

niques available for the measurement of their activities.

Physiology

The great diversity of colorless sulfur bacteria is also reflected in their physiology. This will come as no surprise if we remember that the group encompasses archaebacteria as well as eubacteria, and that the latter group is also very diverse, including common pseudomonads and organisms that might be considered "colorless blue green bacteria," such as species of *Beggiatoa*.

Most of our knowledge of the physiology of these organisms comes from the study of the relatively limited number of bacteria, such as the thiobacilli, that can be grown relatively easily in the laboratory. This is particularly true of our understanding of the biochemistry of sulfur metabolism and, to a lesser extent, of carbon metabolism.

Although the biochemistry of the oxidation of sulfur compounds has received much attention over the last few decades, the pathways involved were not well understood. This was due, in particular, to the fact that the research was formulated around the hypothesis that there would be a single unifying enzymatic pathway for the oxidation of all reduced sulfur compounds. However, it is now clearly established that this is not the case. For example, the facultatively autotrophic *Thiobacillus versutus* and the obligately autotrophic *T. tepidarius* use two entirely different pathways (Fig. 2a and b). It should be noted that not only do the enzymes and electron carriers differ, but their localization in the membranes of the two species appears to be different. This is, of course, important for the mechanism behind the generation of a proton motive force (PMF) in these organisms. Little is known of the electron carriers involved in reverse electron transport for the production of reducing power during autotrophic growth, but all available evidence indicates that the PMF is the driving force for this process. For an extensive review of the state of the art, the reader is referred to Kelly (1988b).

In most obligate and facultative autotrophs, the Calvin cycle serves as the route for carbon dioxide fixation. This is true, for example, for species from the genera *Thiobacillus*, *Thiomicrospira*, *Thiosphaera*, and *Beggiatoa*. Some other species, including those from *Sulfolobus* and *Hydrogenobacter*, possess a carbon dioxide fixation pathway based on a reductive Calvin cycle (Segerer and Stetter, 1989).

Energy and Carbon Sources or Electron Donors

It has been common practice to subdivide the colorless sulfur bacteria in terms of their physiological type as defined mainly by their carbon metabolism. Table 3 defines these physiological types, which will be discussed briefly below. It should be remembered that some genera or species have not been studied in pure culture, and it is not yet known to which of the physiological groups they belong.

Obligate Chemolithotrophs. These highly specialized bacteria require an inorganic source of energy and obtain their cell carbon from the fixation of carbon dioxide. As mentioned above, except in the case of the archaebacteria (which use a reductive carboxylic cycle [König and Stetter, 1989]), the colorless sulfur bacteria do this by means of the Calvin cycle (e.g., Schlegel, 1981). The citric acid cycle in these bacteria seems to be incomplete, and its enzymes probably serve a purely biosynthetic function. Despite their label as "obligate" autotrophs, it has been shown that many of these species actually can use small amounts of exogenous carbon compounds as a supplementary carbon source (Kuenen and Veldkamp, 1973; Matin, 1978), or can even ferment endogenous organic storage compounds such as glycogen (Beudeker et al., 1981; Kuenen and Beudeker, 1982), but these are both secondary metabolic activities, the organisms being primarily dependent on a lithotrophic energy source and carbon dioxide for autotrophic growth. Many *Thiobacillus* species, at least one species each from *Sulfolobus* and *Hydrogenobacter*, and all of the known species of *Thiomicrospira* fall into this group.

Facultative Chemolithotrophs. These bacteria can grow either chemolithoautotrophically with an inorganic energy source and carbon dioxide, or heterotrophically with complex organic compounds providing both carbon and energy, or mixotrophically. Mixotrophy is the simultaneous use of two or more different metabolic pathways for energy and carbon (Gottschal and Kuenen, 1980). In the laboratory, mixotrophic growth is most easily observed

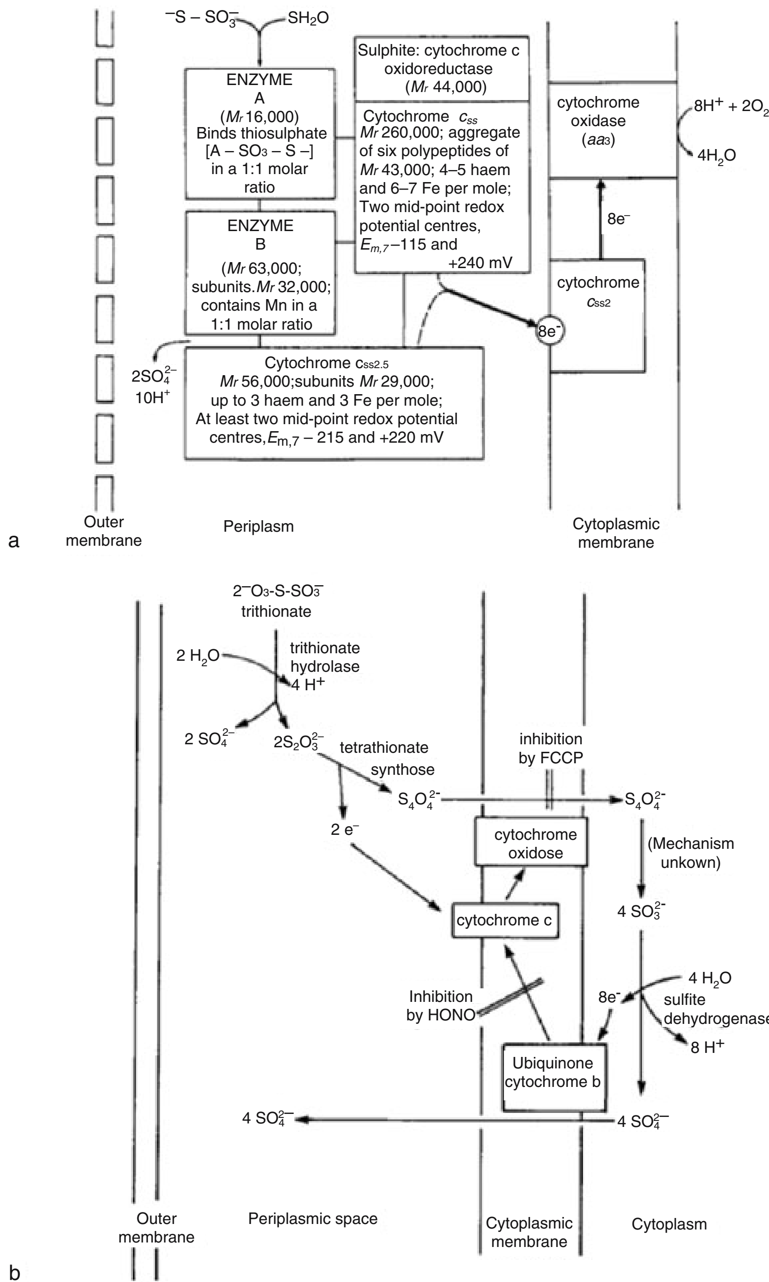


Fig. 2. Pathways of oxidation of reduced sulfur compounds in two different organisms. (a) The periplasmic thiosulfate-oxidizing system of *Thiobacillus versutus* as proposed by Kelly (1988a). The enzyme complex does not produce or metabolize polythionates such as tetrathionate. Thiosulfate is oxidized to sulfate without the formation of sulfur or other intermediates. Thiosulfate metabolism is initiated by its binding to enzyme A. In subsequent steps, sulfate is produced and released, while electrons are finally transferred to an aa_3-type of cytochrome oxidase. (b) The periplasmic and cytoplasmic metabolism of trithionate, thiosulfate, and tetrathionate by *Thiobacillus tepidarius* as proposed by Kelly (1988b). In contrast to the system shown in part a, tetrathionate appears to be an intermediate in the oxidation of both thiosulfate and trithionate. After an initial hydrolysis of trithionate, yielding thiosulfate and sulfate, the thiosulfate is oxidized to tetrathionate. Available evidence indicates a periplasmic location of these systems. Tetrathionate is believed to be transported into the cell and then oxidized to sulfite in the cytoplasm by an unknown mechanism. Sulfite dehydrogenase is responsible for the final oxidation to sulfate, in which cytochrome *b* may be involved. FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-quinolinol-1-oxide.

during continuous culture on limiting mixtures of substrates. The term mixotrophy usually designates simultaneous growth on a mixture of autotrophic and heterotrophic substrates (e.g., on thiosulfate and acetate). However, the simultaneous use of any mixture of substrates requiring (partially) separate metabolic pathways or enzymes, and thus might produce diauxie or biphasic growth in batch culture (e.g., glucose and lactose, succinate and glucose, iron and sulfur, hydrogen and sulfide, acetate and lactate), could be considered as mixotrophy.

Table 3. Classification of the different physiological types of colorless sulfur bacteria.[a]

	Carbon source		Energy source	
Physiological type	Inorganic	Organic	Inorganic	Organic
Obligate chemolithotroph	+[b]	–	+	–
Facultative chemolithotroph (mixotroph)	+	+	+	+
Chemolithoheterotroph	–	+	+	+
Chemoorganoheterotroph (heterotroph)	–	+	–	+

Commonly used synonyms for chemolithotroph include chemolithoautotroph, autotroph, chemoautotroph, and lithotroph.
+, used by the group; –, not used.

Some of the thiobacilli, *Thiosphaera pantotropha*, *Paracoccus denitrificans* (Friedrich and Mitrenga, 1981), and certain *Beggiatoa* species (Nelson and Jannasch, 1983) are typical examples of organisms able to grow on mixtures of reduced sulfur compounds and organic substrates. To some extent, the phototrophic sulfur-oxidizing bacteria might also be considered members of this group since most, if not all, of them are able to grow chemolithoautotrophically and mixotrophically on reduced sulfur compounds in the dark (Kuenen et al., 1985).

CHEMOLITHOHETEROTROPHS. This little-known group of bacteria is characterized by an ability to generate energy from the oxidation of reduced sulfur compounds, but which cannot fix carbon dioxide. Until recently, *Thiobacillus perometabolis* was considered to be a member of this group, but it is now known that under certain conditions, it can grow autotrophically (Katayama-Fujimura et al., 1984). However, unnamed chemolithoheterotrophic species have been isolated, and a few strains of *Thiobacillus* have been well characterized (Tuttle et al., 1974; Gommers and Kuenen, 1988). Some *Beggiatoa* strains may also belong in this group (Larkin and Strohl, 1983). As is clear from the example of *T. perometabolis*, careful testing under a variety of conditions is necessary in order to discriminate chemolithoheterotrophs from the facultative autotrophs as well as from the sulfur-oxidizing heterotrophs.

SULFUR-OXIDIZING CHEMOORGANOHETEROTROPHS. Some heterotrophic bacteria can oxidize reduced sulfur compounds, but do not appear to derive energy from them. However, they may benefit from the reaction by the detoxification of metabolically produced hydrogen peroxide (e.g., some species of *Beggiatoa*, *Macromonas*, *Thiobacterium*, and *Thiothrix*) (Larkin and Strohl, 1983; Dubinina and Grabovich, 1984). The oxidation of thiosulfate to tetrathionate by many heterotrophic bacteria that do not seem to gain energy from the reaction is well documented (Tuttle and Jannasch, 1972; Tuttle et al., 1974; Mason and Kelly, 1988).

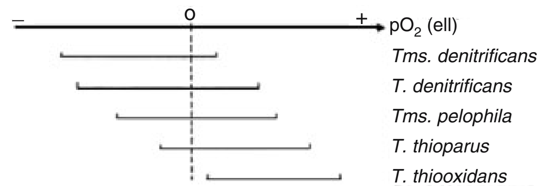


Fig. 3. A "spectrum" showing the response of five different species of colorless sulfur bacteria to redox. The position of each line indicates the range of conditions of redox under which the organism can grow. *T.*, *Thiobacillus*; *Tms.*, *Thiomicrospira*. (Based on Timmer ten Hoor, 1977.)

Electron Acceptors for Aerobic and Anaerobic Growth

Oxygen is universally used among the colorless sulfur bacteria, although the degree of aerobiosis that can be tolerated by different species varies. The response of some of the colorless sulfur bacteria to redox can be demonstrated by means of a "spectrum" as shown in Fig. 3.

Various colorless sulfur bacteria have different ways of growing or surviving anaerobically. One of the best studied is the use of nitrate or nitrite as a terminal electron acceptor, whereby the nitrogen oxides are reduced to nitrogen, a process termed denitrification. This will be discussed in detail in Chapter 23, but a brief consideration of the nitrate-reducing colorless sulfur bacteria is appropriate here.

The denitrifying species tend to be neutrophilic (Table 4), but not necessarily mesophilic,

Table 4. Examples of the neutrophilic, mesophilic species capable of autotrophic growth on reduced sulfur compounds.

	Autotrophy		Denitrification	
Species	Obligate	Facultative	To NO_2–	To N_2
Thiobacillus thioparus	+[a]	–	+	–
T. neapolitanus	+	–	–	–
T. denitrificans	+	–	+	+
T. novellus	–	+	–	–
T. versutus	–	+	+	+
T. intermedius	–	+	–	–
T. perometabolis	–	+	–	–
T. delicatus	–	+	+	–
T. thyasiris	–	+	+	+
Thiomicrospira pelophila	+	–	–	–
Tms. denitrificans	+	–	+	+
Tms. crunogena	+	–	–	–
Thiosphaera pantotropha	–	+	+	+
Beggiatoa sp. (marine)	–	+	–	–
Beggiatoa sp. (freshwater)	–	+	+	+

+, property present; –, property absent.

since at least one of the thermophiles (*Thermothrix thiopara*) can denitrify. A few (e.g., *Thiobacillus thioparus*) can only reduce nitrate to nitrite and require the presence of a nitrite-reducing bacterium for anaerobic growth (Table 4). Strictly speaking, of course, the latter reaction is not truly denitrification, but since the reaction still serves for electron acceptance and survival under anaerobic conditions, these species are appropriately included here.

There are two known obligately chemolithotrophic sulfur bacteria that carry out complete denitrification to nitrogen. *Thiobacillus denitrificans* is relatively versatile in being able to grow under fully aerobic conditions with oxygen, and under fully anaerobic conditions with nitrate or nitrite (Aminuddin and Nicholas, 1973; Ishaque and Aleem, 1973). *Thiomicrospira denitrificans* is more fastidious. It grows well anaerobically with nitrate or nitrite, but can only use oxygen for growth if its concentration is kept extremely low (i.e., below the detection level of normal oxygen electrodes) (Timmer ten Hoor, 1975). These obligate autotrophs are far more efficient at anaerobic (denitrifying) growth on reduced sulfur compounds than the facultative species. Of the latter, only *Thiosphaera pantotropha* has been, thus far, found to retain its sulfur-oxidizing potential under denitrifying conditions, but its μ^{max} while doing so is extremely low (approx. 0.015 h^{-1}) compared with those of *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* (0.06 h^{-1}). Other facultatively autotrophic bacteria lose their sulfur-oxidizing capacity in anaerobic cultures, but are still able to denitrify using organic compounds, or even hydrogen. Among these are *Thiobacillus versutus* and *Paracoccus denitrificans* (Taylor and Hoare, 1969; Friedrich and Mitrenga, 1981). Sulfide-dependent reduction of nitrate to N_2 by *Beggiatoa* tufts has recently been shown using ^{15}N-labelled nitrate (Sweerts et al., 1990).

Of the two sulfur-oxidizing genera of archaebacteria, *Sulfolobus* species appear to be the more dependent on oxygen, although some have been shown to use ferric iron and molybdate as electron acceptors under microaerobic conditions (Brock and Gustafson, 1976; reference is not an exact matchBrierly, 1982). Members of the genus *Acidianus* are able to grow under anaerobic conditions, by using hydrogen as the electron donor and sulfur as the acceptor, thus making these bacteria both sulfur-oxidizing and sulfur-reducing, depending on the conditions (Segerer and Stetter, 1989). Nelson and Castenholz (1981) have reported that some *Beggiatoa* species carry out an anaerobic reduction of intracellularly stored sulfur, using organic compounds such as acetate as electron donors. The ability of these organisms to oxidize sulfide to sulfur under aerobic conditions and then to reverse this reaction anaerobically would permit them to optimally profit from their habitat, where aerobic and anaerobic conditions frequently alternate. They may also actively migrate between aerobic and anaerobic zones.

Even apparently obligately aerobic strains may have mechanisms allowing them to survive during anaerobiosis for a limited length of time. Thus, *Thiobacillus neapolitanus*, a species normally considered to be obligately respiratory, has been shown to be able to ferment internal reserves of polyglucose when confronted with anoxic conditions (Beudeker et al., 1981). As mentioned in the introduction, *T. ferrooxidans* can use ferric iron as an electron acceptor, although it is not yet clear whether this is linked to energy generation.

Ecophysiology as a Function of pH, Temperature and Nutrient Availability

Colorless sulfur bacteria have been found growing at pH 9.0 and pH 1.0, at 4°C and 95°C, and at dissolved oxygen concentrations ranging from air-saturation to anaerobic levels (Table 1). It is obvious that a combination of physical, chemical, and (eco)physiological factors will suit the ecological niche of the organism within a particular microbial community. A number of these will be considered here.

pH Range and Effects. The pH ranges of some of the colorless sulfur bacteria are surveyed in Table 1, and examples of neutrophilic and acidophilic species are listed in Tables 4 and 5. Within these ranges, of course, species often have different pH optima. The outcome of competition for a substrate at different pH values will therefore be dictated to a large extent by the pH optima of the competing bacteria. Thus, Kuenen et al. (1977) found that at pH values above 7.5, *Thiomicrospira pelophila* dominated

Table 5. Characteristics of acidophilic, mesophilic species capable of growth on reduced sulfur compounds and/or iron.

	Autotrophy		Utilization of	
Species	Obligate	Facultative	Sulfur	Iron
Thiobacillus ferrooxidans	+	−	+	+
T. thiooxidans	+	−	+	−
T. albertis	+	−	+	−
T. acidophilus	−	+	+	−
Leptospirillum ferrooxidans	+	−	−[a]	+

Also negative on other sulfur compounds, can use the iron in pyrite.

thiosulfate-limited chemostat cultures, whereas when the pH was below 6.5, *Thiobacillus thioparus* was able to outcompete the other for thiosulfate. At intermediate pH values, the outcome of the experiments was not reproducible, with varying levels of the two populations. Apparently, the substrate affinities of the two species were so similar that other, less well-controlled variables (e.g., iron concentration, minor amounts of wall growth, etc.) became important for the outcome of the competition. Similar pH effects have been observed in the competition between *T. versutus* and *T. neapolitanus* (Smith and Kelly, 1979).

The colorless sulfur bacteria that grow at neutral to slightly alkaline pH values are found in marine and freshwater sediments, soils, and wastewater treatment systems, to name but a few sources. As can be seen from Table 4, representatives of almost all of the genera fall within this group. Many of them have specialized in growth in the gradients where (anaerobic) sulfide-containing zones come into contact with air or oxygen-containing water and will be discussed in the section on gradients. Some colorless sulfur bacteria are extreme acidophiles, able to grow at pH values as low as 1. As Table 5 shows, the group includes mesophilic obligate and facultative autotrophs (e.g., *T. ferrooxidans* and *T. acidophilus*, respectively). The acidophilic colorless sulfur bacteria are abundant in locations such as acid mine-drainage water, and it is therefore interesting that many of them are also able to oxidize (and gain energy from the oxidation of) metals such as iron. Thus, *T. ferrooxidans* is able to grow "mixotrophically" on the iron and sulfur components of pyrite (Arkestein, 1980) or on mixtures of ferrous iron and tetrathionate, gaining energy from the iron and sulfur oxidizing reactions (Hazeu et al., 1986, 1988). There have been a few reports of facultatively heterotrophic growth by *T. ferrooxidans* (e.g., Shafia and Wilkinson, 1969; Lundgren et al., 1964). However, it has since been shown that most of the *T. ferrooxidans* cultures available from culture collections were contaminated with acidophilic facultative autotrophs and heterotrophs (Harrison, 1984), including *T. acidophilus* and *Acidiphilium cryptum*, and it is now generally accepted that *T. ferrooxidans* is an obligate autotroph.

It has frequently been assumed that *T. ferrooxidans* is one of the key species active in pyrite oxidation. In order to assess its likely significance for pyrite oxidation during coal desulfurization, Muyzer et al. (1987) used antibodies raised against *T. ferrooxidans* for an immunofluorescent assay of slurries made from coal from different sources. Unsterilized and sterilized coal samples were inoculated with *T. ferrooxidans*, with a mixed culture of pyrite-oxidizing bacteria from a coal-washing installation, and a mixture of the two. Despite the fact that a DNA-fluorescent stain indicated abundant microbial life in all of the slurries, the only sample in which a significant *T. ferrooxidans* population was detected was the control, which had been sterilized and then inoculated with the pure culture of *T. ferrooxidans*. It appears that in all other cases, other strains (which might include such species as *T. thiooxidans*, *Leptospirillum ferrooxidans*, or *Acidiphilium cryptum*, to name but a few) were able to successfully out-compete *T. ferrooxidans* for a niche in the consortium.

Temperature As pointed out at the beginning of this section, colorless sulfur bacteria can be found growing at temperatures ranging from 4–95°C. However, the majority of the well-studied species are mesophilic. Although it is evident that the majority of natural environments are suitable for the growth of mesophiles, the diversity of the thermophilic organisms is likely to be much larger than suggested by Table 6, particularly in view of the recent discoveries of new thermophilic species among the colorless sulfur bacteria and other metabolic groups. Thus, it is clear that the species discussed in this section should be regarded as indicative rather than definitive. As most of the examples discussed elsewhere in this chapter will be taken from mesophilic bacteria, most of this section will be dedicated to consideration of the thermophiles.

Thermophilic bacteria are generally associated with waters that have been geothermally heated. These range from warm springs, used for bathing since Roman times, through solfataras to submarine hydrothermal vents (e.g., Caldwell et al., 1976; le Roux et al., 1977; Jannasch, 1985). As can be seen from Table 6, the bacteria in this group can be subdivided into two groups, the moderate thermophiles (generally eubacteria),

Table 6. Characteristics of moderately and extremely thermophilic species capable of growth on reduced sulfur compounds.

	Autotrophy		
Species	Obligate	Facultative	Temperature range (°C)
Thiobacillus tepidarius	+	–	20–52
T. aquaesulis	–	+	30–55
Thermothrix thiopara	–	+	72
Sulfolobus acidocaldarius	–	+	60–85
Sulfolobus sp. HVS	+	–	60–95
Acidianus infernus	+	–	60–95
A. brierleyi	–	+	60–95

which grow over the range 45–55°C, and the extreme thermophiles (generally archaebacteria), some of which can grow at temperatures approaching 100°C.

Neutrophilic species make up the moderately thermophilic group. One neutrophile, *Thermothrix (Tx.) thiopara* has a higher optimum growth temperature (72°C). This facultative autotroph was found in neutral (pH 7.0), hot (74°C) springs (Caldwell et al., 1976; Brannan and Caldwell, 1980), where it forms macroscopic streamers as well as microscopic mats on the tufa. The streamers occur at the sulfide: oxygen interface (Caldwell et al., 1983), and the key role that oxygen plays in their development was demonstrated by means of a very simple experiment during which the surface of the hot spring was covered by a sheet of plastic to restrict entry of oxygen from the air. As a result of this, the dissolved oxygen dropped to 0.1 mg l^{-1} from 3 mg l^{-1}, but other parameters such as pH and temperature were unaffected. The *Tx. thiopara* streamers then disappeared from their accustomed positions and reappeared at the edges of the sheet, where the sulfide:oxygen gradient had been reestablished.

The acidophilic archaebacteria of the genera *Sulfolobus* and *Acidianus* represent the colorless sulfur bacteria among the hyperthermophiles. These genera include both obligately and facultatively autotrophic species. They are frequently found in association with sulfidic ores such as pyrite, chalcopyrite, and sphalerite. It has been suggested that the failure to find *Sulfolobus* species around hydrothermal vents, where *Acidianus* does occur, is due to the low salt tolerance of *Sulfolobus* species. *Acidianus* species can tolerate NaCl concentrations of up to 4% (Stetter, 1988). Of course, with growth temperatures between 60–95°C, these strains seem almost "moderate" in comparison to the growth temperatures of the sulfur-reducing *Pyrobaculum* and *Pyrodictium* species (74–110°C).

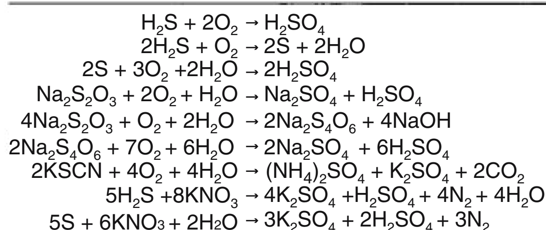


Fig. 4. A model to describe the selection of different physiological types by the ratio of inorganic to organic substrates supplied in the medium. This model may also hold for complex (semi-natural) systems, where the relative turnover rates of the inorganic and organic compounds (or the ratio between the fluxes of these compounds) would determine the selection of different physiological types. For definitions of the various terms, see Table 3.

Nutrient Availability and Ecological Niches. Of the physiological types shown in Table 3, the obligate and facultative chemolithotrophs are the best known, having been the most extensively studied in pure and mixed cultures (e.g., Kelly and Kuenen, 1984; Kuenen, 1989; Kelly and Harrison, 1989; Kuenen et al., 1985; Kuenen and Robertson, 1989a, 1989b). One of the most important environmental parameters affecting the selection of these bacteria in freshwater environments was found by Gottschal and Kuenen (1980) to be the relative turnover rates of inorganic and organic components in the available substrates (Fig. 4). Thus, if the available substrate in energy-limited systems is wholly or predominantly inorganic, obligate autotrophs such as *Thiobacillus neapolitanus* will normally tend to dominate a community. Similarly, abundant organic substrates will generate communities dominated by heterotrophs. On mixed substrates, facultative autotrophs such as *T. versutus* or chemolithoheterotrophs will appear, depending on the ratio between the two types of substrate. If the substrate supply is predominantly organic, the sulfide-oxidizing heterotrophs or other heterotrophs will appear. This model was put to the test by means of a number of competition experiments in two- and three-membered mixed cultures of representatives from the physiological groups. In addition, a number of enrichment cultures inoculated from natural samples containing representatives of all of the physiological types were obtained. All of the experiments essentially showed that the predicted metabolic type became dominant (for example, see Fig. 5a and b). Although mathematical modelling predicted that in some cases pure cultures of only one metabolic type should be obtained, in practice, satellite populations of the others remained (Fig. 6). Clearly, secondary environmental or experimental conditions (e.g., excretion products such as glycollate, fluctuations in substrate or oxygen concentrations, and growth on the wall of the vessel) can result in deviations from the idealized model. It is obvious that a well-mixed chemostat is a model system that is rather remote from the common natural habitats of colorless sulfur bacteria, such as the sulfide:oxygen gradient in a sediment, and the results obtained can only demonstrate the principle. Moreover, the relative turnover rate of the organic and inorganic substrates is only one of the environmental parameters that determines the success of a particular species. Nevertheless, the use of this model (Fig. 4) has now clarified the situation, a practical consequence

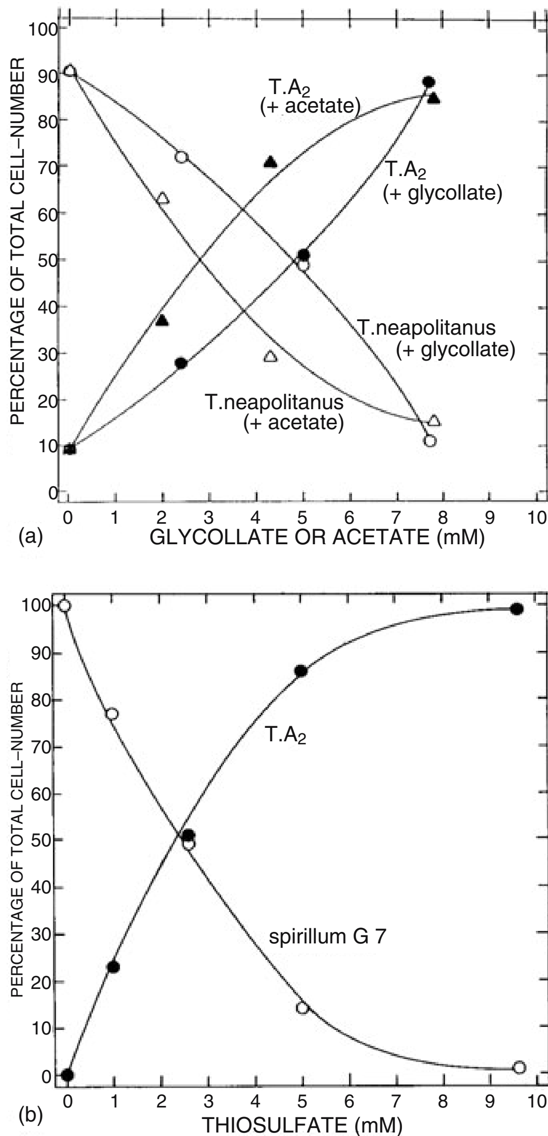


Fig. 5. The effect of organic or inorganic energy sources on competition. (a) The effect of different concentrations of organic substrates on the competition between *Thiobacillus versutus* (T. A_2) and *T. neapolitanus* for growth-limiting thiosulfate in a continuous culture. The influent medium contained 40mM thiosulfate. During growth limitation by thiosulfate, it and the organic additives (where present) were used simultaneously by the mixed culture, and their actual concentrations in the chemostat were below the detection level. The graph shows the ratios of the two species at steady state. Open symbols, *T. versutus*; closed symbols, *T. neapolitanus*; circles, glycollate supplied; triangles, acetate supplied. (b) The effect of thiosulfate on the competition for acetate (10 mM) between *T. versutus* (T. A_2) and a heterotrophic spirillum called G7. For experimental details, see (a). Open symbols, *Spirillum* G7; closed symbols, *T. versutus*. (Based on Gottschal et al., 1979.)

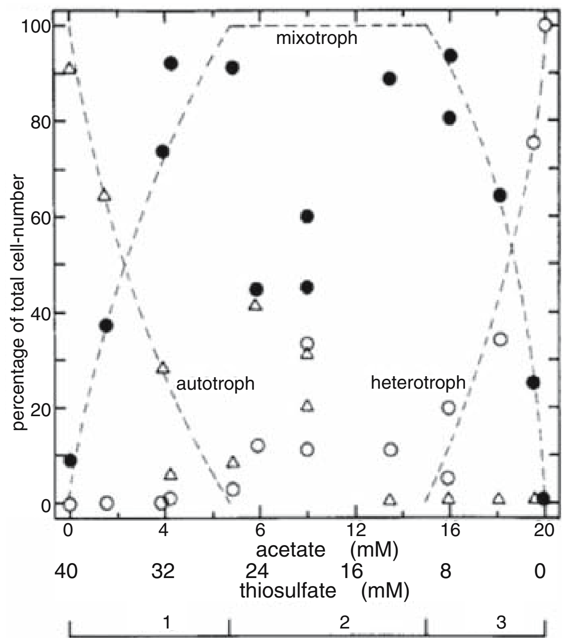


Fig. 6. Competition for acetate and thiosulfate in a chemostat between an autotroph, *T. neapolitanus* (open triangles); a mixotroph, *T. versutus* (closed circles); and a heterotroph, *Spirillum* G7 (open circles). The dotted lines indicate the results predicted from the model, the symbols indicate the actual results. The model held well for the extreme ratios of thiosulfate and acetate. However, although *T. versutus* dominated at intermediate ratios, as predicted, the other two types did not completely disappear. For the experimental details, see Fig. 5a. This model can be used for the selective enrichment of facultative autotrophs in chemostat cultures using an intermediate ratio of acetate and thiosulfate. (Based on Gottschal et al., 1982.)

being that it has shown the way for the selective enrichment of facultatively autotrophic sulfur bacteria from fresh water.

Steady-state conditions are more common in artificial environments than in nature, and therefore in order to test the effect of substrate fluctuations on the selection of the three representative species used in the experiments discussed above (Figs. 5a, 5b, and 6), Gottschal et al. (1981) ran chemostat cultures alternating feeds of acetate and thiosulfate. In two-membered cultures, the mixotrophic *T. versutus* was able to maintain itself on the substrate not used by whichever obligate species was involved, so that both species were subject to alternating periods of growth and starvation. However, in three-membered cultures, the two specialists were able to react more swiftly to the onset of substrate provision because of their constitutive enzymes, while the facultative species, which had to reinduce its autotrophic enzymes each time, disappeared. As with the steady-state experiments, when different mixtures of acetate and thiosulfate alternated, the outcome was deter-

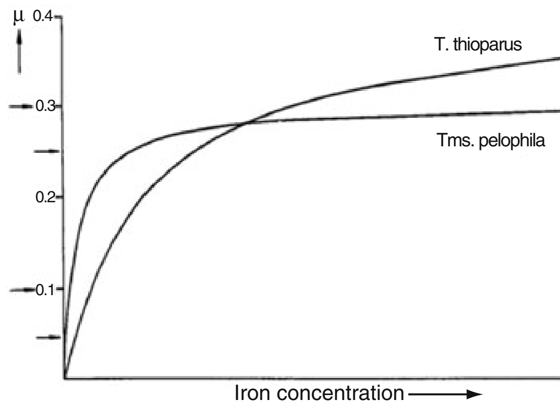


Fig. 7. The specific growth rates (μ) of *Thiomicrospira pelophila* and *Thiobacillus thioparus* as a function of the iron concentration in chemostat cultures at 25°C. The graph was constructed from the results of competition experiments (at the growth rates indicated by the arrows at the y axis). The actual iron concentrations were not determined. (From Kuenen et al., 1977.)

mined by the concentrations involved. Enrichment cultures under this regime yielded a facultative autotroph that was able to avoid the need to induce its carbon dioxide fixation system by accumulating large amounts of PHB during the heterotrophic period.

This work was carried out on aerobic, freshwater chemostat cultures and, as has been discussed in previous reviews (Kelly and Kuenen, 1984; Kuenen, 1989; Kelly and Harrison, 1989; Kuenen et al., 1985), marine enrichments are, for unknown reasons, generally less predictable. For example, mixotrophs did not form the dominant population in thiosulfate/acetate-limited marine cultures (Kuenen et al., 1985). That marine mixotrophs do exist has been shown by the isolation of a facultatively chemolithotrophic marine strain of *T. intermedius* from a thiosulfate-limited culture (Smith and Finazzo, 1981).

Of course, factors other than the availability of electron donors can determine the type of population to be found in any given environment. For example, Kuenen et al. (1977) studied the effect of iron limitation and pH on the outcome of competition between two marine obligate autotrophs, *Thiomicrospira* (*Tms.*) *pelophila* and *Thiobacillus* (*T.*) *thioparus*. As can be seen from Fig. 7, *Tms. pelophila* will dominate mixed cultures of the two species at low iron concentrations, whereas *T. thioparus* will do better when iron is more abundant. One of the characteristics of *Tms. pelophila* is its tolerance of sulfide concentrations high enough to inhibit *Thiobacillus* spp. It has been postulated that sulfide inhibition is caused by the reaction of the sulfide with available iron, forming insoluble ferrous sulfide and thus drastically reducing the concentration of iron available for microbial utilization. If this hypothesis is accurate, the ability of *Tms. pelophila* to grow well at very low iron concentrations would explain its sulfide tolerance.

Taxonomy

Many of the colorless sulfur bacteria were discovered in the early years of microbiology, at a time when scientists were relying mainly on morphological characteristics to identify their organisms, and this fact is still reflected in our approach to their taxonomy. Needless to say, this has caused a certain amount of confusion (see Table 1 for an overview of the genera involved). The problems associated with the identification of some colorless sulfur bacteria have been aggravated because many of the bacteria involved are very specialized (e.g., obligate autotrophs) and, as a consequence, the number of physiological traits that can be screened is limited. This has resulted in relatively trivial features being given undue weight during classification. Taxonomy is a way of establishing identities and relationships in an attempt to create a sense of order among the various forms of life on earth. In ecology, as in other applications of taxonomy, the precise identification of a particular species may not always be as relevant as an accurate description of its physiological characteristics, but the comparison and correlation of data from different sources becomes easier if one can be certain, or even reasonably sure, of the identities of the various bacteria involved. Changes in taxonomic practice largely reflect new developments in available technology as well as improvements in our understanding of which factors indicate relationships, and which are merely resemblances. Taxonomic research into the colorless sulfur bacteria can thus be separated into three distinct, if overlapping phases, which will be discussed sequentially here.

Morphology

The colorless sulfur bacteria, as a group, encompasses rods, spirals, cocci, filamentous cells and archaebacteria, and it comes as no surprise to find that the first of them to be described, *Beggiatoa* (Trevisan, 1842), is also one of the largest. The longest cells reported in the latest edition of *Bergey's Manual* are 50 μm long (Strohl, 1989), but a recent paper described the observation of a marine strain more than 100 μm long (Nelson et al., 1989). Another morphologically distinct genus, *Thiothrix,* was described by Winogradsky in 1888, but it was not until 1904 that Beijerinck described the first of the smaller colorless sulfur bacteria, *Thiobacillus thioparus*.

As may be seen from a survey of the relevant chapters in *Bergey's Manual* a few genera are still, today, based largely on morphological descriptions (e.g., *Thiospira*, *Macromonas*, *Thiovulum*) because pure cultures are either not available, or have only recently been achieved.

In addition to cell size and shape, other morphological details that have been considered important are the appearance of inclusion bodies such as sulfur or poly β-hydroxybutyrate (PHB), number and placement of flagella, colony size, colony form and colony color. One of the dangers associated with too strong a reliance on such features is that all of them can vary depending on the growth conditions. As a single example of this problem, the faculatively autotrophic *Thiosphaera pantotropha* might be considered. When grown autotrophically on thiosulfate, it occurs as small cocci (0.7×0.9 μm), which are generally found singly or in pairs (Fig. 8a). Cultivation in batch culture on rich media in which rapid growth will occur leads to a slightly larger, pleomorphic form (Fig. 8b). In chemostat cultures on mineral medium with acetate, chains of cocci appear. The internal structure of *Thiosphaera pantotropha* also changes with its growth conditions. Thus the normal appearance, with few inclusions, of a Gram-negative organism, which is found during substrate-limited chemostat culture (Fig. 8c), gives way to cells with PHB granules and complex membranous structures (Fig. 8d) when grown under oxygen or nitrogen-limited conditions, or in the presence of hydroxylamine. Cultivation on acetone or propan-2-ol results in the formation of large, crystalline structures (Fig. 8e), while denitrifying growth on sulfide can result in the accumulation of a fine deposit of sulfur in the periplasm (Fig. 8f). The colonial form of this species also varies, with off-white, translucent colonies being produced during growth on mineral medium with acetate or thiosulfate; and larger, thicker, browner colonies being generated during growth on rich media.

Even the obligate autotrophs, which with their more limited range of growth conditions might appear to have less scope for variation, can produce substantial morphological changes. Thus, the number of carboxysomes formed by *Thiobacillus neapolitanus* increases dramatically under CO_2 limitation (Beudeker et al., 1980), and polyglucose inclusions appear under nitrogen limitation (Beudeker et al., 1981).

From all of this, it is clear that while valuable information can be gained from morphological studies on cells or colonies grown under well-defined conditions, this information should be used cautiously and in conjunction with other data. In exceptional circumstances, very distinctive morphology (e.g., in the case of *Beggiatoa* or *Hyphomicrobium*) might be more reliable as an indicator of identity.

Physiological Screening

As more pure cultures became available, it became possible to determine the physiological capabilities of different bacteria, and physiological criteria gradually became an integral part of the taxonomists' armory. For the obligate autotrophs, these might include such tests as optimum pH, growth temperature, ability to denitrify, and (generally very limited) substrate range. In addition to these, the facultative autotrophs are generally subjected to the same range of tests used for heterotrophic bacteria including oxidase, catalase and urease reactions, and the ability to grow on or generate acid from a range of substrates. An extensive study of the *Thiobacillus* species then available resulted in a numerical taxonomy analysis of the genus (Hutchinson et al., 1969) that recognized that "species" such as *Ferrobacillus ferrooxidans* and *Thiobacillus thiocyanoxidans* were actually strains of existing species (*T. ferrooxidans* and *T. thioparus*, respectively). The tests recommended by Hutchinson et al. (1969) for the identification of new *Thiobacillus* species included growth on sulfide, sulfur, thiocyanate, citrate and nutrient broth, the amount of thiosulfate used, sulfur deposition, and the effect of inhibitors such as streptomycin, bacitracin and ampicillin.

In many respects, the range of substrates on which an isolate is tested is defined by the interests of the research group. The reduced sulfur compounds are not included in standard test batteries, and the sulfur-oxidizing abilities of many bacteria are only now being discovered. For example, Friedrich and Mitrenga (1981) tested a number of hydrogen-oxidizing bacteria and found that many of them, including *Paracoccus denitrificans* and some *Alcaligenes* species, were able to grow autotrophically on thiosulfate. Attempts to use thiosulfate as an inhibitor of heterotrophic nitrification by a "*Pseudomonas*" species gave anomalous results until it was realized that the culture was growing mixotrophically, using both the acetate supplied as the primary growth substrate and the thiosulfate added as a possible inhibitor. Subsequent experiments revealed that this "*Pseudomonas*" species was also able to grow autotrophically using reduced sulfur compounds (Robertson et al., 1989).

A problem associated with the use of substrate ranges for taxonomic purposes is that it is difficult to determine how closely related bacteria with the same enzyme system are. Thus, possession of the Calvin cycle enzymes for carbon dioxide fixation or the denitrification pathway enzymes is not considered sufficient grounds for

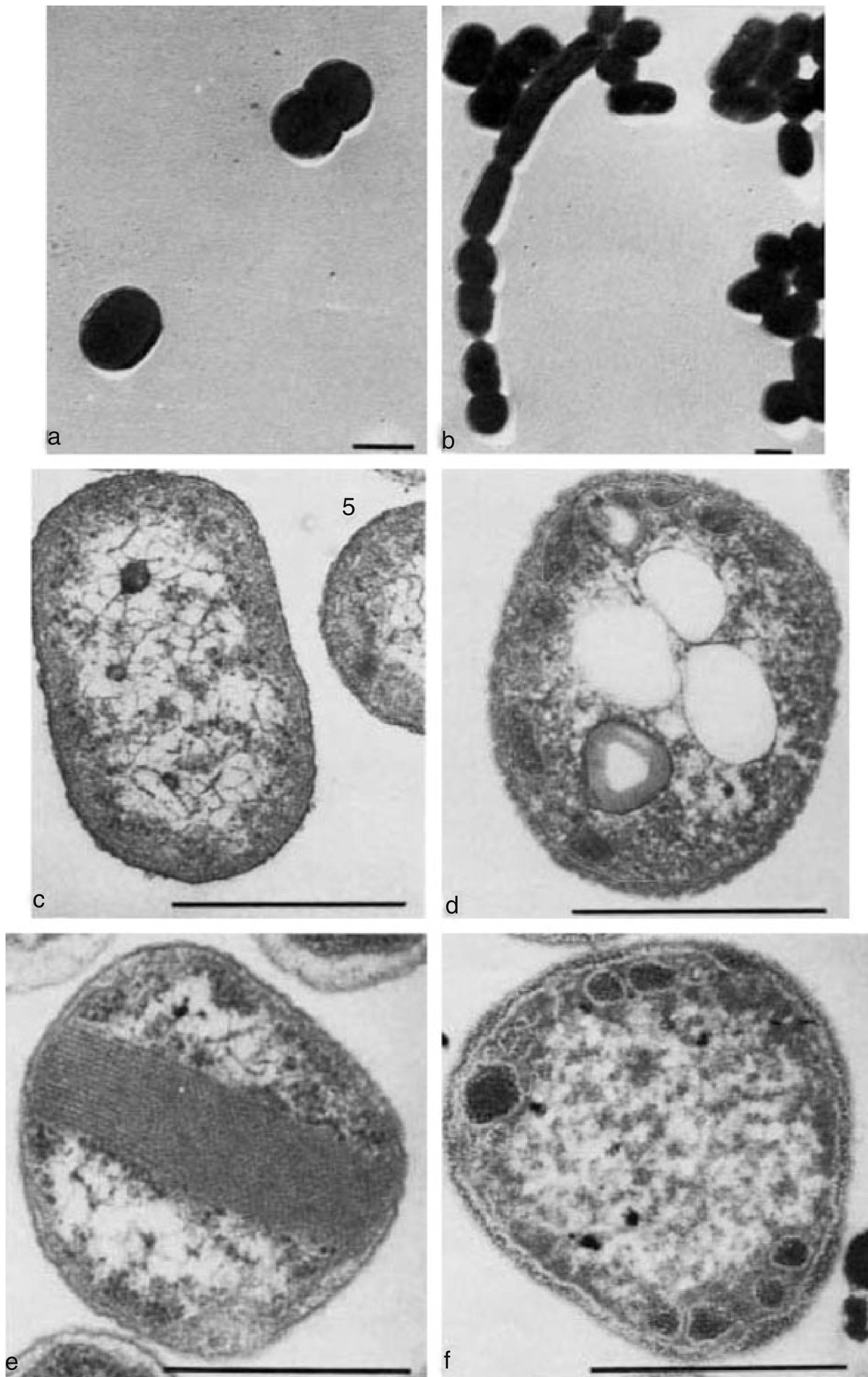


Fig. 8. Variations in the morphology of cells of *Thiosphaera pantotropha* in relation to growth conditions or substrates as seen under the electron microscope. (a) Aerobic, autotrophic growth on thiosulfate, Pt shadowed. (b) Aerobic, heterotrophic growth on a mixture of acetate, fructose, and yeast extract, Pt shadowed. (c) Thin section of cells from an acetate-limited, chemostat-grown culture, stained with ruthenium red to show the membrane structures. (d) Thin section of a cell from an aerobic, acetate-limited chemostat with hydroxylamine, stained with ruthenium red to show the membrane structures. The white bodies are PHB granules. (e) Thin section of an acetone-grown cell showing crystalline inclusions. (f) Thin section of an anaerobic (denitrifying) cell grown on sulfide and stained with silver to show the periplasmic deposits of sulfur. (Fig. 8b from Robertson and Kuenen, 1983b. Fig. 8c from Bonnet-Smits et al., 1988. Fig. 8f, courtesy of H. J. Nanninga. All electron microscopy courtesy of W. Batenberg.) All bars = 0.5 µm.

classifying the relevant bacteria into a single group, and it must be questioned whether the sulfur-oxidizing enzymes are a better indicator, especially since there appears to be several different pathways involved (Kelly, 1988a, 1988b) (see also Fig. 2, above). Certainly, it is recognized that at least one genus, *Thiobacillus*, is very heterogeneous (Kuenen, 1989; Kelly and Harrison, 1989) and will probably require subdivision. It has been suggested that this separation should be made between the obligate and the facultative autotrophs (thus, again on physiological grounds) but, as will be seen in the following section, this is probably not sufficient.

Analytical Techniques

The determination of the GC content of the DNA of bacterial isolates has been used for a long time to determine whether or not strains could be related. It is, to some extent, a negative test because, while widely differing GC values could confirm that two strains were not related, matching GC values do not guarantee that they are the same.

Cellular fatty acid analysis has been used in the taxonomy of the *Thiobacilli* (Agate and Vishniac, 1973; Katayama-Fujimura et al., 1982). Katayama-Fujimura et al. (1982) also included the analysis of ubiquinones and DNA base composition in their study. They initially subdivided the bacteria into groups based on whether they were obligately or facultatively autotrophic, and then on the basis of their possession of menaquinone 8 or 10, (MK-8 or MK-10) and then used the fatty acid analysis to further examine each group. This led to a proposal for the grouping of the different strains, which is shown in Table 7.

Some of the first publications to consider the *Thiobacilli* in relation to other colorless sulfur bacteria involved the phylogenetic analysis of the various species by comparison of their 5S rRNA sequences (Lane et al., 1985; Stahl et al., 1987). This work has now been extended by the use of 16S rRNA analysis (Lane et al., 1990; Oyaizu et al., 1990), and has revealed that there are closer matches between some sulfur-oxidizing bacteria and other apparently unrelated strains such as *Escherichia coli* than between these and other sulfur oxidizers. Table 8 summarizes some of the results from the 5S and 16S rRNA comparisons. The sulfur oxidizing genera *Sulfolobus* and *Acidianus* are archaebacteria and therefore not listed in Table 8.

If the initial separation into obligate and facultative autotrophs employed by Katayama-Fujimura et al. (1982) is removed, it can be seen that the results in Tables 7 and 8 support each other. Thus groups I.1 and I.2 from the menaquinone/fatty acid analysis correspond to group alpha from the 16S rRNA, groups II and III-1 with group beta-1, and groups III-2 and III-3 with beta-2. Of course, the range of bacteria subjected to the menaquinone/fatty analysis was much more limited than that in the 5S and 16S rRNA survey, and more data would be useful. However, such independent agreement must confer additional weight that chemotaxonomy and phylogeny may provide more reliable tools for the classification of these bacteria than physiological or morphological observations.

Habitats

As may be deduced from the range of physiological characteristics discussed above, the colorless sulfur bacteria, in one form or another, are to be found in almost every life-supporting environment where reduced sulfur compounds are found. Because the range of habitats is so wide, the principles underlying the selection of colorless sulfur bacteria in selected situations will be be discussed below. The following section will then deal more generally with the role of the

Table 7. Classification of the *Thiobacillus* species based on analysis of their menaquinone and fatty acid composition.

Autotrophy type	Menaquinone	Hydroxy fatty acid	Species	Group
Facultative	MK-10	None	*T. novellus*	I.1
Facultative	MK-10	3OH 10:0	*T. versutus*	I.1
Facultative	MK-10	3OH 14:0	*T. acidophilus*	I.2
Facultative	MK-8	3OH 10:0	*T. delicatus*	II
Facultative	MK-8	3OH 10:0, 3OH 12:0	*T. perometabolis*	II
Facultative	MK-8	3OH 10:0, 3OH 12:0	*T. intermedius*	II
Obligate	MK-8	3OH 10:0, 3OH 12:0	*T. denitrificans*	III.1
Obligate	MK-8	3OH 10:0, 3OH 12:0	*T. thioparus*	III.1
Obligate	MK-8	3OH 12:0	*T. neapolitanus*	III.2
Obligate	MK-8	3OH 14:0	*T. ferrooxidans*	III.3
Obligate	MK-8	3OH 14:0	*T. thiooxidans*	III.3

MK, menaquinone. The number indicates the number of isoprenoid units. Groupings are as proposed by Katayama-Fujimura et al. (1982).

Table 8. Classification of the colorless sulfur bacteria and examples of apparently related species (group "purple"), also termed *Proteobacteria* (Stackebrandt et al., 1988), as shown by 16S rRNA analysis.[a]

Main group	Subgroup	Species
Alpha	1	*Thiobacillus (T.) acidophilus*, *Acidiphilium rubrum*
	1	*A. cryptum*, *T. novellus*
	2	*Rhodobacter capsulatus*, *T. versutus*
	2	*Paracoccus denitrificans*
Beta	1	*T. denitrificans*, *T. thioparus*
	1	*T. intermedius*, *T. perometabolis*
	1	*Rhodocyclus gelatinosa*
	1	*Vitreoscilla*
	2	*T. tepidarius*, *T. ferrooxidans*
	2	*T. albertis*, *T. thiooxidans*
Borderline		*T. neapolitanus*, *Chromatium vinosum*
Gamma	1	*Thiothrix nivea*, *Riftia symbionts*
	1	*Thiomicrospira pelophila*, *Thiomicrospira* L-12
	1	*Bathymodicius* symbionts
	1	Other symbionts
	1	*Pseudomonas aeruginosa*, *P. putida*
	1	*Beggiatoa alba*, *Beggiatoa* sp.
	2	*Escherichia coli*, *Salmonella*, *Proteus*, *Vibrio*
Delta		*Thiovulum*, *Campylobacter*, *Wollinella*

Atypical strains have been omitted for the sake of simplicity.
Adapted from Lane et al., 1990; and Harrison (1989).

colorless sulfur-oxidizing bacteria in the sulfur cycle, and this discussion of habitats is not intended to be exhaustive.

In natural habitats, the reduced sulfur compounds available tend to be either sulfides (including metallic ores) or sulfur. Thanks to the activities of sulfate-reducing bacteria, especially in anoxic sediments, hydrogen sulfide is very commonly available, and some algal and cyanobacterial mats have been shown to generate organic sulphides (e.g., Andreae and Barnard, 1984). One of the main factors that bacteria growing on hydrogen sulfide have to contend with is the chemical reaction between sulfide and oxygen, and therefore the colorless sulfur bacteria are frequently found in the gradients at the interface between anoxic, sulfide-containing areas and aerobic waters and sediments where, at very low oxygen and sulfide concentrations, they can effectively compete with the spontaneous chemical oxidation reaction. Of course, the rate of chemical oxidation of metal sulfides with oxygen is very low at acid pH levels, so that the acidophilic bacteria need not, therefore, occur predominantly in gradients, as their neutrophilic counterparts must. The same holds for deposits of elemental sulfur, which does not react spontaneously with oxygen at a significant rate. Another habitat in which sulfide-oxidizing bacteria appear to be of some importance is in the complex communities of prokaryotes and eukaryotes around hydrothermal vents, where the sulfide is geologically rather than biologically generated. In the course of research into the life around these vents, it was shown that many invertebrates have symbiotic colorless sulfur bacteria, and this can itself be regarded as a distinct habitat (Cavanaugh et al., 1981). A third example of a type of habitat for these bacteria that is becoming steadily more common is that associated with human activities, largely in connection with waste treatment and industrial leaching of ores for (heavy) metal recovery.

Gradients in Aquatic Systems and Sediments

Sulfide:oxygen gradients occur in stratified water bodies, as well as in soils and sediments. Such gradients can range in size from a few hundred-micrometers-thick in a microbial mat or surface sediment to several meters in a stratified body of water (Sorokin, 1970, 1972; Jørgensen et al., 1979). These gradients can sometimes be distinguished with the naked eye. For example, *Thiovulum* grows as a fine white veil at the interface between sulfide and oxygen (Jørgensen, 1988). Wirsen and Jannasch (1978), studying the effect of the sulfide:oxygen gradient on the formation of these veils in continuous flow cultures, observed that the veils dispersed within minutes of the cessation of the flow of sea water through the culture vessel, and formed again once the flow was resumed, indicating chemotaxis of the swarming form of *Thiovulum* toward critical concentrations of oxygen and sulfide.

The genus *Beggiatoa* contains marine and freshwater species that are typical of life at the aerobic:anaerobic interface. Dense mats of

almost axenic cultures of *Beggiatoa* on sulfide-containing sediments are frequently observed, especially in marine sediments where sulfide production rates can be very high. These mats are characterized by very steep oxygen and sulfide gradients over a few mm (Jørgensen, 1982, 1988). Since *Beggiatoa* oxidizes the sulfide at a very high rate, the overlying aerobic water is effectively "protected" from diffusion of toxic sulfide. The typical conditions for growth in this type of mat have been very difficult to reproduce in the laboratory. Indeed, they are so specialized that it was only recently, when available techniques had improved sufficiently to allow in vitro cultivation on sulfide:oxygen gradients, that the autotrophic potential of marine strains was established unambiguously (Nelson and Jannasch, 1983; Nelson, 1988) (see also Chapter 166). The *Beggiatoa* cells were cultured in closed tubes using a layer of very soft (0.2%) agar over a sulfide-containing plug of harder (1.5%) agar, thus allowing the formation of an upward sulfide gradient. Diffusion from a headspace containing air contributed a downward oxygen gradient. The *Beggiatoa* colony grew as a "plate" that was less than 1 mm thick at the point where the two gradients overlapped. The very rapid oxidation of sulfide allowed the organisms to maintain an extremely low concentration of the two substrates. As a result, chemical oxidation of sulfide was insignificant. For example, the turnover time for sulfide and oxygen was only 3 seconds in *Beggiatoa* gradients, whereas the half life of these two substances in sterile controls was about 20 min. Enzyme analysis and the fixation of $^{14}CO_2$ by these cells confirmed that they were capable of autotrophic growth. The situation regarding freshwater strains is not so clearcut. Schmidt et al. (1987) showed sulfide oxidation rates for a freshwater strain comparable to those obtained with the marine strain discussed above, but further experimentation is necessary in order to establish whether energy for growth can be derived from the reaction.

Another well-known place where gradients occur is within phototrophic mats. Jørgensen and des Marais (1986) studied the zonation around a cyanobacterial mat growing in a hypersaline pond and found that a band of *Beggiatoa* occurred 1.5 mm below the cyanobacteria. The photosynthetic activity of the cyanobacteria generated sufficient oxygen to produce an oxygen peak with a maximum of 1mM at the cyanobacterial band. A steep downward gradient of oxygen overlapped a sulfide gradient at the point where the *Beggiatoa* were growing. In an earlier study, Jørgensen (1982) described the diurnal changes in the sulfide and oxygen gradients and the microbial community to be found in a sulfuretum (a microbial mat in which the total turnover of inorganic and organic compounds is heavily dominated by the sulfur cycle) on the surface of a sediment. It was observed that the mixture of cyanobacteria, phototrophic sulfur bacteria, and *Beggiatoa* was stratified, and that the relative positions of the the three populations among the strata were governed by the level of photosynthetically generated oxygen (Fig. 9). During the night, when the oxygen had been depleted and the oxygen boundary extended to the surface of the sediment, the phototrophic *Chromatium* was found at the surface. However, once photosynthesis began, with the onset of daylight, oxygen began to build up in the sediment, and the *Chromatium* followed the sulfide boundary down, remaining within the anaerobic part of the sediment. The *Beggiatoa* population tended to move with the sulfide:oxygen interface, except during the night when this was in the stagnant water above the surface of the sediment. As *Beggiatoa* is only motile by means of a gliding action, it is restricted to the solid phase.

Other conspicuous colorless sulfur bacteria such as *Thiothrix*, *Thioploca*, and *Archromatium* have all been encountered as typical organisms in such gradients. Furthermore, mixed cultures of *Thiobacillus*-like bacteria sampled from sulfide:oxygen gradients and showing active sulfide-dependent carbon dioxide fixation clearly exhibit chemotaxis toward the interface when transferred to artificial sulfide:oxygen gradients in the laboratory (J. G. Kuenen, unpublished observations).

Hydrothermal Vents

An interesting extension of the model for the selection of freshwater colorless sulfur bacteria discussed above is to be found in the results of research on the mesophilic bacterial communities found around the different hydrothermal vents (Jannasch, 1985, 1988). These vents are a result of the movements of the tectonic plates of the earth's crust. Seawater penetrates deep under the sea floor and is heated geothermally, reaching temperatures as high as 1,200°C. Under these conditions, it reacts with and dissolves various reduced chemicals before being forced to the surface again as hydrothermal fluid, which contains sulfide, CO_2, and methane, as well as various metals and hydrogen. The type of vent that occurs depends very much on the overlying geology, and can be at least partially separated into "bare lava" and "warm" systems. In the bare lava vents, the pressurized hydrothermal fluid reaches the surface of the sea floor at temperatures around 350°C. As it issues from the vents, it reacts with chemicals in the sea water, forming precipitates that often accumulate as "chim-

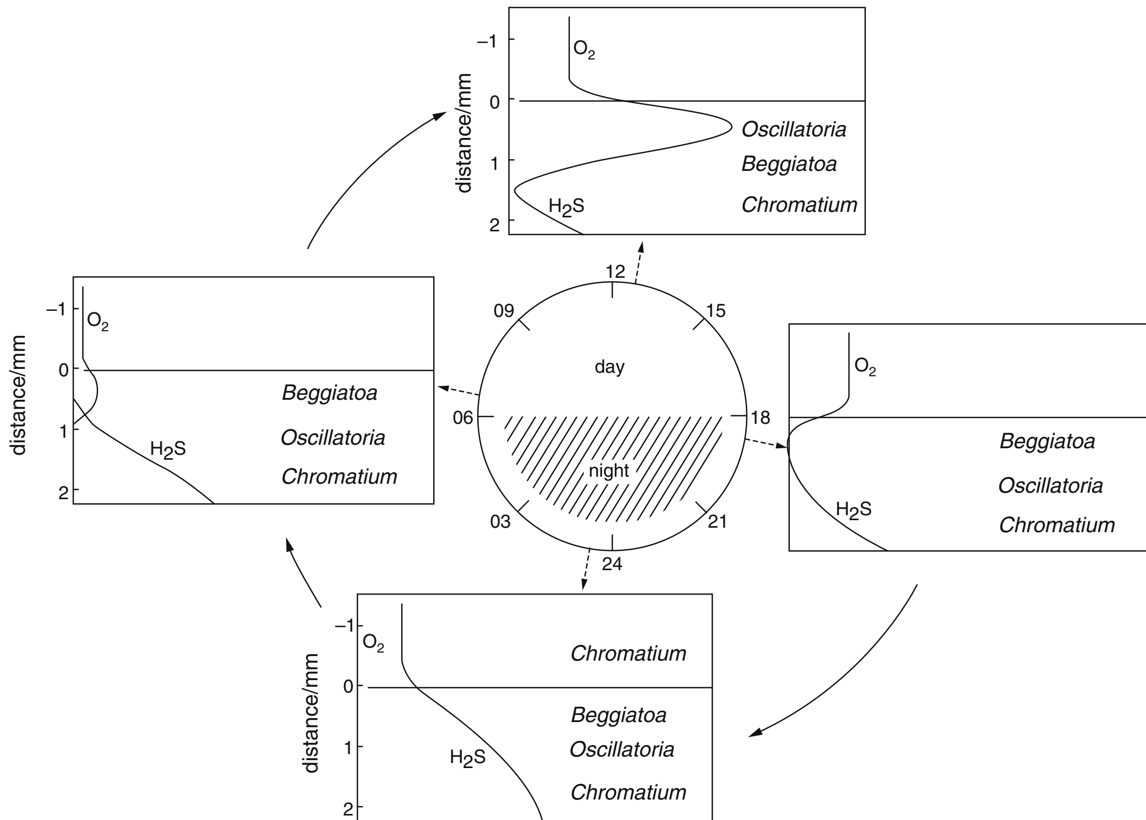


Fig. 9. Diurnal cycle of oxygen and sulfide distribution and of microbial zonation in a marine sulfuretum. The zero line in each box indicates the interface between the sediment and the overlaying water phase. The dominant genera at each stratum are indicated in each box. Diatoms were primarily seen among the *Oscillatoria.* In addition to diurnal changes in light, oxygen, and sulfide, another important factor was that the *Beggiatoa* which are gliding bacteria could not move out of the sediment, whereas *Chromatium,* which is also motile, was able to move into the water phase above. From Jørgensen (1982).

neys." Because the formation of metal sulfides gives the fluid issuing from these chimneys the appearance of smoke, they have become known as "black smokers." The "warm" vents, on the other hand, are the result of the hydrothermal fluid percolating through sediments on its way to the surface, and the solution tends to be much cooler (<25°C) and to have substantial organic content when it reaches the sea water. The waters around these vents support dense communities of bacteria able to grow on the geothermally generated reduced compounds. Thus far, obligately autotrophic sulfur bacteria, especially *Thiomicrospira* species (Ruby et al., 1981; Ruby and Jannasch, 1982), have been found associated with the areas around black smokers where the organic turnover is relatively low, while facultative autotrophs such as *Beggiatoa* appear to dominate around the sedimented vents where the organic turnover is much higher (Jannasch, 1988). Indeed, until recently, it was believed that *Beggiatoa* mats rarely exceeded 1 mm in thickness (Nelson, 1988). However, observations at the Guaymas Basin hydrothermal vents, where the hydrothermal fluid in some areas percolates though 400 m of sediment before reaching the surface, revealed mats of *Beggiatoa* up to 60 cm thick (Nelson et al., 1989). The communities around these vents appeared to be made up of three strains of *Beggiatoa* that had widely differing cell widths (115–122 µm, 40–42 µm, and 24–32 µm). The narrowest of these dominated, almost to the point of a monoculture, in the thickest layers, which were apparently associated with colonies of the vestimentiferan tube worms (see "Symbiosis," below). In addition to sulfide concentration and temperature, the authors suggest that organic excretion compounds from the worms may be an important factor in the development of these mats.

Symbiosis

Geologists studying areas of volcanic activity on the sea bed (1,800–3,700 meters below the surface) were surprised to find that not only were there dense, free-living bacterial populations associated with the vents, but that these perma-

nently dark areas were also occupied by an extensive community of invertebrates (and also, in some areas, fish), most of which were previously unknown (Corliss et al., 1979). Despite the density of the bacterial community, it was difficult to see how a food chain based entirely on suspended bacteria as a source of prey could support the considerable population of very large tube worms, clams and other invertebrates. Investigation of the anatomy of the tube worms (*Riftia pachyptilia*) revealed that they do not have an alimentary tract, but instead possess a large (more than half the weight of the worm) body of tissue, the trophosome, which is very rich in blood vessels. Examination of this tissue under the electron microscope revealed that it also contained a dense, intracellular community of bacteria (Cavanaugh et al., 1981; Cavanaugh, 1983a). The trophosome had already been shown to contain the enzymes necessary for chemoautotrophic growth on reduced sulfur compounds. These enzymes did not occur elsewhere in the tissues of the worm (Felbeck, 1981; Felbeck et al., 1981) and were presumably derived from the bacteria. It appears that this is an example of prokaryotic:eukaryotic symbiosis in which the tube worms rely on organic compounds excreted by the bacteria (see Chapter 215). The blood of the tube worms carries sulfide as well as oxygen from the gills to the trophosome, and has a special sulfide-binding protein that prevents sulfide toxicity. Endosymbionts have also been found in the giant white clams (*Calyptogena magnifica*), among other vent fauna and are not limited to sulfide-oxidizing bacteria, since methylotrophs have also been found (Jannasch, 1988) (see also Chapter 18). As yet, successful attempts to produce cultures of the symbiotic colourless sulphur bacteria have not been reported, but 5S and 16S rRNA analysis has indicated a relatively close relationship with members of the genus *Thiomicrospira* (Lane et al., 1985, 1990), which, as mentioned above, is one of the best-represented genera among the free-living bacterial community at the vents (Ruby et al., 1981; Ruby and Jannasch, 1982; Jannasch, 1988).

Once the occurrence of endosymbiotic bacteria in the animals of the hydrothermal vents had been accepted, many more occurrences were recognized in more mundane locations, including sewage outfalls and sulfide-rich sediments (Southward, 1986; Dando and Southward, 1986). Many of the animals associated with symbionts resemble *Riftia pachyptila* in that they completely lack a mouth and digestive system, whereas others may have only small guts and feeding appendages (Cavanaugh, 1983a, 1983b). Although not all of them have a specialized organ like the trophosome, many endosymbionts do appear to be associated with the gills of the eukaryotic host. For example, intracellular colorless sulfur bacteria have been found in the gill tissues of bivalves such as *Solemya velum* (Cavanaugh, 1983b) and *Thyasyris flexuosa* (Wood and Kelly, 1989). The recent description of a novel *Thiobacillus* species, *T. thyasiris*, (Wood and Kelly, 1989) from the gill tissue of *Thyasyris flexuosa* is probably the first report of the isolation of one of these symbionts. Whether all symbionts are capable of free-living growth (albeit with possible complex nutritional requirements), or whether some are so adapted to their symbiotic way of life that they are no longer capable of independent growth, remains to be seen.

A recent publication (Smith et al., 1989) has illustrated the effect that a localized deposit of organic material in an otherwise oligotrophic environment can have on the indigenous community. The skeleton of a 20 meter-long whale at a depth of 1,240 meters on the sea bed in the Santa Catalina basin was not only covered with mats of *Beggiatoa* resembling *Beggiatoa gigantea*, but it also supported six metazoan species, at least four of which are known in other locations to contain endosymbionts. As well as vent species (*Vesicomya gigas* and *Calyptogena pacifica*), others organisms known from anoxic sediments (*Lucinoma annulata*) and rotting wood (*Idasola washingtonia*) were also observed. None of these prokaryotic or eukaryotic species had been observed in this area before. It was found that the pore water under the skeleton contained around 20 μM sulfide, and the samples of whale bone that were recovered were found to be rich in oil and smelled strongly of sulfide. It would appear from the apparent ages of some of the molluscs present that a single whale carcass is sufficient to support these sulfide-dependent communities for several years.

Artificial Habitats and Application of Sulfur Bacteria

Artificial environments, such as the bioreactors used for industrial wastewater treatment, have provided habitats for bacteria that impose selective parameters not necessarily found in nature. Thus, substrates tend to be more abundant and conditions are generally more stable than in most natural situations. Two categories of artificial habitat where colorless sulfur bacteria are particularly important are wastewater treatment bioreactors and those associated with various leaching activities. Examples of other artificial habitats include industrial sulfur deposits or dumps, mining operations that expose sulfidic ores or sulfur to water or air, coal storage sites, and, last but not least, systems (including sewage

treatment plants) containing various amounts of reduced sulfur compounds.

Waste Treatment

Reduced sulfur compounds can occur in industrial wastes in a variety of forms and from a variety of sources. Thus, sulfide is an inevitable by-product of sulfate reduction associated with methanogenesis (if the effluent from which the methane is being generated contains significant amounts of sulfate) and the oil and gas industries. Thiosulfate and thiocyanate make up a substantial amount of the chemical content of photographic processing waste, and some papermaking processes generate both inorganic and organic sulfides. Of course, the amount of reduced sulfur compounds generated from industrial processes pales into insignificance when the quantity generated from animal wastes is considered, and research into methods of dealing with this is currently underway.

Reduced sulfur compounds present a problem both environmentally, because of their toxicity, and socially, because of their odor. If large amounts of sulfide are released into natural waters, this can result in oxygen depletion, either because of the oxygen demand for biological oxidation or, in the absence of suitable bacteria, by spontaneous chemical oxidation. Many water treatment plants impose surcharges for the treatment of such effluent, and there is obviously considerable pressure on companies to treat their effluent on the site. There are both chemical and physical methods of removing hydrogen sulfide from effluent; these include the use of ion-exchange resins, absorption with aqueous or organic solvents, and chemical oxidation (Gommers, 1988). Many of these simply transfer the problem to another waste stream or involve expensive or complex processes, and they are all expensive, especially for the removal of the last traces of sulfidic compounds.

Colorless sulfur bacteria occur in many sewage treatment systems and, in fact, are inadvertantly used to oxidize reduced sulfur compounds in the waste water. In some cases, this can lead to problems, such as the "bulking" caused by *Thiothrix*. The deliberate use of the biological treatment of sulfide-containing waste using colorless sulfur bacteria has attracted considerable attention of late. The end products (sulfur or sulfate) are not hazardous, and sulfate can be discharged directly into the sea or into brackish estuaries (which already are so high in sulfate that the discharge is insignificant). Moreover, biological treatment systems can be based on existing reactor designs (e.g., fluidized and packed bed reactors) and require very little in the way of new technology.

Another advantage of a biological process is that it can be combined with the treatment of other problems in an effluent. For example, the effluent of a methane reactor will contain ammonia in addition to sulfide. If the ammonia is then converted to nitrate or nitrite by aerobic, nitrifying bacteria, the resulting effluent can then be recycled to provide the electron acceptor for a sulfide-oxidizing reactor immediately after the methane reactor. The microbiological investigation of such a sulfide-oxidizing, denitritrifying reactor revealed the presence of large numbers of facultatively autotrophic colorless sulfur bacteria, which could oxidize sulfide to sulfate while reducing nitrate to nitrogen gas (Robertson and Kuenen, 1983a). In addition to the removal of nitrogen compounds, other advantages associated with the use of denitrifying bacteria rather than aerobic ones include lower production of both biomass and acid.

Combined Sulphide Oxidation and Denitrification. A denitrifying, sulfide-oxidizing reactor system was patented by a Dutch company, Gist brocades, for the post-treatment of effluent from methane-producing reactors (Patent number E.P.A.0051 888). Studies on a laboratory-scale model of this reactor, running on artificial waste water, revealed that sulfide (2–3 kg S/m^3·day), acetate (4–6 kg S/m^3·day) and nitrate (5 kg S/m^3·day) were all effectively removed (Gommers et al., 1988a). The rate-limiting step in the reactor proved to be the oxidation of sulfur to sulfate and, under most loads, the biomass had an overcapacity for both the oxidation of sulfide to sulfur and the conversion of acetate (Gommers et al., 1988b). During experiments in which nitrate depletion occurred, it became evident that in the absence of nitrate, at least one member of the bacterial community was able to reduce any available sulfur, thus illustrating the need for careful monitoring of the electron donor:electron acceptor ratios in such reactors (Gommers et al., 1988b).

The facultatively autotrophic species *Thiosphaera pantotropha* was isolated from a denitrifying, sulfide-oxidizing fluidized bed reactor that was supplied with approximately equivalent amounts of organic and inorganic substrates (Robertson and Kuenen, 1983b), and it initially appeared that the selection of a facultative bacterium would lend support to the model described for the ecological niches of aerobic, fresh-water sulfur-oxidizing bacteria (Fig. 4). However, subsequent attempts to isolate obligate autotrophs from a laboratory-scale model of this system that was being fed with an exclusively inorganic feed also resulted in the isolation of facultative autotrophs (M. Verbeek, W. Bijleveld, L. A.

Robertson, and J. G. Kuenen, unpublished observations). As yet, it is not clear whether obligate autotrophs were present in the inoculum, or the isolation techniques employed were inadequate for any obligate autotrophs present (although they were adequate for the cultivation of known obligate autotrophs), or whether growth in a biofilm in this type of reactor poses an additional selective pressure that favors facultatively autotrophic bacteria. Work has shown that a number of sulfide oxidizers from a wastewater system required cultivation on special membrane filters with sulfide gas before isolated colonies could be obtained (G. C. Stefess, R. de Schrijver, and J. C. de Bruyn, unpublished observations).

The same basic idea, that of using denitrifying colorless sulfur bacteria, was employed in a method proposed by Sublette and Sylvester (1987) for removing H_2S from gas streams by passing them through a reactor containing *Thiobacillus denitrificans*. The bacteria were first immobilized by co-culturing with floc-forming heterotrophs after the authors demonstrated that the presence of the heterotroph had no effect on the sulfide oxidation rate of *T. denitrificans*.

Removal of Sulfide as Elemental Sulfur. As already mentioned, sulfate-containing effluents can be discharged into the sea without significantly increasing the sulfur budget. However, the same is not true if the effluent is discharged into a body of fresh water. To overcome this problem, recovery as elemental sulfur, an intermediate in the oxidation of sulfide to sulfate, would be more appropriate. Research has shown that certain *Thiobacillus*-like bacteria are more inclined to produce sulfur than other species, and that both the dissolved oxygen and the sulfide concentration play an important part in determining whether sulfur or sulfate is the primary end product during sulfide oxidation. Both electron acceptor limitation and high sulfide loads favor sulfur production (Stefess and Kuenen, 1989). A pilot plant based on this principle, using a mixed bacterial biofilm reactor to treat the effluent from a paper mill, is being developed in the Netherlands (Buisman, 1989).

Removal of Organic Sulphides. A problem frequently encountered during the alkaline pulping of wood is the production of organic sulfides, such as methyl mercaptan and dimethyl sulfide. Alkaline pulping is done in order to improve the yield and quality of pulp derived from conifers to be used primarily in the manufacture of paper. Organic sulfides are toxic at even lower concentrations than hydrogen sulfide and have a very low threshold odor. Despite their toxicity, it has proved possible to grow bacteria on high concentrations of organic sulfides by using substrate-limited chemostats (Suylen et al., 1986; Kanagawa and Kelly, 1986; Smith and Kelly, 1988a, 1988b, 1988c). That the ability to oxidize these compounds may be widespread is suggested by the observation that the dominant organism in one set of experiments was a *Hyphomicrobium* species that was later shown to be able to grow as a facultative chemolithotroph on organic sulfur compounds in pure culture (Suylen and Kuenen, 1986; Suylen et al., 1986), whereas the key organism in the other series was a strain of *Thiobacillus thioparus*, an obligate autotroph (Kanagawa and Kelly, 1986). Immobilized cells of *T. thioparus* strain TK-m have now been successfully used on the laboratory scale to deodorize gases containing methyl mercaptan, dimethyl sulfide, dimethyl disulfide, and hydrogen sulfide (Kanagawa and Mikami, 1989; Tanji et al., 1989).

All of the colorless sulfur bacteria mentioned thus far are beneficial in wastewater treatment. However, in oxidation tanks fed with sulfide-containing waste water, the filamentous *Thiothrix* species can cause problems because they are associated with the phenomenon known as "bulking"; this occurs when bacterial aggregations that usually settle easily become loose and flocculent. This can result in blockages or loss of the biomass from the reactor.

Leaching-Associated Activities

Acidophilic bacteria are used in the recovery of metals from poor ores by leaching, and their potential use in the desulfurization of coal is currently being studied. To some extent, coal desulfurization and microbial leaching are the same process, in that in both cases sulfidic ores are oxidized, using similar organisms. However, the desired end products are different, and they are thus generally discussed separately. The aim of coal desulfurization is to produce a solid product (coal) that is as free of sulfur (including sulfur-containing precipitates) as possible, and it is therefore necessary to convert reduced sulfur compounds to soluble forms. In leaching, it is metal recovery that is important, and the presence of jarosite ($M{\cdot}Fe_3(SO_4)_2OH_5$, where M is a monovalent cation such as Na^+ or K^+) and other precipitates in the solid waste is not relevant (although it may constitute an environmental problem around the leaching heaps).

Bacterial Leaching. Bacterial leaching is used in the recovery of metals from ores that are too poor for conventional metallurgical extraction

methods (Tuovinen and Kelly, 1972; Brierley and Lockwood, 1977; Brierley, 1982). Combinations of *T. ferrooxidans* and either *T. thiooxidans* or *T. acidophilus* (previously called *T. organoparus*) and *Leptospirillum ferrooxidans* have been associated with the degradation of pyrite (FeS_2) and chalcopyrite ($CuFeS_2$). The leaching reactions may involve the direct bacterial oxidation of the sulfide ores with oxygen and/or an indirect process during which ferric ions produced by the bacterial oxidation of ferrous iron are used to chemically oxidize the sulfide ores. The ferric ions are thereby reduced to ferrous iron, which, in turn, can be recycled by the bacteria. During this process, other metallic ions such as cupric copper dissolve. Other metals that have been extracted using processes that involve bacteria include zinc, uranium, lead, gold, molybdenum, and, especially, copper.

Dump leaching operations, which are frequently used to extract copper, can be fairly primitive, involving the creation of ore dumps, often in valleys or old open pit mines. As water percolates through the heaped rocks, bacterial activity releases the metals into solution. This solution is then collected in catch basins, the metals recovered, and the liquids recycled to the top of the dump. A somewhat better controlled system is known as heap leaching. During this process, the ore-bearing rocks are crushed to promote contact with the acidified water, and the heaps are built on impermeable bases that prevent seepage into the soil beneath. Aeration systems can be built into the heaps. It is to be expected that as mineral reserves become depleted, and it becomes economically attractive to extract even small amounts of metals in poor ores and spoilage heaps, technological improvements will increase the efficiency of microbial leaching processes and, perhaps, lessen their environmental impact. For a full review of bacterial leaching, the reader is referred to reviews such as Brierley (1982) and the volume edited by Ehrlich and Brierley (1990).

Coal Desulfurization. Research into the use of the pyrite-oxidizing abilities of bacteria, such as *Thiobacillus ferrooxidans* and *Sulfolobus* species, for the removal of sulfur compounds from coal before it is burned, thus reducing sulfur emission into the atmosphere, has been carried out at a number of centers in the last decade. It has been shown that such a process could be effective, especially for low-sulfur coals, using consortia of mesophilic bacteria (Bos et al., 1988; Bos and Kuenen, 1990). Laboratory studies have shown that an optimal process requires two steps. First, a mixed-flow inoculation step, where a fairly dense population of bacteria already growing on pyrite can be brought into contact with fresh, finely ground coal at a pH suitable for growth (around pH 1.8). This inoculation step would then be followed by the use of plug-flow reactors, where the bulk of the pyrite oxidation would take place. At the end of the process, the process water can be recirculated, as can some of the biomass-bearing coal particles, to serve as the inoculum for the fresh coal. A plant design, involving a cascade of Pachuca tanks (Fig. 10), was devised for this type of system (Bos et al., 1988). Pachuca tanks (in their simplest form, an inverted cone with aeration at the narrowest point, at the bottom of the tank) are particularly suitable for this type of process because the upflow of air into the tanks not only provides the bacterial community with the oxygen and carbon

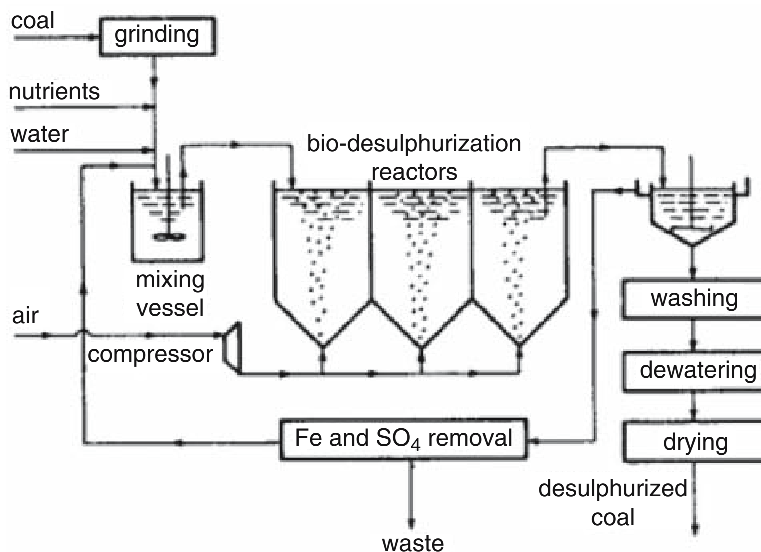


Fig. 10. Simplified scheme for the microbiological desulfurization of coal in Pachuca tanks (bio-desulfurization reactors). After grinding and mixing with water, the coal slurry contains particles less than 100 μm in diameter at a concentration of 20% (w/v). At this particle size, virtually all of the pyrite crystals become accessible to microbial leaching. The total leaching process requires about 10 days. More than 95% of the inorganic sulfur is removed, but little or no organic sulfur is degraded.

dioxide necessary, but it also keeps the slurry well mixed, without any need for complex and expensive stirring mechanisms.

Corrosion

Together with the sulfate-reducing bacteria, many of the sulfuric-acid-producing bacteria, and in particular the acidophiles, have been implicated in many corrosion problems. Indeed a strain of *T. thiooxidans* isolated from a corroding concrete pipe was originally known as *T. concretivorans* (i.e., concrete-eating). In sewage pipes with an aerobic headspace, sulfide may be produced in the anaerobic water phase and then be transferred to the film of water on the aerobic part of the pipe where it may be oxidized to sulfuric acid. In order to dissolve the carbonates in concrete, the pH need only be below 5.0–5.5 and such a pH can be generated by either neutrophilic or acidophilic bacteria. The activities of the acidophiles may also be responsible for steel pipe corrosion (Kuenen and Bos, 1988) as well as many of the pollution problems associated with the acid run-off from mine spoil heaps. These environmental problems are not only associated with the low pH of the water, but also with the toxic concentrations of heavy metals that they may contain. In addition, acidic water containing ferric sulfate may generate precipitates of jarosite, and these can block drainage pipes and cover stream and river sediments.

The Role of Colorless Sulfur-Oxidizing Bacteria in the Sulfur Cycle

Although much is known about the physiology and occurrence of colorless sulfur bacteria, less is known about the quantitative aspects of their activity in nature. Many of the reasons for this are difficulties commonly associated with field work (e.g., heterogeneous samples, unstable gradients, low concentrations of substrates), and are therefore outside the scope of this chapter, but a few difficulties are uniquely associated with the colorless sulfur bacteria.

Commonly used methods for estimating the activity of sulfur-oxidizing bacteria in the field include cell counts, oxidation of (radiolabelled) substrate (sulfide, thiosulfate, or sulfur), product formation (especially sulfuric acid, since this causes pH changes), and $^{14}CO_2$ fixation. Other, more specific techniques include the measurement of substrate-dependent respiration and immunofluorescent microscopy.

Cell Counts

With some of the more conspicuous bacteria (e.g., *Beggiatoa*, *Thiovulum*), it is possible to obtain a rough estimate of numbers based on direct cell counts. However, most of the colorless sulfur bacteria require cultivation before they can be counted. The choice of media and substrates for most-probable-number (MPN) estimates or direct plate counts is especially difficult for the colorless sulfur bacteria. The most obvious problem is that outside the chemostat there is no way of selectively growing facultative autotrophs or chemolithoheterotrophs. They must first be isolated on autotrophic or heterotrophic media, respectively, and then screened for sulfur oxidizing capacity. In addition, low recovery efficiency can be a problem with both plate counts and dilution series. Two other problems are associated with the obligate autotrophs: 1) thiosulfate is frequently used as an energy source in solid media, but this is not always the most suitable energy source. For some bacteria, agar plates containing colloidal sulfur (see Chapter 138) may be more appropriate, while other bacteria may require sulfide. The use of solid sulfide media can present technical problems with regard to toxicity and instability unless one of the less-soluble nontoxic sulfides (e.g., calcium sulfide) is used: 2) some autotrophic species do not give distinct colonies on agar, and moreover, the acidophiles may be inhibited by organic compounds resulting from chemical acid hydrolysis of the agar itself at their required growth pH values. To overcome these agar-associated problems, other techniques (such as the use of silica gel plates or floating filters [de Bruyn et al., 1990] may be more appropriate (G. C. Stefess, R. de Schrijver, and J. C. de Bruyn, unpublished observations). Some of the sulfur-oxidizers may have a requirement for an unidentified growth factor such as a vitamin or mineral.

Activity Measurements

Data on the rates of sulfide oxidation in natural systems are scattered and somewhat variable, possibly because of the difficulty of accurate sampling as well as the reactivity of the compounds involved.

Substrate Uptake and/or Transformation—Chemical and Radioassays. Once cell numbers have been estimated with a degree of confidence, they can only be used to provide an idea of the potential activity of colorless sulfur-oxidizing bacteria within that particular ecosystem. The measurement of substrate transformations (i.e, utilization or accumulation), preferably in situ, can be used as a measure of

actual activity. A major problem associated with the use and measurement of many reduced sulfur compounds, especially sulfide and sulfite, is that they are chemically very reactive and are readily oxidized spontaneously by oxygen. Appropriate controls can, to some extent, overcome this problem, but it must be remembered that in nature biological and chemical reactions compete, and equilibrium reactions causing the exchange of radiolabel in reduced sulfur compounds mean that extra caution must be used in the interpretation of results. Moreover, chemical oxidation rates are influenced by many of the environmental parameters that also affect biological activity (e.g., pH, temperature, chemical constitution of the solutions involved). In a few cases, where dominant populations of known colorless sulfur bacteria occur (e.g., *Sulfolobus* in solfataras, *Beggiatoa* mats), rough estimates have been made of the activity of these organisms. Mosser et al. (1973) found rates for sulfur oxidation to sulfate of 67 and 190 g $m^{-2}·day^{-1}$ for mats of *Sulfolobus acidocaldarius* growing in two hot pools (Moose Pool and Sulfur Cauldron, Yellowstone National Park, respectively). In the Black Sea, a maximum rate of 710 nmol $l^{-1}·day^{-1}$ was observed by Sorokin (1970). For an extended discussion of sulfur oxidation rates in nature, the reader is referred to Kuenen (1975) and to Jørgensen (1988).

Another problem is that the sulfur-oxidizing heterotrophs may also contribute to the turnover of reduced sulfur compounds at natural sites. In some cases, $^{14}CO_2$ fixation can be used to eliminate this but in many locations where mixotrophs or chemolithoheterotrophs are involved, CO_2 may not be the primary source of carbon. This type of experiment could, therefore, sometimes result in underestimates if it is not used in tandem with other measurements. An associated problem is that the specific activity of a given species can vary. For example, Beudeker et al. (1980) found that, when grown under carbon dioxide limitation, the ribulose bisphosphate carboxylase (Rubisco) activity in *T. neapolitanus* was 240 nmol $min^{-1}·mg$ $protein^{-1}$. If, however, thiosulfate was the limiting factor, the enzyme level fell to 72 nmol $min^{-1}·mg$ $protein^{-1}$. Other substrate conversion rates can also vary, especially among species. Thus, it has been found that *T. denitrificans* and *Thiomicrospira denitrificans* oxidize thiosulfate at rates of 0.86 and 2.9 mM thiosulfate g $C^{-1}·h^{-1}$ respectively (Timmer ten Hoor, 1977).

A combination of CO_2 fixation and oxygen and hydrogen sulfide analysis was used to measure microbial activity in Saelenvaan Lake, in Norway. As can be seen from Fig. 11, a peak of CO_2 fixation was found to coincide with the very narrow zone where oxygen and sulfide coexisted.

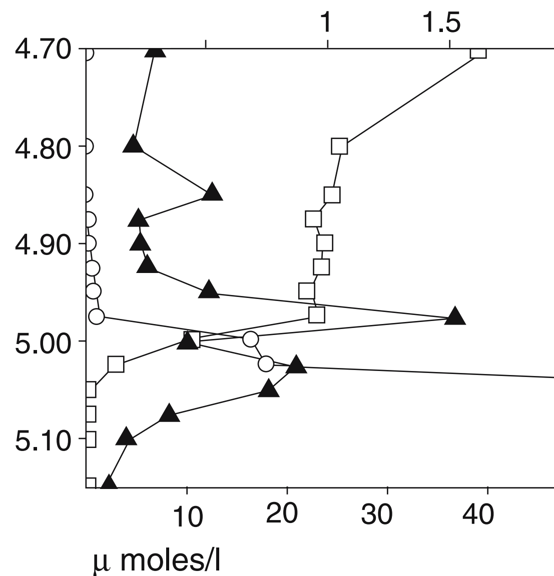


Fig. 11. Profiles of CO_2 fixation, dissolved oxygen, and dissolved hydrogen sulfide concentrations in Saelenvan Lake (Norway) sampled at 5 a.m. on 15 August 1978. The high resolution was due to the use of a special sampling device connected to a pump. CO_2 fixation rates (top horizontal axis) were obtained using $^{14}CO_2$ injected into dark bottles, which were incubated in situ. The left vertical axis is depth in m. The bottom horizontal axis is the concentration of dissolved gas in μmol $liter^{-2}$. Triangles, μmol CO_2 $liter^{-1}·h^{-1}$; squares μmol oxygen $liter^{-1}$; circles, μmol hydrogen sulfide $liter^{-1}$. (From Kelly and Kuenen, 1984.)

It should be noted that the sampling technique was critical for the success of these experiments. A special sampling device with an inlet that removes water from a horizontal area of the column at 1–2 cm intervals (Jørgensen et al., 1979) was necessary—if a less accurate device was used, the very narrow CO_2 fixation zone could not be seen because of dilution by the surrounding water.

Those working on the ecosystems around the hydrothermal vents have, of course, severe difficulties to overcome in making in situ measurements, especially since a new variable, pressure, must be considered (Jannasch, 1985). In order to measure the activity of autotrophic bacteria at these sites, $^{14}CO_2$ fixation was measured in syringes incubated on the sea bed (approximately 250 atmospheres, 3°C) and on board ship (1 atmosphere) at 3 and 23°C. Little or no difference was found between the two samples incubated at 3°C, and the bacteria responsible for the $^{14}CO_2$ fixation were thus obviously barotolerant rather than barophilic. Moreover, $^{14}CO_2$ fixation sharply increased if thiosulfate was added, or when the samples were incubated at 23°C, indicating that mesophilic colorless sulfur bacteria

were responsible (Tuttle et al., 1983; Wirsen et al., 1986).

MICROELECTRODES A technique that has been used with some success in the study of in situ bacterial biofilms and immobilization for biotechnology employs the use of microelectrodes that can be progressively moved through a biolayer, gradually registering the gradients present. The slope of the gradient, combined with data on the diffusion coefficient for the substrate measured, can provide direct information on the flux and turnover of substrates, and thus can give accurate information on in situ activities. These microelectrode systems are frequently linked to a computer that not only controls the rate of passage of the electrode tip through the biolayer, but also records and calculates the results (e.g., Revsbech et al., 1986). Among others, oxygen, pH, sulfide, carbon dioxide, and N_2O microelectrodes are available, but the use of some electrodes (e.g., sulfide, CO_2) is limited by their low sensitivity at commonly used pH values. However, the oxygen electrode has been extensively used, especially in systems where photosynthesis is involved and oxygen supply can easily be controlled by modifying the availability of light (e.g., Jensen and Revsbech, 1989; Revsbech and Ward, 1984). The construction of these electrodes, and their use in various ecosystems, was extensively reviewed by Revsbech and Jørgensen (1986). Their use, in conjunction with some of the other methods mentioned above, may at least provide a means of measuring actual activities in gradients, rather than potential activities in in vitro cultures.

Summary and Conclusion

The carbon metabolism of the colorless sulfur bacteria is the best-known facet of their physiology and biochemistry. New insights into their pathways of sulfur metabolism have done away with the old unifying concept of sulfur metabolism, as it is now clear that there are diverse pathways in the organisms investigated thus far.

With the use of new techniques for cultivating the more fastidious colorless sulfur bacteria (e.g., *Beggiatoa*) in gradient cultures, and with pure cultures of other strains (e.g., *Macromonas*) now available, the way is now open for further research into their (eco)physiology and biochemistry.

It is hoped that microelectrodes, in combination with improved isotope techniques, will also provide more detailed information about the activities of these bacteria in nature.

However, one important question remains—should the colorless sulfur bacteria still be considered a taxonomic group? As discussed throughout this paper, the use as a taxonomic criterion of the ability to gain energy from the oxidation of inorganic reduced sulfur compounds has resulted in the definition of a very heterogeneous group, collectively known as the colorless sulfur bacteria. It is possible that the possession of the relevant pathways for growth on reduced sulfur compounds is of no greater taxonomic relevance than the ability to use the Calvin cycle or to grow on hydrogen. Moreover, it seems likely, in view of the results obtained with 5S and 16S RNA analysis, that we are seeing the result of evolutionary convergence towards the (eco)physiological properties encountered in many of the colorless sulfur bacteria. The extreme heterogeneity of the group is further emphasized as other long-known bacteria are found to also possess the properties of colorless sulfur bacteria. Indeed, the common lack of a test for thiosulfate or sulfide oxidation in routine taxonomic screening has meant that the sulfur-oxidizing potential of species of genera such as *Paracoccus*, *Pseudomonas*, and *Alcaligenes* are only now being recognized.

Despite their morphological and phylogenetic diversity, the colorless sulfur bacteria present a coherent picture in physiological terms. As it is generally the physiological specifications of an organism that define its ecological significance, the reclassification of the colorless sulfur bacteria may present something of a microbiological dilemma because the relationships suggested by the rRNA analysis (Table 8) bear little relation to the ecophysiological activities of the organisms. Thus, in spite of the reallocation of species among different genera, research can only profit from the retaining of physiological, rather than taxonomic, groupings—such as the sulfate reducers, nitrogen fixers, denitrifiers, and colorless sulfur bacteria.

Acknowledgments. We thank Anke de Bruyn, Wim Hazeu, Don Kelly, David Lane, Hiroshi Oyaizu, and Jack Pronk for allowing us access to their results before publication, and to Piet Bos for his constructive comments on the manuscript.

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Prokaryotes (2006) 2:1012–1027
DOI: 10.1007/0-387-30742-7_32

CHAPTER 1.32

Bacterial Stress Response

ELIORA Z. RON

Introduction

Most bacteria live in a dynamic environment where temperature, availability of nutrients, and presence of various chemicals vary. Quick adaptation to these environmental changes is carried out by a series of global regulatory networks that control the simultaneous expression of a large number of genes. There are global regulatory systems that respond to change of temperature, pH, nutrients, salts and oxidation. The level of response by these regulatory networks is proportional to the extent of the change. Since the response level is highest under changes that constitute a stress condition, the control networks are labeled "stress response" systems.

The stress response systems show a high degree of similarity in prokaryotes, and some (e.g., the heat shock response) are also conserved in eukaryotes and archaea. However, the conditions under which the response systems are activated differ significantly from one organism to another. Clearly, the temperatures in which the heat shock response is activated will be much lower for a mesophile than for a thermophile, or the response to salt stress will be completely different in halophiles.

Global Regulatory Networks in Bacteria

The first attempts to study the extent of such regulatory networks were based on proteomic analysis, using O'Farrell two-dimensional (2D) gels, and resulted in the identification of the large group of *Escherichia coli* heat-shock proteins (O'Farrell, 1975; Neidhardt et al., 1981). Later, proteomic-based experiments followed by microarray studies of gene transcription (Hatfield et al., 2003) revealed the size and composition of the various-stress induced stimulons of *E. coli* (VanBogelen et al., 1987b). This induction of large groups of genes in response to a specific environment suggested the existence of global regulatory systems that control the expression of large regulons.

Gene expression can be regulated at the level of transcription or posttranscription. The level of transcription can be regulated by positive control elements—activators—or by negative control elements—repressors. Some of these control elements are specific for one gene, whereas others control a large group of genes, thus creating a regulon. In addition to transcriptional regulation, many posttranscriptional regulatory systems evolved affecting different steps along the way from the gene to the active protein. The posttranscriptional regulatory systems control the stability of the mRNA and the rate of translation initiation. In addition, they can determine the stability of the protein and its activity by carrying out posttranslational modifications. The existence of all of the control elements described here was demonstrated in the global regulatory systems that control the response to heat shock and other environmental and physiological conditions.

Transcriptional regulation is the primary mechanism that regulates gene expression. The process of RNA synthesis and its control was extensively studied in bacteria, especially in *E. coli* and *Bacillus subtilis* (Burgess and Anthony, 2001). The *E. coli* DNA-dependent RNA polymerase is the enzyme responsible for all cellular RNA synthesis. This enzyme consists of a core (subunits $a_2bb'w$) that is capable of elongation and termination of transcription, and an additional subunit (σ), which binds to the RNA polymerase to form the holoenzyme, increases the efficiency of transcription initiation, and determines specific promoter recognition (Burgess et al., 1969). In *E. coli* there are seven known sigma factors: σ^{70} and the vegetative sigma factors, σ^{S}, σ^{32}, σ^{F}, σ^{E}, σ*fecI* and σ^{54} (Helmann and Chamberlin, 1988; Lonetto et al., 1992; Burgess and Anthony, 2001). The sigma factors serve as master regulators mainly by competition for the core RNA polymerase, which is the limiting component of the transcription machinery (Ishihama, 2000). Additional regulation of transcription is exerted by repressors, transcriptional activators, sigma-binding anti-sigma factors, and even by small RNAs (Hughes and Mathee, 1998; Helmann, 1999; Vicente et al.,

1999; Ishihama, 2000; Severinov, 2000; Wassarman and Storz, 2000).

These various control elements regulate the expression of genes during environmental conditions such as starvation, sporulation and additional stress conditions. For example, the *E. coli* stationary phase is regulated by the master regulator σ^S (Lange and Hengge-Aronis, 1991). The levels of σ^S itself are affected by *cis* and *trans* elements—small molecules such as guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and homoserine lactone, and the proteins that react to them, such as cAMP receptor protein (CRP)-cAMP (Hengge-Aronis, 2000). Sigma factor S regulates the induction of more than 50 genes (Hengge-Aronis, 2000). All of these elements create a complex regulatory network that enables the bacterial cell to adapt to the changing environment.

Stress Response, Stimulons and Regulons

In bacteria the stress responses are regulated by several control patterns: 1) Transcriptional control by alternative sigma factors is the most prevalent control pattern. Basically, genes or operons that belong to a specific response regulon contain a promoter that is recognized by a specific, alternative, sigma factor. The function of this sigma factor correlates with the conditions that bring about the response. As an example, in Gram-negative bacteria, the response to elevated temperatures is mediated by two alternative sigma factors (σ32 and σE) whose activities are temperature-dependent. 2) Transcription is controlled by repressor binding to a DNA control element. An example of this control is the HrcA repressor that binds to a conserved inverted repeat control element known as "CIRCE" (for "controlling inverted repeat of chaperone expression") present upstream of operons that code for heat shock proteins in Gram-positive and some Gram-negative bacteria. 3) Transcription is controlled by proteolysis. Well defined is the salt overly sensitive (SOS) response to genotoxic effects, which is mediated by a series of autoregulated proteases. Recently, control by proteolysis has emerged as one of the major systems regulating the availability of alternative sigma factors and other stress-related global processes (Hengge and Bukau, 2003). And 4) transcription is controlled by small RNAs. Recent findings indicate that small RNAs, about 50 of which are present in the *Escherichia coli* genome, control the cellular concentration of RpoS (sigma38), the alternative sigma factor of the starvation (or stationary) response. Small RNAs also control the response to oxidative stress.

A regulon is defined as all genes regulated by the same control pattern, while a stimulon is defined as all the genes whose expression responds to the same conditions. Stimulons are easily delineated by monitoring gene expression in a micro-array or on two-dimensional protein gels. Regulons can only be established following characterization of the molecular basis for the change in gene expression. Clearly, level of overlap between the various regulons and stimulons is high. Thus, the stimulon that responds to shifts to higher temperatures contains genes from at least two regulons (i.e., σ32 and σE). Yet, some of the genes of the σ32 regulon may also be controlled by the HrcA repressor, and so on.

Here, the focus is on two stress response networks—one responding to shifts to higher temperatures (heat shock response) and the other to limitation of carbon source and stationary phase (general stress response).

The Heat Shock Response

The heat shock response was the first global regulatory system to be discovered and is one of the most fundamental. This response is general, found in all living cells examined (Craig, 1985), and is a protective and homeostatic cellular process that increases thermotolerance. It has been studied in many cellular systems such as bacteria, yeast, insects (*Drosophila melanogaster*; Michaud et al., 1997), worms (*Caenorhabditis elegans*; Rose and Rankin, 2001), and mammals (Christians et al., 2002; Li et al., 2002; Srivastava, 2002). The heat shock response is characterized by the induction of a large set of proteins (heat shock proteins—HSPs) as a result of a rapid increase in the environmental temperature. Many of the HSPs are molecular chaperones (e.g., GroEL, GroES, DnaK and DnaJ) and ATP-dependent proteases (e.g., ClpP, Lon (La) and HslVU) that play a critical role in the restoration of protein folding and in protein degradation under normal and stress conditions. Proteins such as GroEL (the bacterial homolog of Hsp 60) and DnaK (the bacterial homolog of Hsp 70) are highly conserved in evolution all the way from bacteria to humans (Boorstein et al., 1994; Gupta, 1995). Although the major proteins in the heat shock response are highly conserved, the regulation of the response varies between different organisms and different bacterial species. Several regulatory systems evolved in bacteria and will be discussed here.

The Hsps are important for protection against environmental stress, and they produce tolerance against high temperature, high salt and

Table 1. Major heat-shock proteins of *Escherichia coli*.

Protein	Function	Molecular weight (kDa)	Theoretical pI	Reference(s)
ClpB	Chaperone	96	5.37	Kitagawa et al., 1991
DnaJ	Chaperone	39	7.98	Bardwell et al., 1986
DnaK	Chaperone	69	4.83	Bardwell and Craig, 1984
GroEL	Chaperone	57	4.85	Neidhardt et al., 1981
GroES	Chaperone	10	5.15	Tilly et al., 1983b
HslR (Hsp 15)	Chaperone	15	9.94	Chuang and Blattner, 1993
Hsp33 (HslO)	Chaperone	33	4.65	Chuang and Blattner, 1993
HtpG	Chaperone	71	5.09	Bardwell and Craig, 1987
IbpA (HtpN, HslT)	Chaperone	16	5.57	Allen et al., 1992
IbpB (HtpE, HslS)	Chaperone	16	5.19	Allen et al., 1992
ClpP	Protease	24	5.52	Maurizi et al., 1990
ClpX	Protease	46	5.24	Gottesman et al., 1984
DegP (HtrA)	Protease	50	8.65	Lipinska et al., 1988
FtsH (HflB)	Protease	71	8.91	Ishihama, 2000
HslU (Clp Y, HtpI)	Protease	49	5.24	Chuang et al., 1993
HslV (Clp Q, HtpO)	Protease	19	5.96	Chuang et al., 1993
Lon (La)	Protease	87	6.01	Gayda et al., 1985
σ^{32} (RpoH, HtpR, Hin, Fam)	Sigma factor	32	5.64	Landick et al., 1991
σ^{20} (RpoD, Alt)	Sigma factor	70	4.69	Burton et al., 1981
σ^{E} (σ^{24}, RpoE)	Sigma factor	22	5.38	Raina et al., 1995
PrpA (PphA)	Phosphatase	25	6.94	Morita et al., 2000
Htp X	Unknown	32	6.60	Kornitzer et al., 1991
Htp Y (HtgA)	Unknown	21	9.44	Missiakas and Raina, 1997
HtrC	Unknown	21	9.33	Raina and Georgopoulos, 1990
PspA	Unknown	25	5.39	Jovanovic et al., 1996
FtsJ	Unknown	23	9.44	Herman et al., 1995

heavy metals (VanBogelen et al., 1987a; Inbar et al., 1993; Hecker and Volker, 1998). Heat-shock proteins also play critical roles in bacterial virulence and in protective systems such as the human immune system (Christians et al., 2002; Li et al., 2002). Several Hsps were found to protect against damage induced by temperature upshifts. Among the characterized proteins are the main cellular chaperone machineries GroE and DnaK, the ATP-dependent proteases Lon (La), HslVU, ClpP, DegP and FtsH (FhlB), and other proteins involved in protein folding, refolding, quality control, and degradation. GroE and DnaK are both multimeric complexes that have ATP-dependent activity (Sherman and Goldberg, 1992; Sherman and Goldberg, 1996; Kandror et al., 1994). The GroE catalytic complex involves GroEL and GroES in a ratio of 1 : 2, creating a football-shape molecular structure (Sparrer et al., 1997). This complex catalyzes protein refolding, and is involved in protein degradation by the ATP-dependent proteases (Sherman and Goldberg, 1992; Sherman and Goldberg, 1996; Kandror et al., 1994). These ATP-dependent proteases degrade abnormal proteins under stress and nonstress conditions, and in addition play major regulatory functions by controlling the degradation of specific proteins (Goldberg, 1972; Maurizi, 1992; Gottesman, 1996; Deuerling et al., 1997; Zhou et al., 2001). The role of these and other *E. coli* Hsps in protection against temperature-induced damage is summarized in Table 1.

Heat shock—a rapid up-shift in the environmental temperature—results in various physical and chemical changes in bacterial proteins and membranes. Presumably, these changes, such as protein unfolding, are detected by cellular systems, which induce the large set of heat shock proteins to cope with the changes and the potential damage. This heat shock response is regulated by several control elements, thus dividing the major stimulon of heat shock proteins into several regulatory groups (regulons).

The heat shock proteins are highly conserved, whereas the control of their expression is highly variable between organisms and even between various bacteria. One of the control elements found in Gram-negative bacteria is a heat shock σ factor that regulates the transcription of the major Hsps. The Gram-negative *E. coli* is a good example of this system because the synthesis of the major Hsps is regulated by the alternative sigma factor called "σ^{32}." In addition, there is a group of proteins induced under conditions of elevated temperature that is regulated by another heat shock sigma factor, σ^{E} (encoded by *rpoE*). In other Gram-negative bacteria, such as the *Agrobacterium tumefaciens* of the Alphaproteobacteria, the control systems are more

complicated. For example, the transcription of GroESL synthesis is stimulated during heat shock by a σ^{32}-like activator, but in non-heat shock conditions, transcription is repressed by the HrcA protein that binds to the CIRCE sequence upstream of the promoter region (Segal and Ron, 1993; Nakahigashi et al., 1999). The control system of HrcA-CIRCE was first described in the Gram-positive *Bacillus subtilis* (Zuber and Schumann, 1994).

The following sections will describe the specific control mechanisms in various bacterial groups. In short, the heat shock response in bacteria is controlled by one or a combination of both of the following control systems: 1) The first system involves alternative sigma factors that act as transcriptional activators by recognizing specific heat shock promoters upstream of heat shock genes. Among these are σ^{32} and σ^{E} of the Gram-negative bacteria, and σ^{B} of the Gram-positive bacteria. 2) The second system utilizes transcriptional repressors. The most conserved and the most ubiquitous among these repressors is HrcA (heat regulation at CIRCE), which binds to a conserved CIRCE present upstream of the heat shock operons. Heat shock operons controlled by HrcA-CIRCE are transcribed by the vegetative sigma factor σ^{A} (= σ^{70}) in Gram-positive bacteria and by the heat shock sigma factor s^{32} in Gram-negative bacteria.

Heat Shock Control Elements in Gram-Negative Bacteria

The first model organism for studying the heat shock response in Gram-negative bacteria was *E. coli*. Most of the heat shock genes of this bacterium are regulated by transcriptional activators, the alternative sigma factors (s^{32} or s^{E}).

Sigma-32-Controlled Genes

The heat shock response of Gram-negative bacteria is regulated mainly by the alternative sigma factors σ^{32} and σ^{E} (Morita et al., 2000). Sigma 32 is a master regulator encoded by the *rpoH* (*htpR* or *hin*) gene that was the first of a group of minor sigma factors discovered in *E. coli* (Grossman et al., 1984; Landick et al., 1984; Yura et al., 1984). This discovery of minor sigma factors led to the general concept of gene regulation by specific sigma factor-dependent transcription. In *E. coli*, at least 34 heat shock genes (out of 51 heat-shock induced loci) are regulated by σ^{32} (Richmond et al., 1999; Morita et al., 2000). The genes were identified by transcription analysis of specific genes, an examination of the synthesis rates of individual proteins, or proteomics and genomics approaches. This regulon includes all the major cytoplasmic Hsps of *E. coli*.

The σ^{32} regulon includes most of the proteins involved in protein folding, repair, and degradation. Such proteins are the heat-shock-induced molecular chaperones ClpB, DnaK, DnaJ, GroEL and GroES, which are involved in protein folding and prevention of protein aggregation (Neidhardt et al., 1981; Tilly et al., 1983b; Bardwell and Craig, 1984; Bardwell and Craig, 1986; Kitagawa et al., 1991; Tomoyasu et al., 2001). The regulon comprises also all of the important cytosolic proteases Lon (La), ClpP, ClpX, HslV (ClpY), and HslU (ClpQ; Goldberg, 1972; Gayda et al., 1985; Maurizi et al., 1990; Chuang et al., 1993b), and the membranal metaloprotease FtsH (HflB; Herman et al., 1995; Tomoyasu et al., 1995). Other important σ^{32}-regulated proteins are HTS (homoserine transsuccinylase), which is a key enzyme in methionine biosynthesis (Biran et al., 1995), proteins involved in protein isomerization (PpiD; Dartigalongue et al., 1998) and HtrM (Raina and Georgopoulos. 1991), and the vegetative sigma factor (σ^{70-}; Burton et al., 1981).

Homologs of *rpoH* were identified in more than twenty species of eubacteria from the alpha, beta and gamma subgroups of proteobacteria (Sahu et al., 1997; Andersson et al., 1998; Emetz et al., 1998; Huang et al., 1998; Karls et al., 1998; Nakahigashi et al., 1998; Nakahigashi et al., 1999; Nakahigashi et al., 2001). In some of these bacteria, the *rpoH* homologs demonstrates translational induction and stabilization upon heat shock, which are very similar to those found in *E. coli* (Nakahigashi et al., 1998).

The general function of the σ^{32} regulon was studied in several bacterial species by analysis of *rpoH* mutants. These mutants were usually found to be temperature sensitive (Zhou et al., 1988; Huang et al., 1998; Nakahigashi et al., 1999). As expected from their temperature-sensitive phenotype, some of the heat-shock proteins are essential at elevated temperature.

The levels of σ^{32} and its activity are temperature-regulated at several levels. At low temperature (30°C), when low amounts of heat-shock proteins are required, the intracellular concentration of σ^{32} is fewer than 50 molecules per cell (Straus et al., 1987; Craig et al., 1991). These low levels are maintained due to transcriptional repression and protein instability. Upon a rapid shift to 42°C, the level increases 15–20-fold within 5 min, and then declines to a new steady state level, 2–3-fold higher than the pre-shift level (Straus et al., 1987). The levels and the time-course of σ^{32} induction are sufficient for the necessary induction of heat-shock-gene expression upon temperature upshift. A relatively modest heat shock activates the translation of *rpoH* transcripts, and transiently stabilizes σ^{32} (Straus et al., 1987; Nagai et al., 1991), whereas a severe

heat shock (a rapid shift from 30 to 50°C) can also activate *rpoH* transcription (Morita et al., 2000). The decrease in the synthesis of heat-shock proteins upon temperature downshift is primarily a result of the decrease in σ^{32} activity (rather than its levels) caused mainly by an excess of the DnaK chaperone machinery (Straus et al., 1989; Taura et al., 1989).

The transcriptional regulation of the *rpoH* gene is very complex. It can be transcribed from at least four promoters, three of them (P1, P4 and P5) are recognized by the vegetative σ^{70}, and the fourth (P3) is recognized by σ^{E} (Erickson et al., 1987; Nagai et al., 1990). P3- and P4-transcription of *rpoH* is negatively regulated by DnaA (Wang and Kaguni, 1989), and P4-and P5-transcription is controlled by an additional negative control system—the cAMP-CRP/CytR nucleoprotein complex (Kallipolitis et al., 1998).

Several findings indicate that the heat shock induced σ^{32} levels are also regulated at the translational level. Expression of *rpoH-lacZ* translational fusion but not transcriptional fusion can be induced. Furthermore, heat induction of the fusion protein occurs even when RNA synthesis is inhibited (Nagai et al., 1991). Recent results based on extensive in vivo and in vitro experiments related to the secondary RNA structure have shown that the translation regulation of RpoH is mediated by the rpoH mRNA's secondary structure (Morita et al., 1999; Morita et al., 2000).

Sigma-32 level is regulated by not only its expression level but also the turnover of the protein. Although this protein is unstable during normal growth at 30°C (or even at 42°C), significant stabilization occurs immediately upon temperature upshift from 30°C to 42°C and continues for 4–5 minutes (Straus et al., 1987). The protein instability involves the DnaK chaperone machinery. Mutants in DnaK, DnaJ, or GrpE markedly stabilize σ^{32} under nonstress conditions (Tilly et al., 1983a; Tilly et al., 1989; Straus et al., 1990), indicating this involvement of these proteins in σ^{32} turnover. The initial studies suggested that the membrane-associated metalloprotease FtsH (HflB) is responsible for σ^{32} degradation (Herman et al., 1995; Tomoyasu et al., 1995). However, later studies were able to show that the cytosolic proteases Lon (La), HslVU and ClpP are also involved in σ^{32} degradation (Wawrzynow et al., 1995; Kanemori et al., 1997; Kanemori et al., 1999; Morita et al., 2000). Although the relative significance of each protease is difficult to determine in σ^{32} degradation, the latter three proteases seem to play a significant role in the degradation, possibly even equivalent to that of FtsH (Kanemori et al., 1997). Presumably during heat shock the DnaK machinery and the proteases become occupied by the misfolded and unfolded proteins that accumulate because of the denaturing effect of temperature increase. Consequently, levels of the proteolytic machinery are insufficient to bring about σ^{32} degradation and it accumulates and activates the transcription of the heat shock genes. Since the DnaK chaperones and the proteases have σ^{32} promoters, their synthesis is increased and therefore a few minutes after the temperature upshift, the level of the proteases and chaperones is high enough to destabilize σ^{32}, bringing the level of the heat shock proteins to a new steady state.

The final level of σ^{32} regulation is activity regulation (Morita et al., 2000). This regulation operates mainly by creating ternary complexes of (DnaK-ADP)-DnaJ-σ^{32} that sequester the σ^{32} that competes with the RNA polymerase core enzyme (Gamer et al., 1992; Gamer et al., 1996; Liberek et al., 1992; Liberek and Georgopoulos, 1993). Then, GrpE binds to the ternary complex and stimulates ADP release and complex dissociation by triphosphate (TP) binding. This cycle of binding and release appears to play an important role in σ^{32} activity (and possibly stability) in vivo (Gamer et al., 1992; Morita et al., 2000).

The σ^{32} control system has been well characterized in *E. coli* and other Gammaprotebacteria. However, σ^{32}-like heat shock transcriptional activators have recently been demonstrated in other bacteria, such as *Agrobacterium tumefaciens* of the Alphaprotebacteria (Nakahigashi et al., 1995; Nakahigashi et al., 1998; Nakahigashi et al., 1999; Segal and Ron, 1995a). The σ^{32} of the Alphaprotebacteria is different from that of *E. coli*, and the heat shock promoters are also different in the two groups of Gram-negative bacteria (Nakahigashi et al., 1999; Segal and Ron, 1995a; Fig. 1). The physiological difference of the two sigma factors may be more important: while the *E. coli* σ^{32} is unstable and tightly controlled by proteolysis carried out by the FtsH protease, the alphaprotebacterial σ^{32} is a stable protein, whose activity is affected mainly by a DnaK-mediated control (Nakahigashi et al., 2001).

Genes Controlled by σ^{E}

Another alternative sigma factor involved in the heat-shock response is σ^{E} (σ^{24}), which was found to be an essential gene in *E. coli* at all temperatures (De Las Penas et al., 1997). Presumably the σ^{E} regulon protects cells against extracytoplasmic stress-derived damage. Genes belonging to the σ^{E} regulon are important for bacterial pathogenesis: the mucoid phenotype of *Pseudomonas aeruginosa* in cystic fibrosis infections is controlled by AlgU, an analogue of σ^{E} (Yu et al., 1995), and *rpoE* mutants of *Salmonella typhimu-*

Putative promoters

Heat shock promoter in α-subdivision	CTTG	<17/18>	CYTAT-T
Heat shock promoter in γ-subdivison	TCTC-CCTTGAA	<13/14>	CCCAT-AT
Vegetative promoter in γ-and α-subdivsions	TTGACA	<17>	TATAAT

Promoter recognition domains

Sigma factor domain		2.4	4.2
α-Subdiviaion	σ-32	IKA**SIQ**EYILRSWS**L**VK**MG**TT	YGVS**R**ERVRQ**I**EK**R**AMKKLR
γ-Subdivision	σ-32	IKA**EIH**EYVLR**N**WRIVK**VA**TT	YGVS**A**ERVRQ**L**EK**N**AMKKLR
		* * * * * *	* * *
α-Subdiviaion	σ-70	IRQAITRSIADQARTIRIPVHM	F**S**VTRERIRQIEAKALRNV**K**
γ-Subdivision	σ-70	IRQAITRSIADQARTIRIPVHM	F**D**VTRERIRQIEAKALRNV**R**
			* *

Fig. 1. Putative heat shock promoters and promoter recognition domains of σ-32 and σ-70 in alpha-purple and gamma-purple proteobacteria. (From Segal and Ron [1995a] and Nakahigashi et al. [1999].)

rium are defective in growth inside cells (Humphreys, 1999).

The *E. coli* σ^E controls the expression of genes encoding periplasmic folding catalysts, proteases, biosynthetic enzymes for the lipopolysaccharide component lipid A, and other proteins whose functions are involved with the cell envelope. Members of this regulon include periplasmic proteins that are involved in protein metabolism: the protease DegP (HtrA) and the periplasmic peptidyl prolyl isomerase FkpA (Erickson and Gross, 1989; Strauch et al., 1989; Dartigalongue and Raina, 1998).

As mentioned above, σ^E activates transcription of *rpoH* under conditions of severe heat shock, and because it has a σ^E promoter, it also regulates itself. The response is regulated by RseA (an inner membrane antagonist of σ^E), RseB (a periplasmic protein that binds to the periplasmic face of RseA), and the proteases DegS and YaeL. Envelope stress promotes RseA degradation, which occurs by a proteolytic cascade initiated by DegS. There is evidence that one σ^E-inducing stress (OmpC overexpression) directly activates DegS to cleave RseA (Alba and Gross, 2004).

HrcA-CIRCE-Controlled Genes

The HrcA-CIRCE repression system is the major system regulating the operons coding for chaperones in Gram-positive bacteria. This system is comprised of an inverted repeat *cis* element and a *trans* protein-repressor encoded by the *hrcA* gene. The first reported inverted repeat upstream to the *groE* operon was found in *Mycobacterium tuberculosis* in 1989 (Baird et al., 1989). Recognition of this element as a widespread heat-shock control element for the *groE* and *dnaK* operons took several years. Several lines of direct evidence for the role of CIRCE as a negative *cis* element were obtained (Narberhaus, 1999): 1) deletion of the inverted repeat relieved the repression of a reporter gene fusion (*amyS*; Van Asseldonk et al., 1993); 2) placement of CIRCE behind a foreign promoter reduced the expression of the downstream gene (Zuber and Schumann, 1994); and 3) site-directed mutation, or the removal of three or four nucleotides in one arm of the inverted repeat, resulted in an elevated transcription of the downstream genes at normal growth temperature (Zuber and Schumann, 1994; Babst et al., 1996). Transcription remained derepressed when the inverted repeat was restored by compensating mutations in the second arm of the inverted repeat. Therefore, the CIRCE is not only a potential stem and loop structure (because its sequence by itself is required for repression), but also a binding site for a sequence-specific repressor protein that binds to the CIRCE.

The elucidation of CIRCE as a potential repressor-binding site initiated a search for the

counterpart repressor. Major steps towards tracking the repressor were accomplished by two observations (Narberhaus, 1999): 1) a deletion of *orf39*—the first gene of the *dnaK* operon of *B. subtilis* resulted in an elevated levels of *groE* transcript (Schulz et al., 1995); and 2) *B. subtilis* mutants affected in the regulation of *groE* and *dnaK* operons were mapped to *orf39* (Yuan and Wong, 1995a). Moreover, production of Orf39 from a plasmid that carries a functional copy of *orf39* restored the repression activity in one of the mutants (Yuan and Wong, 1995a). The binding of Orf39 to CIRCE was shown by gel retardation (Narberhaus, 1999), and the name "HrcA" ("heat regulation at CIRCE") was given to this protein after disruption of the equivalent gene in *Caulobacter crescentus* (Roberts et al., 1996).

For several years, the HrcA-CIRCE system was found only in Gram-positive bacteria and was considered as a Gram-positive heat-shock control element. However, since the first discovery of the CIRCE element in the Gram-negative Alphaproteobacterium *A. tumefaciens* (Segal and Ron, 1993), many CIRCE elements were identified in other Gram-negative bacteria. The inverted repeat was detected in a large number of phylogenetically distant bacteria, including Gram-negative bacteria of the Alpha-, Beta-, and $Gamma_1$-purple proteobacteria. The only groups where it is probably not present at all are the $Gamma_2$ and $Gamma_3$ purple bacteria, which also include the Gram-negative model organism *E. coli* (Segal and Ron, 1998; Ron et al., 1999). The inverted repeat (TTAGCACTC-N9-GAGTGCTAA) is highly conserved in all of the studied genes (R. Segal and Ron, 1996; Segal and Ron, 1998).

In contrast to Gram-positive bacteria where CIRCE-regulated genes are transcribed with the vegetative sigma factor (σ^A), in *A. tumefaciens* the *groEL* operon is HrcA-CIRCE controlled, but is transcribed mainly by σ^{32} (Nakahigashi et al., 1999). In *A. tumefaciens*, it was possible to show, using 2D gels, that GroE proteins are the only proteins whose synthesis is repressed by the HrcA-CIRCE system (Rosen et al., 2002b). In *Bradyrhizobium japonicum*, two *groESL* operons were found: *groESL*$_1$ is σ^{32} regulated while *groESL*$_2$ is CIRCE-HrcA-σ^{96} dependent (σ^{96} recognizes the housekeeping promoter of *B. japonicum*; Munchbach et al., 1999a). The control of chaperone expression by the HrcA-CIRCE system seems to be more ancient than the σ^{32}-dependent transcription of heat-shock genes because it is found in all the bacteria except two small groups that lost it during evolution, whereas σ^{32}-dependent transcription is found only in Gram-negative bacteria (Ron et al., 1999).

Minor Regulatory Elements

Expression of at least ten genes in *B. japonicum*, seven of which code for small Hsps, is under the control of ROSE (repression of heat-shock gene expression; Narberhaus et al., 1998; Munchbach et al., 1999b). This negatively *cis*-acting DNA element confers temperature control to a s^{70}-type promoter. ROSE elements are not restricted to *B. japonicum* but are also present in *Bradyrhizobium* sp. (*Parasponia*), *Rhizobium* sp. strain NGR234, and *Mesorhizobium loti* (Nocker et al., 2001). The latest model for ROSE activity suggests that ROSE controls heat-shock protein expression by a temperature-dependent secondary structure of ROSE mRNA that controls the access of the ribosome to the ribosome binding site (Nocker et al., 2001).

Proteome analysis of *A. tumefaciens* and in its mutants deleted for *rpoH*, *hrcA* or in both, showed that the heat-shock induction of 32 (out of 56) heat shock proteins is independent of RpoH and HrcA. These results indicate the existence of additional regulatory factors in the *A. tumefaciens* heat-shock response (Rosen et al., 2001; Rosen et al., 2002b). These uncharacterized regulatory elements may also involve ROSE because *A. tumefaciens* belongs to the Rhizobiaceae group.

An additional unique posttranscriptional control mechanism demonstrated in *A. tumefaciens* involved a specific cleavage of the *groESL* operon transcript. The resulting *groES* transcript is rapidly degraded, whereas the *groEL* transcript is stable, leading to a differential expression of the two genes of the operon—as could be detected by quantitative analysis of the protein expression, using 2D-gels (Segal and Ron, 1995b; Rosen et al., 2002b). This mRNA processing is temperature dependent and constitutes the first example of a controlled processing of transcripts in bacteria.

The General Stress Response in *E. coli*

The "general stress response" is induced during carbon starvation or entry into stationary phase. In *E. coli* these conditions result in a variety of physiological and morphological changes that, presumably, ensure survival during periods of prolonged starvation. Although this general stress response was believed to involve the induction of 30–50 proteins (Lange and Hengge-Aronis, 1991), this stimulon now appears to be much larger and involve almost 500 genes, most of which are induced by osmotic shock. About half are induced by stationary phase or acidic stress, and many are induced by more than one, or all of

these stresses (R. Hengge, personal communication). The general stress response is also important in quorum sensing (Schuster et al., 2004).

The genes coding for the general stress response in *E. coli* are transcribed by an alternative sigma factor, σS (RpoS), which recognizes a consensus promoter upstream of the general stress genes. The promoter specificity of σS has been difficult to determine, as the promoter it recognizes appears quite similar to those recognized by the vegetative σ70. The specific σS promoter elements were recently characterized (Becker and Hengge-Aronis, 2001; Gaal et al., 2001; Hengge-Aronis, 2002; Lee and Gralla, 2002; A. Typas and R. Hengge-Aronis, personal communication) and the results suggest that the selectivity is provided by the K173 (the lysine in position 173 of the amino acid sequence) in σS (which is glutamate in σ70). σS binds to the C(-13) and the distal upstream (UP) element –35 of the promoter.

The *E. coli* RpoS is a highly unstable protein, whose degradation is inhibited by various stress signals, such as carbon starvation, high osmolarity and heat shock. As a consequence, these stresses result in the induction of σS-regulated stress-protective proteins (Bouche et al., 1998). Proteolysis of σS requires the response regulator RssB (a direct recognition factor with phosphorylation-dependent affinity for σS, which targets σS to the ClpXP protease; Zhou et al., 2001; Pruteanu and Hengge-Aronis, 2002). Recognition of σS by the RssB/ClpXP system involves two distinct regions—region 2.5 of RpoS is a long α-helix which binds phosphorylated RssB. This binding exposes a second region of RpoS, located in the N-terminal part, which is a binding site for the hexameric ring of the ClpX chaperone (Studemann et al., 2003).

Recent studies demonstrate the involvement of small, noncoding RNAs (Vogel et al., 2003) in the proteolysis of σS. These small noncoding RNA sequences are abundant—around 50 such sRNAs were described in *E. coli*. The levels of many of these sRNAs vary with changing environmental conditions, suggesting a potential regulatory function. At least three sRNAs were found to affect the regulation of RpoS translation (Repoila et al., 2003). DsrA and RprA stimulate RpoS translation in response to low temperature and cell surface stress, respectively, whereas OxyS represses RpoS translation in response to oxidative shock. However, in addition to regulating RpoS translation, DsrA represses the translation of HNS (a global regulator of gene expression), whereas OxyS represses the translation of FhlA (a transcriptional activator), allowing the cell to coordinate different pathways involved in cell adaptation.

Control of the Heat Shock Response and the General Stress Response in Gram-Positive Bacteria

Although the stress gene and proteins in Gram-negative and Gram-positive bacteria are highly conserved, regulation of these genes is very variable. The presence of HrcA-CIRCE control elements has been noted in only some Gram-negative bacteria, and a comparison of Gram-negative with Gram-positive bacteria reveals major differences. Table 2 shows the factors affecting regulation of major stress genes in Gram-positive bacteria and in two Gram-negative bacteria belonging to the Alphaproteobacteria and Gammaproteobacteria. The data indicate that the expression of a stress protein can be under the control of different regulons, and also show difference in control elements between the various bacteria.

Many of the genes that in Gram-negative bacteria belong to the heat shock regulon (as their expression is controlled by the heat shock tran-

Table 2. Regulation of major stress genes.

Gene	Function of gene product	Bacteria	Regulon	Transcription during stress	Control element	Stability of gene product
DnaK	Chaperone	Gram positive	Heat shock	σA (σ70)	CIRCE	
		Alphaproteobacteria	Heat shock	σ32		
		Gammaproteobacteria	Heat shock	σ32		
GroEL	Chaperone	Gram positive	Heat shock	σ70	CIRCE	
		Alphaproteobacteria	Heat shock	σ32	CIRCE	
		Gammaproteobacteria	Heat shock	σ32		
rpoH	Activator—σ32	Alphaproteobacteria	Heat shock	σ32		Stable
		Gammaproteobacteria	Heat shock	σ32, σE		Unstable
lon, clpP	Proteases	Gram negative	Heat shock	σ32		
		Gram positive	General stress	σB		

Abbreviation: CIRCE, a conserved inverted repeat control element or "controlling inverted repeat of chaperone expression."

scriptional activator σ32) constitute part of the general stress response in Gram-positive bacteria. The only genes that are truly "heat shock genes" in Gram-positive bacteria are the genes coding for the major chaperones—Hsp10 and Hsp60 (GroES and GroEL) and the Hsp70 group (DnaK, DnaJ and GrpE).

Heat Shock Response

The model organism for studying the heat shock response in Gram-positive bacteria is *B. subtilis*. In contrast to *E. coli*, where most heat shock proteins are exclusively under the control of the alternative sigma factor σ32, Gram-positive bacteria have no heat-shock specific sigma factor. Rather, the heat-shock response of these bacteria involves the induction of the major chaperones, which is regulated by the HrcA-CIRCE control elements (Zuber and Schumann, 1994; Hecker and Volker, 1998), and several groups of proteins regulated by specific control elements, all of which are discussed below. Another major difference is that some of the proteins, which are part of the heat shock regulon in *E. coli* (such as the Clp proteases), are part of general stress proteins (GSPs) in *B. subtilis*, whose induction is regulated by the alternative sigma factor σ^B.

HRCA-CIRCE CONTROLLED GENES. This system, consisting of the HrcA repressor which binds to the CIRCE inverted repeat, was already described in the section The Heat Shock Response and General Stress Response in Gram-Negative Bacteria. Though in Gram-negative bacteria this system controls only the *groESL* operon, its role in Gram-positive bacteria is much more central. In the Gram-positive bacteria, the genes coding for Hsp70 (DnaK) and the proteins functionally associated with it are also under the control of HrcA-CIRCE. Thus, this control element regulates the expression of the genes coding for all the major chaperones. Notably, in these bacteria, the genes coding for the group of Hsp70 chaperones are usually organized in one operon: *grpE-dnaK-dnaJ*. In the group of low G+C Gram-positive bacteria, such as *B. subtilis*, this operon also contains the gene coding for the HrcA repressor and is *hrcA-grpE-dnaK-dnaJ*(R. Segal and Ron, 1996).

In *B. subtilis*, the operons regulated by the HrcA-CIRCE system (*groESL* and *dnaK* operons) are always transcribed during heat shock by the vegetative sigma factor σ^A (Yuan and Wong, 1995b). This situation is different from the Gram-negative bacteria, in which all the heat shock operons, including the *groESL* operon (which contains the CIRCE element) are transcribed by the specific heat shock σ32. Recently GroE itself has been shown to autogenously regulate the transcription of the *groE* and *dnaK* operons by the finding that the GroE chaperonin machine modulates the activity of the HrcA repressor (Mogk et al., 1997).

GENES CONTROLLED BY ADDITIONAL REPRESSORS. In *Streptomyces coelicolor* and *Streptomyces albus*, the *groESL*$_1$ operon and the *groEL*$_2$ gene are regulated by tandem CIRCE elements, whereas the *dnaK* operon encodes its own autoregulatory repressor (Bucca et al., 1995; Bucca et al., 1997). Heat-inducible transcription of the *dnaK* operon (*dnaK*, *grpE*, *dnaJ* and *hspR*) initiates from the vegetative promoter. Disruption of *hspR* led to high and constitutive transcription levels of the *dnaK* operon but had no effect on the *groE* expression level (Bucca et al., 1997). Similar to the GroE modulation of HrcA activity, DnaK protein forms a specific ATP-independent complex with the *Streptomyces* HspR repressor, and this interaction is necessary for HspR to bind a dnaKp fragment in gel-shift assays (Bucca et al., 2000). The *dnaK* heat-induction model suggested by Bucca et al. suggests a decrease in the availability of DnaK because of the accumulation of heat-damaged proteins (Bucca et al., 2000). This model has many similarities to the heat induction of the σ^{32}-dependent transcription in *E. coli*, a model that will be discussed in detail below.

Another heat-shock control element found in *S. albus* is the RheA, which represses the transcription of *hsp18* (encoding a small heat-shock protein) by binding specifically to the *hsp18* promoter (Servant and Mazodier, 1996; Servant et al., 1999). Transcription analysis of *rheA* in the *S. albus* wildtype and in *rheA* mutant strains suggested that RheA represses transcription not only of *hsp18* but also of *rheA* itself (Servant et al., 1999).

The General Stress Response

SIGMA B-CONTROLLED GENES. Sigma B was found to control a stress-starvation regulon that comprises a very large set of general stress genes (for reviews, see Hecker et al. [1996] and Hecker and Volker [1998]). These σ^B-dependent genes are strongly induced by heat, ethanol, acid or salt stress, as well as by starvation for a carbon source, phosphate and oxygen (Bernhardt et al., 1997; Bernhardt et al., 1999; Hecker and Volker, 1998; Buttner et al., 2001). Recent experiments (Petersohn et al., 2001) using gene arrays containing all currently known open reading frames of *B. subtilis* suggest that as many as 125 genes are under the control of σ^B. At least 24 of these also seem to be subject to a second, σ^B-independent stress induction mechanism. Most of the σ^B-dependent general stress proteins are probably located in the cytoplasm, but 25 contain at least one membrane-spanning domain, and at

least 6 proteins appear to be secreted. This very large stress regulon seems to give a basal level of protection against a large variety of stress conditions.

Two groups of signals were found to trigger the induction of *sigB*, the gene that codes for σ^B. The first group contains extracellular signals (i.e., glucose, oxygen, or phosphate, but not amino acid, starvation) that result in a drop of the ATP level (Maul et al., 1995). (Amino acids trigger the induction of ppGpp and keep the ATP pool constant.) The second group of stimuli includes physical stress-factors, such as heat, salt and acid stress, but not oxidative stress (Hecker and Volker, 1998). This group of stimuli induces the synthesis of σ^B via a two-component system (RsbS and RsbT) that changes the balance of a complex network of anti-sigma (RsbW) factor and its agonist (nonphosphorylated RsbV) to activate σ^{B-} (Akbar and Price, 1996; Yang et al., 1996). For the expression of some genes, the involvement of σ^B is essential, whereas for others it seems to be nonessential because it can be replaced by alternative stress-induction mechanisms (Hecker and Volker, 1998).

Not much is known about many of the 125 GSP genes (Petersohn et al., 2001), and their physiological role in the complex general stress response is not understood. The identified GSPs can be assigned to five main groups (Hecker and Volker, 1998): 1) Group 1 is the σ^B-dependent genes that encode subunits of stress-inducible proteases. ClpP, ClpC, and ClpX are probably essential for the renaturation or degradation of misfolded or denatured proteins that accumulate in the cell upon exposure to stress conditions (Gottesman, 1996; Gerth et al., 1998). Null mutants of *clpC*, *clpP* and *clpX* are extremely sensitive to heat, salt or ethanol stress, and much more sensitive than mutants of *sigB* (Kruger et al., 1994; Msadek et al., 1994; Gerth et al., 1998). 2) Group 2 is the σ^B-dependent genes that encode general oxidative stress-protective proteins (such as *katE*, which encodes catalase; Engelmann et al., 1995) and the DNA-protecting protein Dps (Antelmann et al., 1997b). Other σ^B-dependent proteins (such as thioredoxin ClpC, ClpP and the fifth and sixth gene products of the *clpC* operon [*sms* and *yacK*]; Kaan et al., 1999) may also be involved in adaptation to oxidative stress (Hecker and Volker, 1998). 3) The third group is proteins with a putative role in the adaptation to salt or water stress. A proline-uptake system encoded by a functional copy of *opuE* is required by *B. subtilis* for the use of external proline as an osmoprotectant (Hecker and Volker, 1998). However, the physiological role of σ^B in the expression of *opuE* is still unclear because exogenously provided proline was used as an osmoprotectant in a *sigB* mutant (Von Blohn et al., 1997). YtxH and GsiB are homologous to plant-desiccation proteins, which are involved in water-stress protection, and YkzA is a homolog of the *E. coli* OsmC, which is involved in osmo-adaptation (Mueller et al., 1992; Volker et al., 1994; Maul et al., 1995; Varon et al., 1996). 4) Group 4 is a heterogeneous group of proteins: their role in adaptation to stress is yet to be determined. One of these proteins, GspA (Antelmann et al., 1995), is also induced upon amino acids starvation (Eymann and Hecker, 2001) and seems to be involved in the expression of *hag*, which encodes flagellin, or UDP-glucose pyrophosphorylase, which participates in cell-wall metabolism (Varon et al., 1993). Some proteins seem to participate in nicotinamide adenine dinucleotide (NAD) synthesis (e.g., *nadC* and *nadE* gene products) or might catalyze reduced NAD phosphate (NADP[H])-dependent reactions (Antelmann et al., 1997a; Antelmann et al., 1997b; Hecker and Volker, 1998; Scharf et al., 1998). And 5), the fifth group consists of a large number of proteins that, so far, show no significant similarity to known proteins (Petersohn et al., 2001).

Several of the general stress operons in Gram-positive bacteria were found to be regulated by more than one control element. The *B. subtilis clpC*, *clpP* and *trxA* operons are under the control of the vegetative sigma factor σ^A and the stress sigma factor σ^B (Kruger et al., 1996; Gerth et al., 1998; Scharf et al., 1998). Although both promoters were used under a number of stress conditions, the induction pattern of the genes varied for the different genes and the particular stress condition. A *cis* element that contains a heptameric tandem consensus sequence was found upstream of the *clpC*, *clpE*, and *clpP B. subtilis* operons and was shown to be the binding site of the CtsR repressor (Kruger and Hecker, 1998; Derre et al., 1999a; Derre et al., 1999b). CstR was lately found also in *Listeria monocytogenes* (Nair et al., 2000).

Complexity of the Stress Response Networks

Regulation of bacterial stress response involves various positive and negative control elements that often interact with each other. Some heat shock proteins are directly regulated by only one control element, but other genes and operons are regulated by several control elements (e.g., *E. coli pspABCE* [Jovanovic, 1996], *A. tumefaciens groESL* [Segal and Ron, 1995b; G. Segal and Ron, 1996; Nakahigashi et al., 1999], and *B. subtilis clpC* [Kruger et al., 1996; Gerth et al., 1998; Scharf et al., 1998]). However, the stress

response is always a complex response that regulates itself. As an example, the heat-shock response is induced by damaged proteins, whose cellular concentration increases with temperature. Yet, since the heat shock stimulon contains the genes coding for proteases and chaperones, their induction at increased temperatures reduces the concentration of the damage proteins, thus reducing the level of induction of the heat shock response.

Because the regulatory elements of these complex stress response networks are associated with each other, any impairment of the cellular steady state at one point may affect the whole network, directly or indirectly. Therefore, the study of these global regulatory networks requires global analysis methods (Rosen and Ron, 2002a). Such methods for transcriptome and proteome analysis are now available and have been implemented in this field. For comprehensive understanding, more than one method should be used. Analysis of mRNA levels is required to define all the genes whose transcription is affected by environmental conditions or regulatory genes. This analysis, however, is insufficient because the expression and activity of the stress genes are controlled at posttranscriptional, higher regulatory levels. Thus, global analysis at the protein level (i.e., proteomics studies) also must be performed. These studies define the final cellular level of the various proteins, as well as their modifications, some of which may be controlled by stress conditions. One important protein modification shown to play a role in global regulatory networks is protein phosphorylation, usually at one or a few amino acids. Recently, a new group of highly phosphorylated proteins has been identified. These proteins accumulated during several stress conditions and may be involved in the degradation process (Rosen et al., 2004). In eukaryotic systems, protein phosphorylations are known to be involved in protein labeling and in many signal transduction pathways. In bacteria, the number of known phosphorylated proteins is much lower. However, several phosphorylated proteins are involved in the heat shock response of various bacteria, as will be shown in the following examples. The heat shock transcriptional activation of the *lonD* gene of *Myxococcus xantus* is controlled by a two-component histidine-aspartate phosphorylation system (Ueki and Inouye, 2002). The general-stress sigma factor of *B. subtilis* (σ^B) is regulated by several regulatory kinases and phosphatases (the Rsb proteins), which catalyze the release of σ^B from an anti-σ^B factor (Akbar and Price, 1996; Yang et al., 1996; Akbar et al., 2001; Zhang et al., 2001). Another heat shock protein (Hsp70 of *Mycobacterium leprae*) was found to be phosphorylated at threonine-175 (Peake et al., 1998), which results in an increased affinity for a model polypeptide substrate. One of the best-studied examples of stress-controlled protein modification was already discussed above, in the section The General Stress Response of E. coli. The phosphorylated form of RssB (a stationary phase response regulator) targets the alternative transcriptional activator σ^S for degradation by ClpXP (Bouche et al., 1998; Zhou et al., 2001). In view of these examples, protein modification will probably be demonstrated as one of the important control elements in global regulatory networks.

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Prokaryotes (2006) 2:1028–1049
DOI: 10.1007/0-387-30742-7_33

CHAPTER 1.33

Anaerobic Biodegradation of Hydrocarbons Including Methane

FRIEDRICH WIDDEL, ANTJE BOETIUS AND RALF RABUS

Introduction

Hydrocarbons, by definition, are constituted only of the two elements, hydrogen and carbon, and thus do not contain functional groups. As a result, most hydrocarbons exhibit poor solubility in water and low chemical reactivity at room temperature (except for hydrocarbons with triple bonds). Nevertheless, relative to several electron acceptors, in particular oxygen, hydrocarbons are energy-rich, and they are exploited as growth substrates by numerous microorganisms. Whereas the aerobic microbial degradation of hydrocarbons was known since the beginning of the twentieth century and has been studied microbiologically and biochemically in much detail (see the chapter on Hydrocarbon-Oxidizing Bacteria in this Volume), first major insights into the anaerobic degradation of hydrocarbons have been achieved only in the 1990s.

Hydrocarbons are naturally widespread organic compounds that are formed either as metabolites by bacteria, archaea, plants and animals (Birch and Bachofen, 1988; Widdel and Rabus, 2001), or as abiotic (thermogenic) transformation products of dead buried biomass (Tissot and Welte, 1984). The conversion of the polar compounds of biological origin to hydrocarbons is a defunctionalization and reflects an overall thermodynamic principle. The eliminated inorganic compounds H_2O, CO_2 or NH_4^+ together with the pure C-H compounds are energetically more stable than the parental organic compounds with -OH, $-COO^-$, $-NH_3^+$ or other polar groups (Widdel and Rabus, 2001). The energetically final, most stable state of carbon is represented by CH_4 and CO_2. It is likely that many reactions leading to hydrocarbons already took place during the early history of life and that hydrocarbons therefore represent relatively "old" substrates from an evolutionary point of view. Hence, hydrocarbon-degrading bacteria and archaea with various pathways may have already evolved before oxygen became abundant as an electron acceptor in the biosphere. The study of anaerobic microorganisms that degrade hydrocarbons is of interest from various points of view: A) The long-term fate of hydrocarbons as globally abundant forms of organic carbon in anoxic sediments, gas hydrates, petroleum reservoirs and other subsurface environments is of geochemical interest. A process of high global relevance in this respect is the anaerobic oxidation of the most abundant hydrocarbon, methane, which is also a potential greenhouse gas. B) The exploitation of petroleum is always connected with its mobilization and an increased exposure to microbial degradation. It is likely that certain hydrocarbons serve as substrates for sulfate-reducing bacteria in oil field waters and lead to the production of undesired sulfide. Knowledge of the involved organisms and their growth conditions may be valuable for the development of measures against this process. C) If deep aquifers (groundwater) or the sea floor are contaminated by oil spills, aerobic hydrocarbon degradation is usually limited by the availability of dissolved oxygen. It is therefore of interest to determine the extent to which oil hydrocarbons, in particular those with noticeable water solubility, can be degraded with other electron acceptors of higher solubility such as sulfate or nitrate. D) Since biochemical reactions always occur at functional groups, a hydrocarbon has to be functionalized before it can be channeled into the metabolism. Such reactions overcome unusually high activation energies (usually those of apolar C-H bonds) and thus include very reactive intermediates, but nevertheless occur in a controlled, highly specific manner. They are, therefore, biochemically intriguing. Knowledge of reaction mechanisms and principles may, in the longer run, be of interest in the development of new chemical catalysts by biomimetic approaches.

Anaerobic oxidation of the smallest hydrocarbon, methane, is apparently performed by close relatives of methanogens; these relatives represent special lines of descent within the Euryarchaeota of the archaeal domain. In contrast,

the anaerobic utilization of non-methane hydrocarbons occurs in various Deltaproteobacteria of the bacterial domain that utilize nitrate, ferric iron or sulfate as electron acceptor. Principles of anaerobic hydrocarbon metabolism have been elucidated in a number of representative microorganisms. Whereas the aerobic activation of hydrocarbons always involves reactive oxygen species derived from O_2 to introduce hydroxyl groups, the anaerobic activation employs rather different mechanisms, depending on the chemical nature of the hydrocarbon. There is evidence for at least five principally different mechanisms for an anaerobic activation of hydrocarbons; these are 1) the assumed activation of methane in a "reverse methanogenesis," 2) the radical-catalyzed addition of alkanes and many alkylbenzenes to fumarate yielding substituted succinates, 3) the dehydrogenation of ethyl- and propylbenzene yielding aryl-substituted secondary alcohols, 4) the hypothesized addition of a carboxyl or methyl group to unsubstituted aromatic hydrocarbons, and 5) the hydration of double bonds (assumed) and triple bonds (demonstrated experimentally) in alkenes and acetylene, respectively. The anaerobic activation reactions are usually very sensitive towards oxygen. In comparison to the aerobic activation reactions, the anaerobic ones are relatively slow. Also anaerobic hydrocarbon degraders usually exhibit much slower growth than their aerobic counterparts.

For convenience, the following presentation of the anaerobic degradation of various hydrocarbons follows their chemical classification in textbooks and monographs, i.e., begins with methane and other alkanes and leads via alkenes and alkynes to aromatic hydrocarbons.

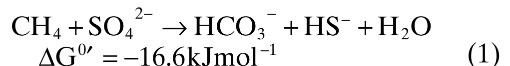
The focus of this chapter is on the presentation Microbiology and Biochemistry. Ecological aspects are presented in the case of the anaerobic oxidation of methane because the results from biogeochemical studies in various marine habitats are fundamental for our understanding of this process. Actually, the process has been discovered in situ rather than via microbiological experiments in vitro. Within the scope of this article, ecological aspects are not presented for the oxidation of various other hydrocarbons, except for some physiological aspects of anaerobic growth on crude oil. An important habitat-related topic in this context is contamination of aquifers and anaerobic in situ remediation. For information in this area, the reader is referred to other articles (for overview and recent articles, see Beller [2000a], Elshahed et al. [2001], Gieg and Suflita [2002], Hunkeler et al. [2002], Reusser et al. [2002], and Richnow et al. [2003], and Townsend et al. [2003]).

Anaerobic Degradation of Methane

Studies in the Habitat

Methane is of microbial or thermogenic origin and is the most abundant and chemically most stable hydrocarbon (Crabtree, 1995). Microbial origin is usually evident from low $^{13}C/^{12}C$ ratios and the absence of other gaseous hydrocarbons (ethane, propane, butane). Thermogenic methane (like other thermogenic hydrocarbons) either results from chemical transformation reactions (catagenesis, metagenesis; Tissot and Welte, 1984) of buried organic carbon, or from the interaction of water, iron(II)-containing rock, and carbon dioxide at several hundred degrees Centigrade (Holm and Charlou, 2001). Large reservoirs of methane hydrate with a mass exceeding that of conventional fossil fuel reservoirs by at least a factor of two lie buried in deep, sulfate-depleted zones of sea sediments (Kvenvolden, 1999). Despite permanent upward migration and new thermogenic or microbial production, little of the methane ever escapes into the hydrosphere. Most methane is scavenged by anaerobic microbial oxidation.

First evidence for an anaerobic oxidation of methane (AOM) came from geochemical studies in marine sediments (Martens and Berner, 1974; Barnes and Goldberg, 1976; Reeburgh, 1976). In these, methane diffusing upwards from deep sites often disappears nearly completely before any contact with oxygen is possible. The only electron acceptor that can account for the disappearance of methane is sulfate, resulting in the reaction



$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \Delta G^{0\prime} = -16.6 \text{kJmol}^{-1} \qquad (1)$$

The zone of AOM in depth profiles of subsurface sediments is usually evident from a concave-up curvature of the methane concentration coinciding with an increased sulfate reduction rate (Alperin and Reeburgh, 1985; Iversen and Jørgensen, 1985). In zones where biogenic methane with its naturally low $^{13}C/^{12}C$ ratio disappears, isotopically light dissolved inorganic carbon and precipitated carbonates have been detected (Reeburgh, 1980; Ritger et al., 1987; Paull et al., 1992). This finding as well as the formation of radiolabeled CO_2 upon injection of ^{14}C-methane into samples from anoxic sediments further provided evidence for AOM (Reeburgh, 1980; Iversen and Jørgensen, 1985; Hansen et al., 1998).

Various habitats with intense AOM have been detected at submarine cold seeps. The most striking ones so far are surface sediments above methane hydrates at Hydrate Ridge (Cascadia Margin, Oregon) in the Northeast Pacific

(Boetius et al., 2000) and the gas seeps of the Northwestern Black Sea shelf (Michaelis et al., 2002). In these habitats, microbial life appears to be based exclusively on AOM. In Hydrate Ridge sediments, AOM occurs above gas hydrates lying a few centimeters below the sea floor. Sulfide produced by AOM nourishes chemolithotrophic sulfide-oxidizing aerobic bacteria, including those in symbioses with bivalves. In the Black Sea, AOM occurs in the anoxic part, far below the chemocline. Since AOM leads to a significant increase of inorganic carbon and alkalinity, calcium ions from seawater (usually near 10 mM) tend to be precipitated according to the net equation

$$CH_4 + SO_4^{2-} + Ca^{2+} \rightarrow CaCO_3 + H_2S + H_2O \quad (2)$$

The absence of a reoxidation of hydrogen sulfide to sulfuric acid in the direct vicinity may explain the massive deposition of carbonate plates and chimney-like structures in the anoxic part of the Black Sea (Figs. 1 and 2). Acidification by aerobic reoxidation of hydrogen sulfide would counteract such formation of precipitates.

Labeling studies with ^{14}C-methane in cultures of methanogenic archaea suggested that methanogenesis is reversible to a certain, small extent during net formation of methane (Zehnder and Brock, 1979; Harder, 1997). The occurrence of such "mini-reversibility" led to the view that a net AOM can be slowly catalyzed by methanogens themselves if an electron sink is available. Hoehler et al. (1994) proposed that AOM is performed by archaea and sulfate-reducing bacteria (SRB) in a consortium where the former produce a free, extracellular intermediate that is

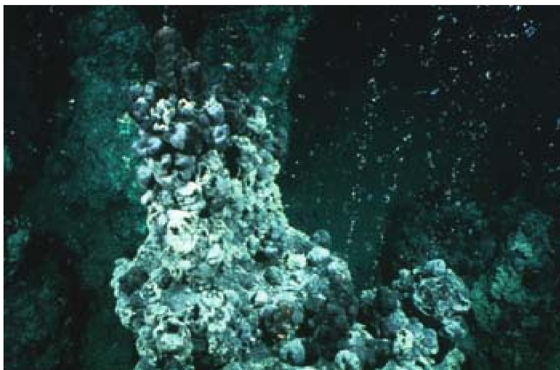


Fig. 1. Chimney-like structure at a cold methane seep on the northwestern Black Sea shelf (water depth, 250 m). The upper chimney-like structure (diameter approx. 0.3 m) consists of carbonate precipitates (presumably $CaCO_3$ and other alkaline earth carbonates) enclosed by massive biofilms that are partly dark due to precipitated ferrous sulfide. Bubbles of excess methane that has not been oxidized are rising from the chimneys. Courtesy of project GHOSTDABS, Walter Michaelis, University of Hamburg.

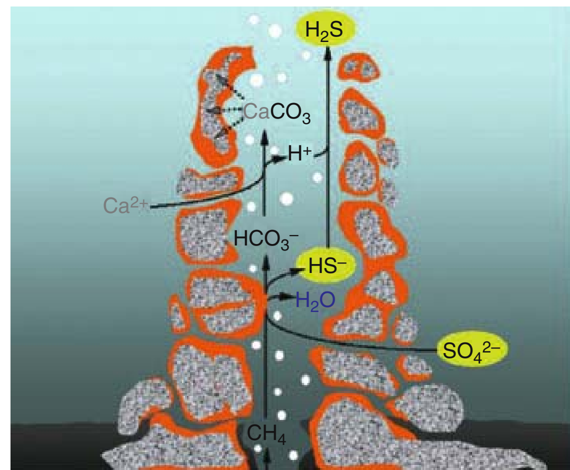


Fig. 2. Scheme of the anaerobic oxidation of methane showing the formation of calcified chimney-like structures as it occurs at the Northeastern Black Sea shelf. Methane-oxidizing mats are indicated in orange and precipitated carbonates in grey (grained).

scavenged by the latter. Molecular hydrogen has been most frequently supposed to function as such an intermediate. Further evidence for the existence of methanotrophic archaea was provided by the finding of strongly ^{13}C-depleted isoprenoid, presumably archaeal lipids and 16S rRNA gene sequences representing a distinct phylogenetic cluster related to the Methanosarcinales (Elvert and Suess, 1999; Hinrichs et al., 1999; Pancost et al., 2000; Hinrichs and Boetius, 2002). Microscopy of whole-cell hybridization assays with 16S rRNA-targeted fluorescent probes revealed consortia of archaeal and bacterial cells belonging to the Methanosarcinales and the *Desulfosarcina-Desulfococcus* branch of the Deltaproteobacteria, respectively (Boetius et al., 2000; Fig. 3). The incorporation of light (^{13}C-depleted) methane-derived carbon has subsequently been shown for each of the apparent partners, the archaea and the bacteria (Orphan et al., 2001; Orphan et al., 2002; Elvert et al., 2003). Presently, the two most abundant phylogenetic groups of methane-oxidizing archaea that have been distinguished by molecular probing in situ are the ANME-1 (Michaelis et al., 2002) and ANME-2 (Boetius et al., 2000) groups; the latter are more closely related to the Methanosarcinales than the former. Both groups are associated with apparently sulfate-reducing bacteria of the *Desulfosarcina*/*Desulfococcus* group (Knittel et al., 2003). A third phylogenetic group of archaea associated with sulfate-reducing bacteria of the *Desulfobulbus* group has been recently discovered at an Arctic mud volcano (T. Lösekann, personal communication). The associations between archaea and bacteria exhibit

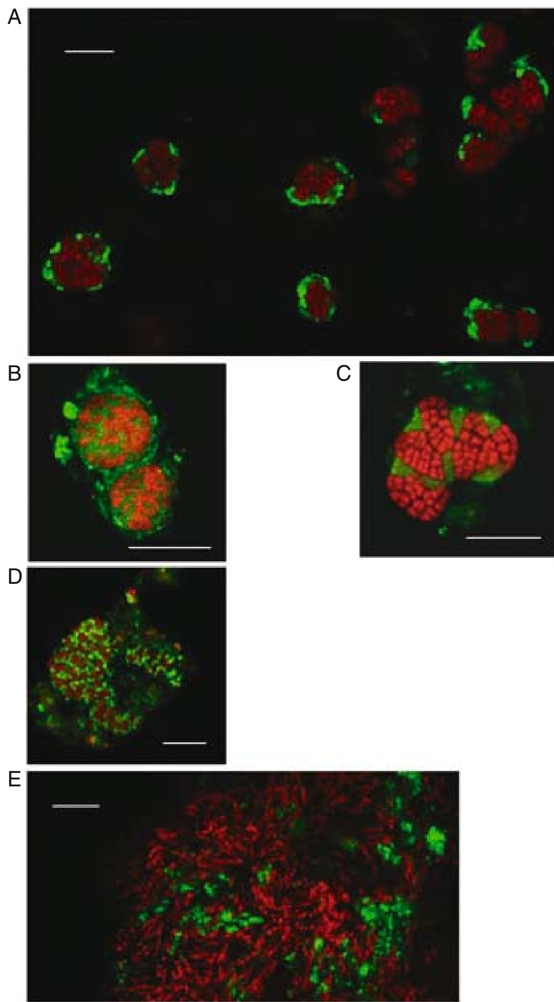


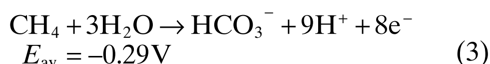
Fig. 3. Fluorescent in situ hybridization (16S rRNA-targeted) of methane-oxidizing archaea in association with sulfate-reducing bacteria. Probes for archaea exhibit red and probes for sulfate-reducing bacteria green fluorescence. (A), (B) Consortia grown as “shell-type.” (C), (D) Consortia grown as “mixed type”. (E) Consortia grown as “biofilm”. Origin: (A), (E) mat from a cold methane seep in the Black Sea (shown in Fig. 1); (B), (C), (D) sediment above methane hydrate of Hydrate Ridge (Cascadia Margin), NE Pacific. Scale bars, 10 μm. All photographs by courtesy of K. Knittel, T. Lösekann, and A. Gieseke, Bremen.

diverse structures (Fig. 3), but usually one type is dominant in a certain habitat. Three main structures of consortia may be distinguished: First, in a shell-type association, an inner core of densely packed archaeal cells is surrounded by the bacterial cells. Second, in a mixed type of association, archaeal and bacterial cells are more randomly or evenly throughout the consortia. And third, a biofilm-type of association is characterized by archae and bacteria growing seperately in dense microcolonies.

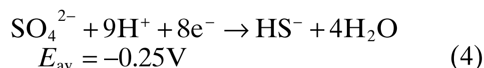
Physiological and Metabolic Studies

The reaction of AOM could be demonstrated in vitro with sediment samples from a marine gashydrate area that is naturally enriched with consortia of archaea and bacteria (Nauhaus et al., 2002). The measured substrate-product balance was in accordance with the above stoichiometric equation. This and the absence of sulfide formation in methane-free control experiments showed that electron donors other than methane were not relevant in the investigated sediment and that microbial communities are fueled merely by methane and sulfate. Furthermore, the measured molar ratio demonstrated that the portion of methane that is channeled into the synthesis of cell carbon (approximately according to $CH_4 + CO_2 \rightarrow 2\,[CH_2O]$) and not used for sulfate reduction must be very small and not quantifiable within the accuracy limits of the measurements, according to the relatively low free energy gain. For various in situ conditions, a free energy change between -10 kJ mol^{-1} and -40 kJ mol^{-1} was estimated, depending on the concentrations of substrates and products in their depth profiles. It is unclear whether AOM under the thermodynamically least favorable conditions can still be connected with growth. Results so far suggest that microorganisms performing AOM grow very slowly and have lower growth yields than, for instance, sulfate-reducing bacteria growing on conventional substrates such as acetate or lactate (which assimilate approx. 10% of their carbon substrate).

The intermediate channeled from methane oxidation into sulfate reduction is still a matter of discussion. In vitro feeding studies with the conventional methanogenic substrates, H_2, formate, acetate, or methanol, in the absence of methane suggested that none of these compounds is an intermediate during AOM (Nauhaus et al., 2002). Indeed, their functioning as an intermediate has been viewed critically also from a theoretical perspective on the basis of diffusion limits and kinetic predictions (Boetius et al., 2000; Valentine et al., 2000; Spormann and Widdel, 2000; Sørensen et al., 2001). Hence, the transfer of reducing equivalents from methane utilization into sulfate reduction probably does not occur via an intermediate that is a typical methanogenic growth substrate. Three principal possibilities for such a transfer can be envisaged. First, a syntrophic interaction may occur by a transfer only of reducing equivalents via electron shuttles (or hydrogen shuttles other than free H_2) that are associated with the cell surfaces. The formal half-reactions and their redox potentials (average of different steps) under in situ conditions (for sites with intense AOM) would be



$$CH_4 + 3H_2O \rightarrow HCO_3^- + 9H^+ + 8e^- \quad E_{av} = -0.29V \tag{3}$$



$$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O \quad E_{av} = -0.25V \tag{4}$$

and a shuttle system with a midpoint potential in this range would be most favorable (Widdel and Rabus, 2001; Nauhaus et al., 2002). In this case, the archaeal partner would form the methane-derived CO_2 which may be assimilated by the sulfate-reducing bacterium in an autotrophic mode of growth. Second, a syntrophic interaction may occur by a transfer of a methane-derived carbon compound, which according to the experiments is not acetate or methanol. In this case, the bacterial partner (viz., the sulfate reducer) would form the methane-derived CO_2. And, third, methane oxidation as well as sulfate reduction may both take place in the archaeal cells. Growth of the sulfate-reducing partner could be explained by scavenging and utilization of a certain amount of reduced, so far unknown metabolites, i.e., as a kind of metabolic parasitism or commensalism. This model is favored by the finding that archaeal cells in some sediments with AOM are not closely associated with bacterial cells (Orphan et al., 2002) with bacterial cells (Orphan et al., 2002).

The hypothesis that AOM is biochemically in principle a reversal of methanogenesis was supported by the analysis of genes and biochemical components in samples from habitats where methane-oxidizing microbial communities are abundant (Hallam et al., 2003; Krüger et al., 2003). From these habitats, genes were retrieved that were very similar to those encoding the three subunits of methyl-coenzyme M reductase (MCR; composition, $\alpha_2\beta_2\gamma_2$), the terminal enzyme in methanogenesis; there were different types of evidence that these genes belong to the characteristic archaea (ANME-1 and ANME-2 groups) that are commonly associated *in situ* with AOM. The deduced proteins were phylogenetically related to MCR subunits of the Methanosarcinales, but clearly represented own lines of descent. Furthermore, from one of the analyzed habitats, a methane seep area of the Black Sea (Fig. 3), mat-like biomass with ANME-1 archaea was obtained that was sufficient for the purification of proteins. The dominant protein consisted of three subunits with N-terminal amino acid sequences matching those of the retrieved genes (Krüger et al., 2003). This protein harbored a nickel factor that was apparently a heavier (951 Da) variant of factor F_{430} (905 Da), the unique nickel porphinoid in MCR (two molecules F_{430} per molecule of MCR; Thauer, 1998). Hence, special archaea seem to catalyze the initial reaction of of AOM by an enzyme that shares an evolutionary origin with MCR in conventional methanogens, but has been optimized for "reverse methanogenesis."

In this context, the mechanism for the activation of methane as the least reactive hydrocarbon is of particular biochemical interest. A hypothetical activation mechanism may be derived from a reaction model of MCR (in methanogenesis) that is based on the crystal structure (Ermler et al., 1997) and theoretical considerations (Pelmenschikov, 2002; R. K. Thauer, personal communication). According to this model, methyl-coenzyme M (CoM-S-CH_3) in methanogens reacts with F_{430} in its Ni(I) state to yield a methyl radical (·CH_3) while the coenzyme moiety (i.e. the thiolate) is bound to F_{430}, now being in its Ni(II) state. The methyl radical subsequently yields free CH_4 via abstraction of an H-atom from the thiol group of coenzyme B (H-S-CoB), thus leaving a thiyl radical (·S-CoB). The latter scavenges the Ni(II)-bound coenzyme M to form the heterodisulfide (CoM-S-S-CoB). Vice versa, methane in AOM could first react with the coenzyme B thiyl radical yielding a methyl radical and coenzyme B, as depicted in the hypothetical reaction sequence in Fig. 4. Methane activation in this way would resemble, to certain extent, the anaerobic activation of other hydrocarbons which may also involve a thiyl radical for the initial attack (see the sections on Aerobic Degradation of Non-Methane Alkanes and Toluene in this Chapter). An alternative hypothetical mechanism is the direct reaction of methane at the nickel center yielding a Ni-CH_3 species as initial activation product.

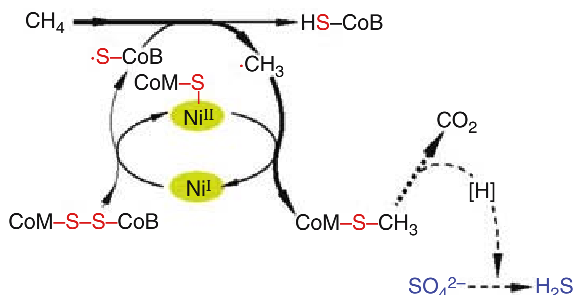


Fig. 4. Hypothesized reaction mechanism for the anaerobic activation of methane as a reversal of the final step in methanogenesis. The heterodisulfide (CoM-S-S-CoB) is reductively cleaved by Ni(I) as a strong reductant and forms a thiyl radical (·S-CoB) as a highly reactive intermediate; first, a short-lived disulfide radical anion (CoM-·S-S-CoB) may be formed (Pelmenschikov, 2002; R. K. Thauer, personal communication). Bold lines indicate the pathway of methane.

Anaerobic Degradation of Non-Methane Alkanes

Alkanes are major constituents of petroleum and natural gas. The possibility of an anaerobic oxidation of *n*-alkanes of various chain lengths has been repeatedly investigated since the 1940s (for references see, Aeckersberg et al., 1991) to understand the technically undesirable process of sulfide formation during oil production. Sulfide formation in oil field waters had been recognized in the 1920s as the activity of sulfate-reducing bacteria (Bastin et al., 1926). *n*-Alkanes as major oil constituents were suspected to serve as growth substrates for sulfate-reducing bacteria. Early reports of an anaerobic *n*-alkane degradation by bacteria that had been isolated with "conventional" polar substrates, for instance, sulfate-reducing *Desulfovibrio* or denitrifying *Pseudomonas* species, could not be repeated (for overview, see Aeckersberg et al. [1991] and Ehrenreich et al. [2000]). Definitely anaerobic degradation of *n*-alkanes with sulfate or nitrate was demonstrated first by quantitative measurement of degradation balances with novel isolates that differed from any of the previously known species of sulfate-reducing and denitrifying bacteria (Aeckersberg et al., 1991; Aeckersberg et al., 1998; Ehrenreich et al., 2000; Rueter et al., 1994; So and Young, 1999). Isolates are listed in Table 1. Furthermore, *n*-alkane degradation to methane and carbon dioxide in the absence of nitrate and sulfate was shown in methanogenic enrichment cultures with defined compounds (Zengler et al., 1999a; Anderson and Lovley, 2000) and with contaminated aquifer sediment enriched with petroleum (Townsend et al., 2003). Degradability of branched or cyclic alkanes under anoxic conditions has been studied to a lesser extent, but there is evidence that also these compounds can be activated and degraded (Bregnard et al., 1997; Rios-Hernandez et al., 2003; Wilkes et al., 2003).

The detection of succinates with alkane-derived alkyl chains in a sulfate-reducing enrichment culture (Kropp et al., 2000) and a denitrifying *Azoarcus* strain (Rabus et al., 2001) growing with *n*-dodecane or *n*-hexane, respectively, suggested that alkanes are activated by an addition to fumarate with formation of a new C-C bond. Hence, this was an analogy to the earlier elucidated activation reaction of toluene yielding benzylsuccinate (see the section on Toluene in this Chapter). Furthermore electron paramagnetic resonance (EPR) spectroscopy showed the presence of an organic radical in *n*-hexane-grown cells but not in *n*-hexanoate-grown cells (Rabus et al., 2001). This further supported the hypothesis that alkane activation resembles, in principle, anaerobic toluene activation, which most likely involves a glycyl radical (see the section on Toluene in this Chapter). A general scheme for such type of anaerobic hydrocarbon activation is shown in Fig. 5. However, in contrast to toluene, the alkanes are activated and added to fumarate at their secondary carbon atoms yielding methylalkylsuccinates. Interestingly, the alkylsuccinates occurred as two diastereomers. Formation of stereoisomers is exceptional among enzymatic reactions. Assuming that the reaction at fumarate is stereoselective, as in toluene activation (see the section on Toluene in this Chapter), the formation of the stereoisomers may be due to relaxed stereospecificity at the alkane carbon; this suggests that the diastereomers are nonracemic. An observation that cannot be explained mechanistically is the lack of a deuterium label at carbon-2 of the succinate moiety in (1-methylpentyl) succinate, if this is formed from *n*-hexane and 2,3-d_2-fumarate. If the addition were analogous to that of toluene (see the section on Toluene in this Chapter), this label should be retained. Also energetically, the activation of alkanes and toluene are not fully comparable. The energy to be overcome during activation of an alkane at the secondary carbon atom is by 33 kJ mol^{-1} higher than in the case of toluene. A hypothetical alkane activation at the primary carbon atom would be even by 51 kJ mol^{-1} higher than toluene activation. The net reaction of hydrocarbon addition to fumarate is exergonic (ΔG^0 between −35 kJ and −39 kJ mol^{-1}; Rabus et al., 2001). For the sulfate-

Table 1. Reported bacterial isolates with the capacity for the anaerobic oxidation of saturated hydrocarbons.

Genus and species or strain designation	Affiliation (16S rRNA-based)	Hydrocarbon used for isolation	Range of *n*-alkanes utilized	References
Sulfate-reducing bacteria				
Hxd3	Deltaproteobacteria	*n*-Hexadecane	C_{12}–C_{20}	Aeckersberg et al., 1991
HD3	Deltaproteobacteria	*n*-Decane	C_6–C_{14}	Rueter et al., 1994
Pnd3	Deltaproteobacteria	*n*-Pentadecane	C_{14}–C_{17}	Aeckersberg et al., 1999
AK01	Deltaproteobacteria	*n*-Hexadecane	C_{13}–C_{18}	So and Young, 2000
Denitrifying bacteria				
Azoarcus sp. (HxN1)	Betaproteobactria	*n*-Hexane	C_6–C_8	Ehrenreich et al., 2000
OcNl	Betaproteobactria	*n*-Octane	C_8–C_{12}	Ehrenreich et al., 2000
HdNl	Gammaproteobacteria	*n*-Hexadecane	C_{14}–C_{20}	Ehrenreich et al., 2000

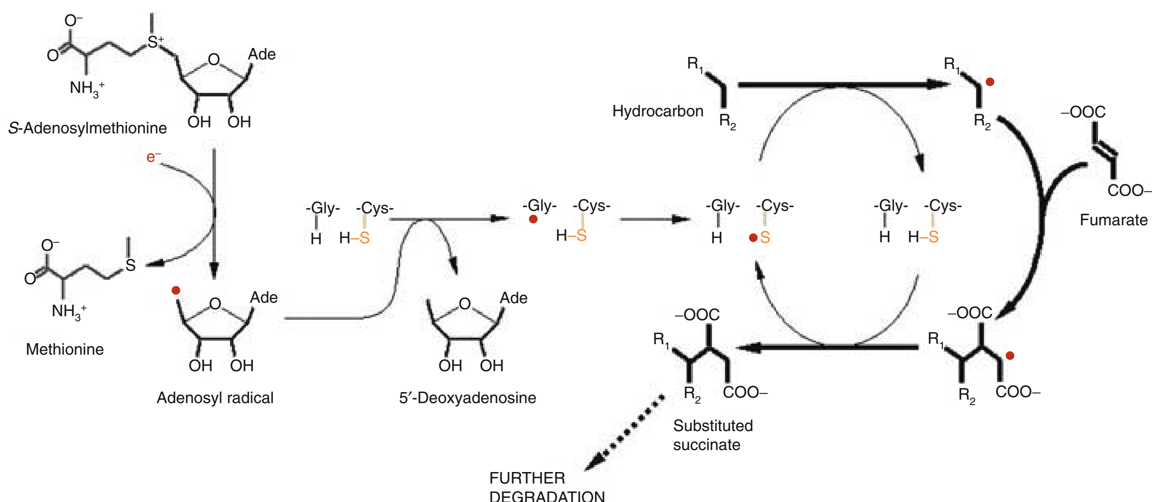


Fig. 5. Suggested general reaction scheme for the activation of saturated hydrocarbons and alkylbenzenes. The adenosyl radical is generated by an activating system in a reductive one-electron step that converts *S*-adenosylmethionine to methionine and the adenosyl-radical. By analogy to known glycyl radical enzymes in different metabolic reactions (Becker et al., 1999; Stubbe, 2000; Himo, 2002), the radical is first stored at a glycyl moiety in the polypeptide chain of the hydrocarbon-activating enzyme. The radical then abstracts a hydrogen atom from a cysteinyl moiety to generate a thiyl radical. The thiyl activates the hydrocarbon which adds to fumarate. The newly formed radicalic carbon compound recombines with the hydrogen atom thus leading to the free activation product (substituted succinate) and the reactive enzyme for the next round. R_1 = alkyl or aryl; R_2 = H or CH_3.

reducing bacterium, strain Hxd3 (Aeckersberg et al., 1991; Aeckersberg et al., 1998), the initial reactions of *n*-alkanes have been proposed to present a modified route with succinate addition at the third carbon position (Rabus et al., 2001), or an alternative, novel route (So et al., 2003).

The pathway for the further degradation of alkylsuccinates (Fig. 6) has been suggested on the basis of labelling studies and identified metabolites. The decisive finding that led to the proposition of the pathway was the observation that the deuterated 3-d_1-(methylpentyl)succinate formed in a labelling experiment yielded exclusively 3-d_1-4-methyloctanoate which was detected as further metabolite (Wilkes et al., 2002). This could be only explained by a deuterium/carboxyl-CoA exchange (opposite migration) followed by a loss of the other (free) carboxyl group. The exchange reaction would be analogous to the methylmalonyl-CoA mutase reaction involved in the formation or degradation of propionate. The assumed alkylmalonyl-CoA formed during anaerobic degradation of alkanes presents an acid with a β-carbonyl function and can easily undergo decarboxylation (or transcarboxylation) to yield the CoA derivative of a methyl-branched monocarboxylic acid. The position of the methyl branch does not interfere

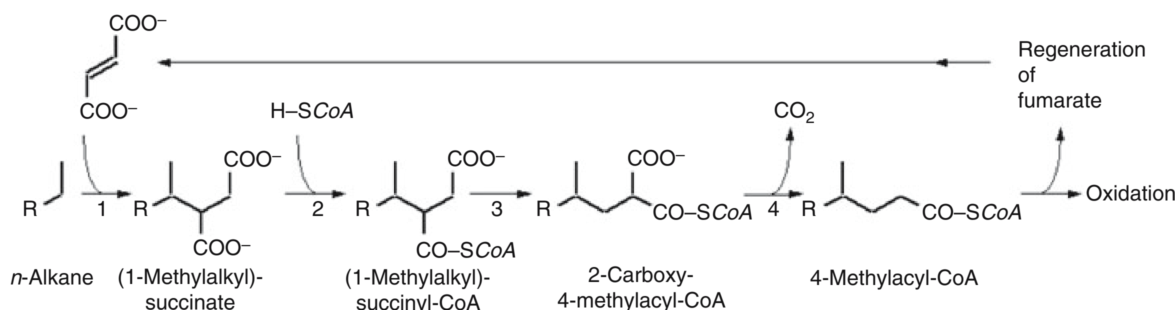


Fig. 6. Proposed reactions for the anaerobic activation and further metabolism of *n*-alkanes. (1-Methylalkyl)succinate generated by the radical-catalyzed reaction with fumarate (1) is presumably activated to become the coenzyme A thioester (2), which obviously undergoes carbon skeleton rearrangement (H/carboxyl-CoA exchange) (3). The resulting 3-ketoacid structure can undergo decarboxylation (or transcarboxylation) and lead to an activated fatty acid (4) that is oxidized by conventional β-oxidation. A propionyl fragment (not shown) from β-oxidation may be used for the regeneration of fumarate. (For details, see Wilkes et al., 2002.)

with conventional β-oxidation. The proposed pathway also offers a sequence for the regeneration of fumarate as the cosubstrate for the next round of alkane activation (Wilkes et al., 2002).

Anaerobic Degradation of Alkenes

Living organisms produce numerous alkenes, widespread compounds being the plant hormone ethene, carotenoids without functional groups, or the structurally large group of alkenoic terpenes that belong to the quantitatively most important secondary metabolites of plants. Alkenes are usually not present in petroleum and natural gas; they undergo saturation or aromatization during the long-term geochemical transformation processes (diagenesis and catagenesis).

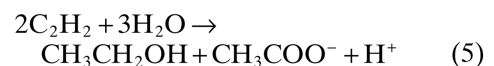
An anaerobic degradation of alkenes containing one or more double bonds has been repeatedly documented. A methanogenic coculture has been enriched on 1-*n*-hexadecene (Schink, 1985a), and a denitrifying bacterium has been isolated with 1-*n*-heptadecene as organic substrate (Gilewicz et al., 1991). Also, some of the denitrifying and sulfate-reducing bacteria originally isolated with *n*-alkanes are able to grow with 1-alkenes (see references in Table 1). Squalene, an isoprenoid alkene with six isolated double bonds, was slowly degraded in a methanogenic enrichment culture (Schink, 1985a). Strains of a nutritionally versatile denitrifying bacterium, *Alcaligenes defragrans*, have been isolated with the monounsaturated hydrocarbons *p*-menth-1-ene, α-pinene, 2-carene, and the diunsaturated hydrocarbon α-phellandrene (Foss et al., 1998). Two denitrifiers, strains pCyN1 and pCyN2, that were isolated with the aromatic monoterpene hydrocarbon *p*-cymene, also utilized several number of alkenoic monoterpenes (Harms et al., 1999a).

In contrast to monoterpenes, carotenoids appear to be very recalcitrant under anoxic conditions in the dark. Carotenoids (including those with functional groups) are preserved in anoxic sediments over thousands of years (Overmann et al., 1993). Strong adsorption to a matrix may contribute to this stability in sediments. Accordingly, β-carotene did not support growth of methanogenic enrichment cultures (Schink, 1985a).

The initial reactions of alkene activation are still unknown. Most anaerobes capable of degrading alkenes were directly enriched with these compounds and never were able to utilize saturated hydrocarbons (Schink, 1985a; Gilewicz et al., 1991; Foss et al., 1998). This suggests that there are specific alkene activating enzymes, and that the double bond is essential for the catalytic activation mechanism. Reactions appear in principle possible at the carbon atom next to the double bond, or at one of the double-bonded carbon atoms. In case of a reaction at the carbon adjacent to the double bond, hypothetical radical or cationic intermediates can be stabilized via delocalization. Comparative substrate tests with various menthadienes (monoterpene alkenes with two double bonds) suggested that sp^2-hybridization of the ring carbon atom next to the methyl group is a prerequisite for degradability of the hydrocarbon by *Alcaligenes defragrans* (Hylemon and Harder, 1999); however, there are no hints so far that the methyl group is the site of direct enzymatic attack. If a reaction (e.g., addition of a proton) occurred at an isolated double bond of an alkene, the intermediate (e.g., carbenium ion) would not be stabilized. Nevertheless, a bacterial enzyme has been purified and heterologously expressed that hydrates the isolated terminal double bond in the isopropyl side chain of limonene, an alkenoic monoterpene, yielding α-terpineol (Savithiry et al., 1997). In denitrifying *Alcaligenes defragrans*, a cometabolic conversion of the monoterpene isolimonene to isoterpinolene, viz., a shift of the terminal double bond to an energetically more stable position, has been observed (Heyen and Harder, 1998). The double bond involves a tertiary (branched) carbon atom such that a catalysis by protonation with formation of the relatively stable carbenium ion at a tertiary carbon is likely. (For more detailed aspects of reactions at double bonds, see Buckel [1992] and Spormann and Widdel [2000].)

Anaerobic Degradation of Alkynes (Acetylene)

Natural carbon compounds with triple bonds between carbon atoms (e.g., mycomycin) are rare: reactive secondary metabolites in some fungi and a few other organisms where such substances may sometimes function as deterrents. These compounds contain in addition polar groups; pure hydrocarbons with triple bonds have not been detected among the natural hydrocarbons. It is therefore remarkable that not only aerobic but also anaerobic bacteria such as *Pelobacter acetylenicus* can be readily enriched and isolated with acetylene (Schink, 1985b; Rosner and Schink, 1995). The anaerobic bacteria ferment acetylene to ethanol and acetate as end products according to



$$2C_2H_2 + 3H_2O \rightarrow CH_3CH_2OH + CH_3COO^- + H^+ \quad (5)$$

Growth on acetylene is fast (doubling time, 5–5.5 h). The natural significance of the capacity for acetylene degradation is unknown.

Acetylene is chemically reactive, and the fast metabolism of acetylene in *Pelobacter acetylenicus* indicates also substantial biochemical reactivity. Degradation of acetylene in *P. acetylenicus* occurs via hydratation to acetaldehyde, as observed in aerobic acetylene degrading bacteria (DeBont and Peck, 1980; Kanner and Bartha, 1982):

$$C_2H_2 + H_2O \rightarrow CH_3CHO \qquad (6)$$

One part of the acetaldehyde is then oxidized to acetate; the reducing equivalents are transferred to another part of the acetaldehyde (Schink, 1985b). Acetylene hydratase, which has been purified (Rosner and Schink, 1995), involves tungsten as a high-valent metal. It is part of tungstopterine-guanine dinucleotide (Meckenstock et al., 1999). The monomeric enzyme (molecular mass 83 kDa) contains in addition an Fe_4S_4 cluster. In a hypothesized model, acetylene binds to the tungsten(IV) site, followed by the addition of water to the triple bond. The reaction of the enzyme appeared to be independent of the redox state of the iron-sulfur cluster.

Anaerobic Degradation of Aromatic Hydrocarbons

Aromatic hydrocarbons are, besides or next to saturated ones, the main constituents of petroleum (Tissot and Welte, 1984). Many aromatic petroleum hydrocarbons are substituted by one or more alkyl chains of varying length and thus exhibit much structural diversity. Alkyl-substituted aromatic hydrocarbons may be regarded as chimeras of pure aromatic and aliphatic structures. So far known, the degradation of alkyl-substituted aromatic hydrocarbons makes use of the reactivity of the aliphatic carbon atom that is adjacent to the aromatic ring (in case of alkylbenzenes termed the benzyl carbon). Since this possibility does not exist for unsubstituted aromatic hydrocarbons (benzene, naphthalene, etc.), their degradation has to occur via different mechanisms.

Benzene

Evidence for anaerobic degradation of benzene, the most stable aromatic hydrocarbon, was first provided by studies with enriched populations in or from sediments with different electron acceptors (Grbić-Galić and Vogel, 1987; Edwards and Grbić-Galić, 1992; Phelps et al., 1998; Rooney-Varga et al., 1999; for overview, see Coates et al., 2002). A sediment-free sulfate-reducing consortium was analyzed on the basis of 16S rRNA gene sequences (Phelps et al., 1998); the bacterium supposed to be responsible for benzene degradation affiliates with purified strains of sulfate-reducing bacteria that degrade naphthalene, *m*-xylene, or ethylbenzene (Galushko et al., 1999; Kniemeyer et al., 2003). The first pure cultures shown to degrade benzoate anaerobically were two denitrifying *Dechloromonas* strains originally enriched and isolated with 4-chlorobenzoate and humic substances (Coates et al., 2001).

In the benzene-utilizing sulfate-reducing enrichment culture, benzoate was detected as an intermediate; however, isotope labeling showed that the carboxyl group was not derived from CO_2 in the medium. On the basis of substrate adaptation and inhibition studies, a coenzyme B_{12}-catalyzed methylation of benzene to toluene was suggested for *Dechloromonas* (Coates et al., 2002); toluene can indeed also serve as a growth substrate for this organism. Methylation of benzene, possibly with *S*-adenosylmethionine as the methyl donor, had been shown before as a biochemical reaction in human bone marrow (Flesher and Myers, 1991). Such a reaction can be envisioned as a biochemical analogue of a Friedel-Crafts type electrophilic substitution. An electrophilic enzymatic attack on benzene would circumvent an initiation of benzene activation by the energetically problematic radical cleavage of a C-H bond. In case of benzene (and naphthalene), the C-H bond is even more stable than that of methane (McMillen and Golden, 1982; March, 1992; Widdel and Rabus, 2001).

Naphthalene and Phenanthrene

Hints about anaerobic naphthalene degradation originally again came from studies with enriched communities under conditions of sulfate reduction (Coates et al., 1996; Zhang and Young, 1997). Complete degradation of naphthalene was demonstrated by quantitative growth experiments with a novel type of sulfate-reducing bacterium in pure culture (Galushko et al., 1999). Experiments with radiolabeled substrate revealed naphthalene oxidation by pure cultures of denitrifying bacteria (Rockne et al., 2000). Identification of 2-naphthoic acid as a metabolite in sulfate-reducing cultures enriched with naphthalene suggested an initial activation via carboxylation to 2-naphthoate (Zhang and Young, 1997; Meckenstock et al., 2000). This finding is in agreement with the observation that the sulfate-reducing isolate can utilize 2-naphthoate but not 1-naphthoate (Galushko et al., 1999). In principle, also a methylation as suggested in the case of benzene appears possible. Identification of other metabolites in a sulfate-reducing enrichment culture indicated further metabolism of 2-naphthoate (presumably as activated acid) via subsequent reduction of the two rings yielding

decahydro-2-naphthoate or an activated form (Meckenstock et al., 2000).

Degradation of the tricyclic aromatic hydrocarbon phenanthrene under anoxic conditions was demonstrated in sediment cultures with ^{14}C-labeled substrate (Coates et al., 1996; Zhang and Young, 1997). Mass spectrometric analysis indicated formation of a phenanthrene carboxylate isomer as initial product, again suggesting substrate carboxylation (Zhang and Young, 1997).

Toluene

Anaerobic biodegradation of hydrocarbons has been most intensely studied with toluene. Toluene-degrading anaerobes are apparently widespread and are relatively easily enriched from various aquatic sediments. Soon after the demonstration of anaerobic toluene degradation in various sediments and enrichment cultures (for references, see Widdel and Rabus, 2001), a steadily increasing number of pure cultures with the capacity for anaerobic toluene degradation was recognized or isolated de novo (Table 2). Most isolates are denitrifiers that belong to the genera *Azoarcus* and *Thauera*. Other isolates reduce iron(III), sulfate, or fumarate in a binary (syntrophic) culture. Furthermore, an anoxygenic phototroph utilized toluene together with CO_2 for the light-driven cell synthesis (Zengler et al., 1999b). Methanogenesis from toluene has been shown so far only in enrichment cultures (e.g., Grbić-Galić and Vogel, 1987; Beller and Edwards, 2000b). The apparent abundance of anaerobic bacteria with the capacity for toluene degradation may be explained by the occurence of toluene as a natural product in various habitats. Toluene does not only originate from petroleum and petroleum products, but also is formed by anaerobic microbial degradation of phenylacetate, a fermentation product of phenylalanine (Fischer-Romero et al., 1996). In comparison to other aromatic or saturated hydrocarbons, toluene allows relatively rapid growth (for denitrifiers, doubling time 6 h), which has been in favor of biochemical studies. Indeed, the study of anaerobic toluene degradation has provided important clues to help our understanding of anaerobic hydrocarbon metabolism.

Table 2. Reported bacterial isolates with the capacity for the anaerobic oxidation of aromatic hydrocarbons.

Genus and species or strain designation	Affiliation (16S rRNA-based)	Hydrocarbon used for isolation	Hydrocarbons utilized	References
Sulfate-reducing bacteria				
Desulfobacula toluolica	Deltaproteobacteria	Toluene	Toluene	Rabus et al., 1993
PRTOL1	Deltaproteobacteria	Toluene	Toluene	Beller et al., 1996
oXyS1	Deltaproteobacteria	*o*-Xylene	Toluene, *o*-xylene, and *o*-ethyltoluene	Harms et al., 1999a
mXyS1	Deltaproteobacteria	*m*-Xylene	Toluene, *m*-xylene, and *m*-ethyltoluene	Harms et al., 1999a
EbS7	Deltaproteobacteria	Ethylbenzene	Ethylbenzene	Kniemeyer et al., 2003
NaphS2	Deltaproteobacteria	Naphthalene	Naphthalene	Galushko et al., 1999
Iron(III)-reducing bacteria				
Geobacter metallireducens	Deltaproteobacteria	Acetate	Toluene	Lovley et al., 1989
Denitrifying bacteria				
Azoarcus sp. (T)	Betaproteobacteria	Toluene	Toluene, *m*-xylene	Dolfing et al., 1990
Thauera aromatica (K172)	Betaproteobacteria	Phenol	Toluene	Anders et al., 1995
Thauera aromatica (T1)	Betaproteobacteria	Toluene	Toluene	Evans et al., 1991
Azoarcus tolulyticus (To14)	Betaproteobacteria	Toluene	Toluene, *m*-xylene	Song et al., 1999
Azoarcus tolulyticus (Td15)	Betaproteobacteria	Toluene, *m*-xylene	Toluene, *m*-xylene	Song et al., 1999
ToN1	Betaproteobacteria	Toluene	Toluene	Rabus and Widdel, 1995
mXyN1	Betaproteobacteria	*m*-Xylene	Toluene, *m*-xylene	Rabus and Widdel, 1995
EbN1	Betaproteobacteria	Ethylbenzene	Toluene, ethylbenzene	Rabus and Widdel, 1995
EB1	Betaproteobacteria	Ethylbenzene	Ethylbenzene	Ball et al., 1996
PbN1	Betaproteobacteria	*n*-Propylbenzene	Ethylbenzene, *n*-propylbenzene	Rabus and Widdel, 1995
CyN1	Betaproteobacteria	*p*-Cymene	*p*-Cymene, toluene, various alkenoic monoterpenes	Harms et al., 1999b
CyN2	Betaproteobacteria	*p*-Cymene	*p*-Cymene, various alkenoic monoterpenes	Harms et al., 1999b
Phototrophic bacteria				
Blastochloris sulfoviridis	Alphaproteobacteria	Toluene	Toluene	Zengler et al., 1999

The identification of benzylsuccinate in a toluene-degrading sulfate-reducing enrichment culture (Beller et al., 1992) and a denitrifying strain (Evans et al., 1992) was an important discovery and basis for the elucidation of the anaerobic metabolism of toluene (Biegert et al., 1996; Beller and Spormann, 1997; Heider et al., 1999; Spormann and Widdel, 2000; Boll et al., 2002) and other hydrocarbons. It could be shown that benzylsuccinate was not a by-product, but the direct initial intermediate formed from toluene and fumarate in a carbon-carbon addition reaction that does not require electron carriers or ATP as cosubstrates (Biegert et al., 1996). Activation by addition to fumarate was subsequently demonstrated in other metabolic types of anaerobic toluene-degrading bacteria (Zengler et al., 1999b; Beller and Edwards, 2000; Kane et al., 2002). Stereochemical analysis in denitrifying bacteria showed formation of *R*(+)-benzylsuccinate (Beller and Spormann, 1998; Leutwein and Heider, 1999). Experiments with deuterium-labeled toluene revealed that the hydrogen/deuterium atom that has to be removed before toluene can add to fumarate is retained in the product (Beller and Spormann, 1998).

Structural and mechanistic properties of the toluene-activating enzyme, benzylsuccinate synthase, have been elucidated by genetic (Coschigano et al., 1998; Leuthner et al., 1998b; Achong et al., 2001) and enzymatic (Leuthner et al., 1998b) approaches. One of the structural genes (*bbsA*; Fig. 7) revealed a region with high similarity to genes encoding pyruvate formate-lyase (PFL) and type III ribonucleotide reductase (RNR; Coschigano et al., 1998; Leuthner et al., 1998b); the latter two enzymes are known to involve glycyl radicals in the polypeptide chain. Benzylsuccinate synthase from *Thauera aromatica* (strain K172) is a heterohexamer ($\alpha_2\beta_2\gamma_2$) with a native molecular mass of 200 kDa (Leuthner et al., 1998b). The presence of a glycyl radical was further confirmed by protein fragmentation upon exposure to oxygen (Leuthner et al., 1998b), and by a characteristic EPR signal in *Thauera aromatica* strains (Krieger et al., 2001; Duboc-Toia et al., 2003). Furthermore, the gene encoding the putative activating (radical-generating) enzyme was detected on the basis of sequence similarities with the activator genes of PFL and RNR (Coschigano et al., 1998; Leuthner et al., 1998b).

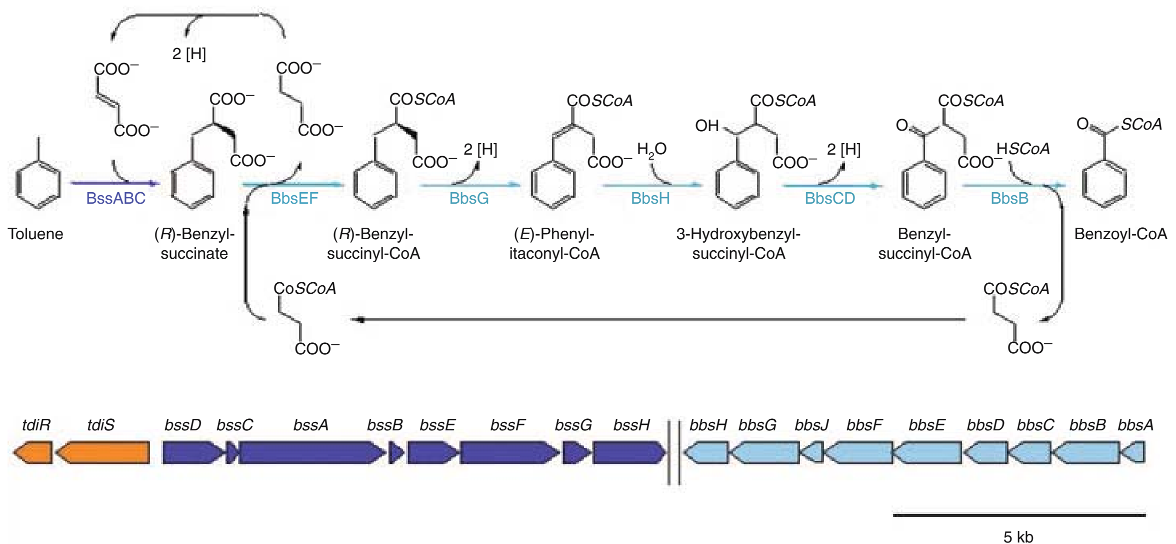


Fig. 7. Reactions and genes involved in anaerobic toluene degradation to the level of benzoyl-CoA in denitrifying *Azoarcus* strain EbN1. The biochemical reactions are supposed to be the same in other denitrifying, iron(III)-reducing, sulfate-reducing, syntrophic (associated with methanogens) and phototrophic bacteria. Fumarate as cosubstrate for toluene activation by benzylsuccinate synthase is recycled. Benzoyl-CoA is further oxidized via reductive dearomatization and ring cleavage (Haarwood et al., 1999) to carbon dioxide (not shown). Reducing equivalents ([H]) are used for the reduction of the electron acceptor. Enzyme names of gene products (in bold) are as follows: BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA:benzylsuccinate CoA-transferase; BbsG, benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 3-hydroxyacyl-CoA dehydrogenase; and BbsB, benzylsuccinyl-CoA thiolase. Identified genes involved in this pathway are clustered into two groups. Dark blue: genes for benzylsuccinate synthase and associated proteins. Light blue: genes for enzymes involved in the β-oxidation-like reaction sequence leading from benzylsuccinate to benzoyl-CoA. Orange: genes for a toluene-specific two-component regulatory system which may mediate coordinated (simultaneous) expression of both gene clusters in response to toluene.

The occurrence of a glycyl radical in biochemical reactions was detected in pyruvate-formate lyase (Becker et al., 1999) and class III ribonucleotide reductase (Stubbe, 2000). Benzylsuccinate synthase presents a third class of glycyl radical enzymes. The activating enzymes of PFL and RNR generate the glycyl radical via cleavage of *S*-adenosylmethionine by one-electron reduction yielding methionine and an adenosyl radical; the latter abstracts an H atom from a glycyl residue leading to a glycyl radical (viz., -NH-·CH-CO- in the polypeptide chain); this is supposed to be a storage radical which then generates a thiyl radical (-*S*·) as the reactive form (Fig. 5). In a theoretical study, calculations of energy requirements of partial reactions suggested that formation of the benzyl radical is the rate-limiting step in the formation of benzylsuccinate (Himo, 2002).

RNA analysis demonstrated that expression of genes related to benzylsuccinate formation is induced by toluene and confirmed their previously observed arrangement in an operon (Leuthner et al., 1998b; Coschigano, 2000). In both strains of *Thauera aromatica*, genes were identified that exhibited significant sequence homology to sensor/regulator proteins of two-component systems and were suggested to function in regulation of toluene metabolism (Coschigano et al., 1997; Leuthner and Heider, 1998a).

Benzylsuccinate is further metabolized, somewhat in analogy to β-oxidation of fatty acids, to succinyl-CoA and benzoyl-CoA (Leutwein and Heider, 1999; Leuthner and Heider, 2000). The promotor controlling transcription of the operon for the enzymes of benzylsuccinate metabolism in *T. aromatica* (*bss* operon) was recently identified (Leuthner and Heider, 2000). A scheme is shown in Fig. 7. Benzoyl-CoA undergoes reductive dearomatization, ring cleavage, and reactions resembling those in β-oxidation of fatty acids (Haarwood et al., 1999).

A large DNA contig of *Azoarcus* strain EbN1 with the entire genetic blueprint for anaerobic toluene degradation has been elaborated (Kube et al., 2004; Fig. 7). Five genes of unknown function separate the predicted *bss* and *bss* operons. The absence of genes encoding for regulatory proteins in the proximity of the *bss* operon and the occurrence of similar sequence motifs in the promotor regions of the *bss* and *bss* operons suggest a coordinated regulation of both operons. The regulation in response to toluene is probably mediated by a two-component regulatory system (TdiSR).

Xylenes and *p*-Cymene

Among alkylbenzenes with two or more alkyl substituents, xylenes (dimethylbenzenes) are the most relevant ones in oil and as chemicals. Several strains that can degrade toluene can also grow with *m*-xylene (Spormann and Widdel, 2000; Widdel and Rabus, 2001). There is evidence that anaerobic degradation of *m*-xylene proceeds, in analogy to that of toluene, via *m*-methylbenzylsuccinate to *m*-methylbenzoyl-CoA (Spormann and Widdel, 2000). Further degradation of *m*-methylbenzoyl-CoA would be possible by reactions analogous to those of benzoyl-CoA because the methyl group does not interfere with reactions of a regular β-oxidation. Degradation of *o*- and *p*-xylene, which appear to be poorly and rarely utilized by anaerobic bacteria (Häner et al., 1995; Harms et al., 1999b), may be also initiated by reaction with fumarate which then leads to *o*- and *p*-methylbenzoyl-CoA, respectively. Upon ring cleavage, however, the methyl groups would prevent one round of regular β-oxidation and thus require additional mechanisms for complete substrate oxidation.

Furthermore, degradation of higher dialkylbenzenes has been observed, among which *p*-isopropyltoluene (*p*-cymene) as a plant hydrocarbon is probably the naturally most significant compound (Harms et al., 1999a). *p*-Isopropyltoluene was rapidly utilized by denitrifying strains, suggesting an effective mechanism to bypass the blockage (due to alkyl branching) of β-oxidation after dearomatization of the assumed intermediate *p*-isopropylbenzoyl-CoA and thiolytic ring cleavage. *p*-Isopropylbenzoate (*p*-cumate) has been detected as a metabolite, suggesting that the methyl group is the site of the initial enzymatic attack. One of the isolated strains, pCyN1, could also utilize toluene. However, the capacities for *p*-cymene and toluene degradation in strain pCyN1 were separately induced by the substrates, suggesting distinct enzymatic systems at least for parts of the metabolic pathways (Harms et al., 1999a).

Ethylbenzene and Propylbenzene

Pure cultures of anaerobes isolated with and shown to degrade ethylbenzene are denitrifying *Azoarcus* strains (Rabus and Widdel, 1995; Ball et al., 1996) and a gas-vesicle containing type of sulfate-reducing bacterium (Kniemeyer et al., 2003).

Ethylbenzene oxidation in the denitrifiers was shown to proceed via dehydrogenation to 1-phenylethanol and acetophenone, which is probably followed by carboxylation and activation yielding benzoylacetyl-CoA, and subsequently by thiolytic cleavage to acetyl-CoA and benzoyl CoA (Rabus and Widdel, 1995; Ball et al., 1996; Champion et al., 1999; Johnson and Spormann, 1999; Kniemeyer and Heider, 2001a; Kniemeyer and Heider, 2001b), as depicted in Fig. 8. Ethyl-

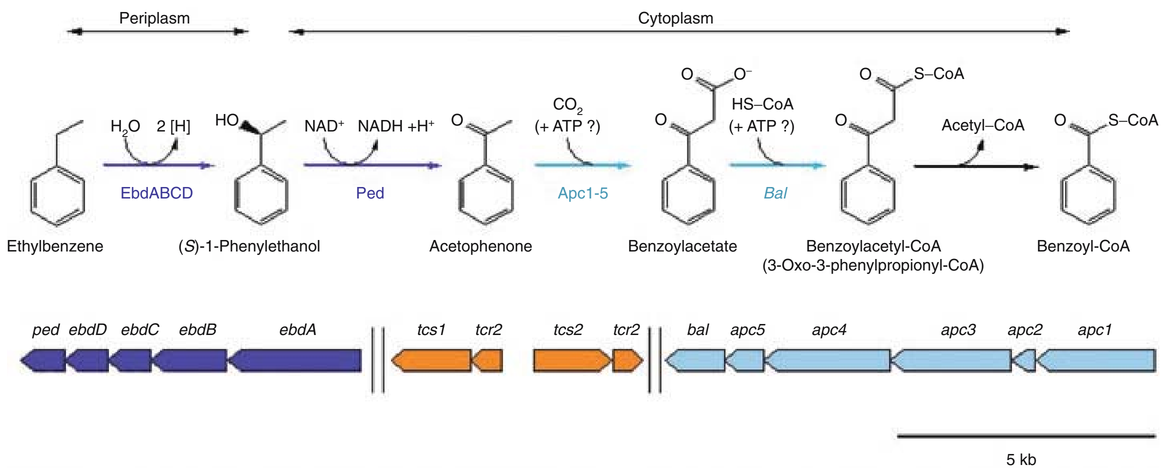


Fig. 8. Reactions and genes involved in anaerobic ethylbenzene degradation to the level of benzoyl-CoA in denitrifying strain EbN1. The biochemical reactions are supposed to be the same in other denitrifying bacteria. Benzoyl-CoA is further oxidized via reductive dearomatization and ring cleavage (Haarwood et al., 1999) to carbon dioxide (not shown). Reducing equivalents ([H]) are used for the reduction of nitrate to dinitrogen. Enzyme names of gene products (in bold) are as follows: EbdABCD, ethylbenzene dehydrogenase; Ped, (*S*)-1-phenylethanol dehydrogenase; Apc1-5, acetophenone carboxylase; and Bal, benzoylacetate CoA-ligase. The last reaction step is probably catalyzed by a benzoylacetyl-CoA thiolase. Identified genes involved in this pathway are clustered into two groups. Dark blue: genes for the upper part of the pathway from ethylbenzene to acetophenone. Light blue: genes for the lower part of the pathway from acetophenone to benzoyl-CoA. Orange: genes for two-component regulatory systems, one of which has been suggested to be specific for ethylbenzene degradation (Tcs2/Tcr2) and the other one for acetophenone (Tcs1/Tcr1) degradation; accordingly, both parts of the pathway may be regulated independently.

benzene dehydrogenase, which produces (*S*)-1-phenylethanol in *Azoarcus* strains (Johnson and Spormann, 1999; Johnson et al., 2001; Kniemeyer and Heider, 2001b), is a novel molybdenum/iron-sulfur/heme protein localized in the periplasm (Kniemeyer and Heider, 2001a). The natural electron acceptor is unknown. The capacity for ethylbenzene degradation was shown to be induced (Champion et al., 1999; Rabus and Heider, 1998). From the versatile alkylbenzene-utilizing *Azoarcus* strain EbN1, genes involved in anaerobic ethylbenzene metabolism have been identified on a large contig (Rabus et al., 2002). The genes for the “upper” pathway leading from ethylbenzene to acetophenone and for the “lower” pathway for the further metabolism of acetophenone are organized in two distinct operons. Two tentative two-component regulatory systems are encoded between these catabolic operons and are proposed to mediate the sequential, independent regulation of the pathways.

There is evidence from alkylbenzene-degrading communities (Elshahed et al., 2001) and a pure culture (Kniemeyer et al., 2003) that the initial anaerobic reaction of ethylbenzene in sulfate-reducing bacteria differs completely from the reaction in denitrifiers and is analogous to toluene activation. The formation of (1-phenylethyl)succinate and 4-phenylpentanoate indicated a radical-catalyzed addition of ethylbenzene to fumarate and subsequent carbon rearrangement and decarboxylation, in analogy to the anaerobic metabolism of alkanes (Fig. 6). In accordance with such a pathway for ethylbenzene instead of initial dehydrogenations, the sulfate-reducing strain was unable to oxidize 1-phenylethanol and acetophenone, which are intermediates and growth substrates in ethylbenzene-degrading denitrifiers. In the “low-potential” metabolism of sulfate-reducing bacteria, activation via a reductively generated radical (Fig. 5) is apparently easier to achieve than dehydrogenation (Fig. 8), which has a relatively high redox potential (1-phenylethanol/ethylbenzene, $E^{0\prime}$+0.03 V; estimated by analogy using thermodynamic data of other alcohols/hydrocarbons).

Azoarcus strain PbN1 utilizes *n*-propylbenzene in addition to ethylbenzene. The pathway of propylbenzene oxidation is assumed to proceed in analogy to that of ethylbenzene (Rabus and Widdel, 1995; Fig. 8) and may even involve the same enzymes for activation and subsequent steps (Kniemeyer and Heider, 2001a).

2-Methylnaphthalene

Alkylnaphthalenes (and higher polycyclic aromatic hydrocarbons) occur in great structural variety in crude oil, but little is known about degradation of these compounds. Alkylnaphtha-

lenes are expected to undergo activation easier than naphthalene (as alkylbenzenes are easier to activate than benzene) and to exhibit initial reactions comparable to those of alkylbenzenes. Indeed, identification of naphthyl-2-methylsuccinic acid as metabolite in a sulfate-reducing enrichment culture growing on 2-methylnaphthalene is in support of an activation mechanism analogous to that of toluene (Annweiler et al., 2000).

Anaerobic Microbial Growth with Crude Oil

As in the study of aerobic biodegradation of hydrocarbons (see the chapter on Hydrocarbon-Oxidizing Bacteria in this Volume), also the study of their anaerobic biodegradation is frequently connected with questions concerning microbial growth with and utilization of crude oil or derived industrial products such as fuels and organic solvents. A broad and relevant topic in this respect is the natural or stimulated in situ bioremediation of hydrocarbon-polluted environments. Even though aerobic hydrocarbon degradation is faster and probably includes a broader range of hydrocarbons than anaerobic degradation, the latter is of interest in subsurface environments where oxygen has no or limited access. Indeed, research of anaerobic in situ bioremediation of petroleum-derived hydrocarbons has developed into an own, vast area of research since the late 1980s. Coverage of this is beyond the scope of this chapter, and detailed information and references are given in other articles (e.g., Beller, 2000a, Elshahed et al., 2001; Gieg and Suflita, 2002; Reusser et al., 2002; Hunkeler et al., 2002; Richnow et al., 2003; Townsend et al., 2003). Here, we summarize some physiological aspects of anaerobic microbial growth with crude oil.

Crude oil (often also referred to as petroleum) is a highly complex mixture of saturated aliphatic and aromatic hydrocarbons as the main constituents (on the average 85% by weight; Tissot and Welte, 1984). Other constituents are aliphatic and aromatic compounds containing sulfur (e.g. alkylated thiophenes and benzothiophenes), monocarboxylic acids (so-called naphthenic acids), and resins and asphaltenes which are carbon- and hydrogen-rich polymeric substances with O- and S-atoms. Alkenes are usually absent; alkenes from biomass have been saturated or (in the case of appropriate structure) aromatized during the maturation of petroleum (Tissot and Welte, 1984).

Anaerobic enrichment cultures reducing sulfate (Rueter et al., 1994; Rabus et al., 1996) or nitrate (Rabus et al., 1999) and pure cultures reducing sulfate (Rueter et al., 1994; Wilkes et al., 2000) or nitrate (Rabus and Widdel, 1996; Wilkes et al., 2003) have been shown to grow with crude oil as the only source of organic substrates with simultaneous depletion of hydrocarbons. The enrichment cultures with crude oil (in freshwater medium) and nitrate favored the development of members of the Betaproteobacteria; crude oil and sulfate (in marine medium) enriched for sulfate-reducing Deltaproteobacteria related to genera with complete substrate oxidation and unrelated to *Desulfovibrio*. Of the saturated hydrocarbons, *n*-alkanes were consumed mostly in the C_6-C_{14} range. Of the aromatic hydrocarbons, toluene, xylenes and ethylbenzene were completely or largely consumed. Hence, in particular the lighter hydrocarbon fraction of crude oil seemed to be degraded under anoxic conditions. Estimations based on the amounts of oil added to cultures and sulfate or nitrate reduced by them suggested that up to about one tenth (wt/wt) of the crude oil was anaerobically degraded. However, the fraction that can be oxidized anaerobically may be higher since also hydrocarbons other than *n*-alkanes and alkylbenzenes can be also utilized anaerobically, especially by populations from highly contaminated sites (Bregnard et al., 1997; Lovley, 2000; Rabus and Widdel, 2001; Rios-Hernandez et al., 2003; Townsend et al., 2003; see also the section on Anaerobic Degradation of Aromatic Hydrocarbons). Nevertheless, anaerobic degradation of crude oil seems to occur to a lesser extent and at a much slower rate than aerobic degradation.

Anaerobic biodegradation of hydrocarbons, especially by sulfate-reducing bacteria, offers an explanation for the specific depletion of hydrocarbons in certain petroleum reservoirs despite the absence of oxygen (Connan et al., 1996). Nevertheless, petroleum in many reservoirs is still rich in the hydrocarbons that can be degraded anaerobically. This raises the question as for the circumstances that allow or prevent anaerobic hydrocarbon degradation in oil reservoirs (Rabus et al., 1996). First, microorganisms that were originally present in the sediments with biogenic carbon (that finally gave rise to petroleum) may have died off as the temperature increased with the depth of burial. But also reservoirs with temperatures favorable for bacterial growth (probably below 80°C) may harbor sterile oil if it has uplifted from deeper, hotter reservoirs (Wilhelms et al., 2001). Second, sulfate-reducing microorganisms as the apparently most significant anaerobic degraders of oil may be strongly limited by sulfate that may have been mostly depleted already before oil maturation. Third, diffusion of hydrocarbons from oil phases to water phases which are both in a rock matrix may be too slow for maintenance of microbial

life. In situ growth on petroleum hydrocarbons may be favoured only in connection with migration events and upon introduction of seawater and microorganisms from present marine habitats, for instance during oil production.

For the evaluation of the extent of an anaerobic biodegradation in contaminated deep aquifers or petroleum reservoirs, analyses of isotope ratios and polar metabolites are potentially useful. In cultures with crude oil (Fukui et al., 2000; Wilkes et al., 2000) or defined hydrocarbons (Meckenstock et al., 1999; Morasch et al., 2001), the residual portion of the degradable hydrocarbon became enriched in the heavier isotopes, ^{13}C (or ^{2}H), i.e. the lighter isotopomers were preferentially degraded. Isotope fractionations in hydrocarbon-contaminated aquifers can thus be taken as evidence for an anaerobic biodegradation in situ (Bolliger et al., 1999; Richnow et al., 2003). During anaerobic growth with crude oil in pure and enriched cultures (Wilkes et al., 2000, 2003) as well as during degradation of mixed hydrocarbons in contaminated subsurface sediment (Beller, 2000; Gieg and Suflita, 2002), formation of metabolites from the anaerobic degradation pathways was observed. However, metabolites such as alkyl- or arylsuccinates or alkylated benzoic acids may be also formed cometabolically, and the range of hydrocarbons activated in this way can be wider than the range that is definitely mineralized with coupling to growth (Wilkes et al., 2000, 2003).

Cultivation

The most commonly used electron acceptors for the study of the anaerobic degradation of hydrocarbons are sulfate and nitrate, and the following description of cultivation media and methods therefore focuses on these electron acceptors. However, the media can be modified for other purposes, for instance by omitting sulfate or nitrate to study methanogenesis from nonmethane hydrocarbons. The range of hydrocarbons utilized with sulfate as electron acceptor is apparently wider than the range utilized with nitrate. However, denitrifying anaerobic hydrocarbon degraders usually grow much faster, have higher growth yields, and are easier to handle in the laboratory. Denitrifying hydrocarbon degraders are, therefore, usually the first choice for enzymatic studies.

The use of defined, transparent media that do not contain organic nutrients other than the hydrocarbon substrate is recommended. An exception is the addition of ascorbate as a mild, compatible reductant (scavenger of traces of oxygen) to pure cultures of denitrifiers if this does not serve as a growth substrate (which is usually the case). Components that undergo chemical changes or volatilization in the heat are added from separately sterilized stock solutions after autoclaving and cooling of the main medium.

Oxygen is excluded as far as possible during preparation of the medium. An anoxic chamber is useful but not obligatory. With appropriate gassing devices and tubes with fitted stoppers and fixing caps (to avoid loss of stoppers) as described for the cultivation of sulfate-reducing bacteria (Widdel and Bak, 1992), all steps can be done at a normal laboratory bench.

Stock solutions are prepared as follows.

Trace Element Mixtures	A	B
Distilled water	987 ml	1000 ml
HCl (25% = 7.7 M)	13 ml	none
EDTA, disodium salt	none	5.2 g
H_3BO_3	10 mg	10 mg
$MnCl_2 \cdot 4H_2O$	5 mg	5 mg
$FeSO_4 \cdot 7H_2O$	4000 mg	2100 mg
$CoCl_2 \cdot 6H_2O$	190 mg	190 mg
$NiCl_2 \cdot 6H_2O$	24 mg	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg	10 mg
$ZnSO_4 \cdot 7H_2O$	200 mg	144 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg	36 mg
pH adjustment	none	6.0

"A" is for sulfate-reducing bacteria; the mixture is kept acidic (by the added HCl). "B" is for nitrate-reducing bacteria; the indicated pH is adjusted with NaOH. Use only fresh, greenish crystals of $FeSO_4 \cdot 7H_2O$; brownish grains indicate weathering and oxidation. The trace element solutions are autoclaved (preferentially anoxically under N_2).

Bicarbonate Solution

Dissolve 84 g $NaHCO_3$ in distilled water to a final volume of 1000 ml. Saturate the solution with CO_2 (by shaking in a stoppered bottle under a head space of CO_2) and autoclave in closed tubes or bottles with fixed stoppers (butyl rubber or Viton) under a head space of CO_2 (1/4 of total volume).

Vitamin Mixture	
Sodium phosphate (10 mM; pH 7.1)	100 ml
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
D(+)-Pantothenic acid, calcium salt	5 mg
Pyridoxine dihydrochloride	15 mg

Thiamine Solution	
Sodium phosphate (10 mM; pH 3.4)	100 ml
Thiamine chloride dihydrochloride	10 mg

Vitamin B12 Solution	
Distilled water	100 ml
Cyanocobalamin	5 mg

Filter-sterilize (pore size, 0.2 μm) all the above vitamin solutions and store in the dark (preferentially in brown glass bottles) at 4°C.

Sodium Sulfide Solution

Dissolve 48 g $Na_2S \cdot 9H_2O$ in 100 ml (final volume) of distilled water. Use only colorless, clear crystals of sodium sulfide. Sodium sulfide is autooxidizable. In the case of large crystals, opaque or milky surface layers (oxidation products) may be removed by brief rinsing with distilled water on a plastic sieve. Dissolve the sodium sulfide by stirring under an N_2 atmosphere and autoclave the solution in closed tubes or bottles with fixed stoppers (butyl rubber or Viton) under a head space of N_2 (≥1/4 of total volume).

Sodium Ascorbate Solution

Distilled water	40 ml
Ascorbic acid	9 g
NaOH (1.0 M)	40 ml

Add the NaOH solution slowly while stirring and cooling in an ice water bath, preferentially in a device that allows gassing with N_2 to avoid access of air. Add further NaOH dropwise so as to achieve a final pH of 8–9. Dilute with distilled H_2O to a final volume of 100 ml. Filter-sterilize and store anaerobically under a head space of N_2 in the dark at 4°C.

Preparation of Media

Depending on the physiological type of microorganisms to be cultivated and the salinity of the original source, one of the following basal mineral media is first prepared. For many marine isolates, the full marine medium can be replaced by the saltwater medium that tends to form less inorganic precipitates. For microorganisms from brackish habitats, also other concentrations of NaCl, $MgCl_2$ and $CaCl_2$ can be tested and applied.

Basal Salt Media	A	B	C
NaCl	0.5 g	20.0 g	26.0 g
$MgCl_2 \cdot 6H_2O$	0.5 g	3.0 g	5.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g	0.15 g	1.4 g
NH_4Cl	0.3 g	0.3 g	0.3 g
KH_2PO_4	0.2 g	0.2 g	0.1 g
KCl	0.5 g	0.5 g	0.5 g
For sulfate-reducing bacteria:			
Na_2SO_4	3.0 g	3.0 g	4.0 g
For nitrate-reducing bacteria:			
$NaNO_3$	1.0 g	1.0 g	1.0 g

"A" (freshwater medium) is for microorganisms from freshwater habitats. "B" (saltwater medium) can be used for marine microorganisms that do not require high magnesium and calcium ion concentrations; the advantage of the relatively low concentration of these ions is that the pH can be increased without significant formation of precipitates. "C" (full marine medium) is used for marine microorganisms with unknown salt demands or which require high magnesium and calcium ion concentrations (as in natural seawater); a certain disadvantage is the formation of significant precipitates with increasing pH. Omit Na_2SO_4 and $NaNO_3$ in case of methanogenic cultures or growth tests with electron acceptors other than sulfate or nitrate. Prepare media in special flasks with tubes for anoxic sterile gassing and a closable outlet that allows distribution of the complete medium to smaller cultivation tubes or bottles (Widdel and Bak, 1992). Dissolve the salts in distilled water up to a final volume of 1000 ml and autoclave. Cool solution under an N_2-CO_2 mixture to prevent redissolution of oxygen. Then, add the following sterile stock solutions (amounts per liter of medium).

Trace element mixture	1.0 ml
$NaHCO_3$ solution	30.0 ml
Vitamin mixture	1.0 ml
Thiamine solution	1.0 ml
Vitamin B12 solution	1.0 ml

Reductant for sulfate-reducing bacteria:

Na_2S solution 5.0 ml

Reductant for nitrate-reducing bacteria:

Sodium ascorbate solution 3.0 ml

Do not use the sodium ascorbate solution in enrichment cultures. Adjust the pH to 7 with sterile 1 M Na_2CO_3 or H_2SO_4 solution. Distribute the completed medium in culture tubes and bottles and store anoxically under a small head space of an N_2-CO_2 mixture. Add the hydrocarbon of interest individually to each tube or bottle. Procedures are described in the following section.

Cultivation with Hydrocarbons

GASEOUS HYDROCARBONS. The gas is released from steel bottles via gauges. Aseptic addition is guaranteed by passing the hydrocarbon gas through a sterile cotton or membrane filter. The gaseous hydrocarbons may be injected through stoppers to the culture head space by means of syringes with hypodermic needles. The syringes should be preflushed with the gas to remove oxygen. The added amount is obvious from the added volume (at 25°C, a volume of 24 ml of the pure gas at ambient pressure [101 kPa] is approx. 1 mmol). The application of high overpressure to gaseous hydrocarbons (with the exception of methane) is usually not necessary. In the case of methane, an increased pressure clearly stimulates anaerobic methane-oxidizing communities (Nauhaus et al., 2002). A safe device has been described that allows application of high pressure to methane in glass tubes (Nauhaus et al., 2002).

LIQUID HYDROCARBONS. These can be sterilized by filtration through solvent-resistant cellulose filters (pore size, 0.2 μm) or be autoclaved in tightly closed bottles with a head space (approx. 1/2 of bottle volume); in the case of volatile hydrocarbons, the weight should be controlled to reveal the tightness of the closure. For storage (as well as for autoclaving), screw caps with Teflon-coated sealing disks are useful (Fig. 9A, B). A special glass flask has been used for sterilization and aseptic, anoxic storage of crude oil without loss of volatile components (Rabus and Widdel, 1996; Fig. 9B).

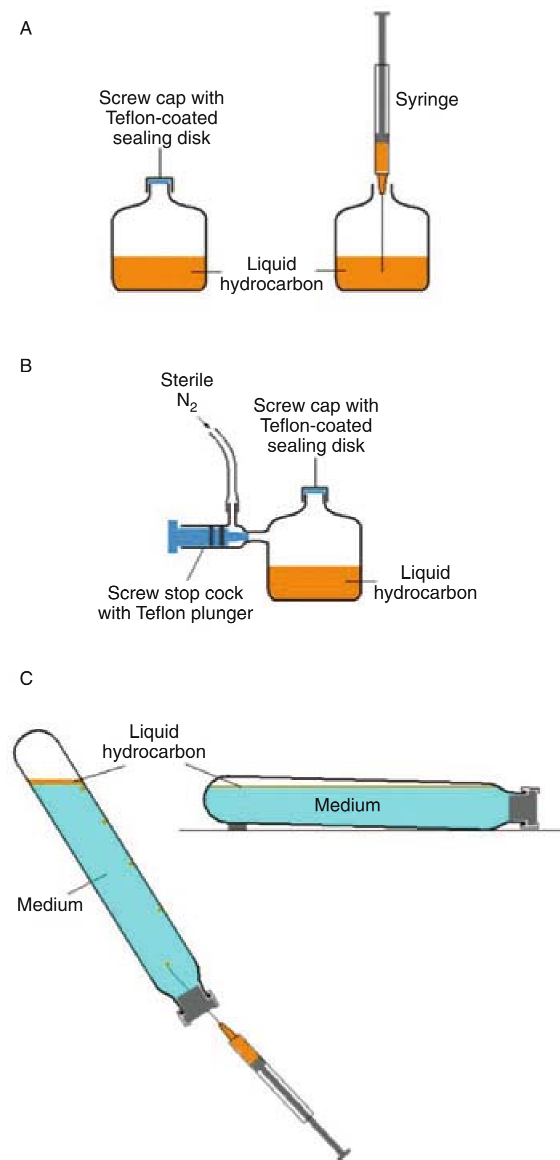


Fig. 9. Storage of (sterile) hydrocarbons and their addition to anoxic culture medium. (A) Use of a simple bottle. (B) Advanced method using a special bottle (with inner surfaces only of glass and Teflon) for anoxic autoclaving and storage. (C) Injection of hydrocarbon into culture tube while contact with the stopper is avoided, and incubation in near-horizontal position.

Hydrocarbons from stocks may be taken up with anoxic (N_2-gassed) syringes and added to the cultures, for instance by injection through the stoppers (Fig. 9A, C). The syringes should have plungers with plastic or Teflon sealing. Rubber-sealed plungers are affected by liquid hydrocarbons such that they stick to the syringe cylinder.

Cultivation with liquid hydrocarbons poses problems because of their poor solubility (viz., hydrocarbons usually float on the medium surface), their frequent toxicity, and their tendency to adsorb to stoppers or even to deteriorate stoppers gradually.

The low solubility problem can be minimized by providing a large contact area between the medium and the overlying hydrocarbon phase. For this purpose, tubes or bottles (preferentially flat bottles) are incubated horizontally (Fig. 9C). This enlarges the surface area and minimizes diffusion distances between the hydrocarbon phase and the bacteria in the medium.

The toxicity can in principle be minimized by adding extremely small amounts to keep the hydrocarbon concentration below saturation. However, such amounts are often below one milligram per liter and therefore yield only marginal cell densities. It is therefore much easier to provide such hydrocarbons from a dilute solution (often 1–15%, v/v) in an inert hydrophobic carrier. The overlying carrier phase then acts as a reservoir of the hydrocarbon substrate that is permanently provided at a nontoxic concentration. Mineral oil (pharmaceutical grade; not useful for cultures that degrade long-chain alkanes), 2,2,4,4,6,8,8-heptamethylnonane, or pristane have been applied as carriers.

Adsorption of hydrocarbons to stopper material can be minimized or prevented in several ways. Stocks of sterile hydrocarbons can be kept in bottles with screw caps with Teflon-coated sealing disks. For culture tubes and bottles, Teflon-coated stoppers may be used. Even if needles penetrate these stoppers, the areas exposed to the hydrocarbon remain relatively small and adsorption is much slower than at an unprotected stopper surface. In any case, stoppered culture tubes and bottles containing hydrocarbons should be kept in near-horizontal position so that the hydrocarbon phase is not in contact with the stopper. This is achieved by keeping the orifice always lower than the medium level (Fig. 9C). If the tube or bottle containing the hydrocarbon phase is initially in an upright position (which is usually the case), inversion to the horizontal position necessarily brings the hydrocarbon phase in contact with the stopper. Shaking the tube or bottle (causing a transient water-hydrocarbon emulsion) while it is being inverted can avoid adherence of large hydrocarbon droplets to the stopper. An elegant approach is to add the hydrocarbon to the horizontal bottle through the stopper by means of an anoxic syringe; this can be done in such way that the hydrocarbon ascends to the medium surface without coming into contact with the stopper (Fig. 9C).

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Prokaryotes (2006) 2:1050–1079
DOI: 10.1007/0-387-30742-7_34

CHAPTER 1.34

Physiology and Biochemistry of the Methane-Producing Archaea

REINER HEDDERICH AND WILLIAM B. WHITMAN

The methane-producing Archaea or methanoarchaea are distinguished by their ability to obtain all or most of their energy for growth from the process of methane biosynthesis or methanogenesis. To date, no methanogens have been identified that can grow without producing methane, and these Archaea are all obligate methane producers that are uniquely specialized for this lifestyle. Methanogenesis is an anaerobic respiration, but its complexity and commitment of resources far exceeds that found in other common respiratory processes. For instance, it requires the biosynthesis of six unusual coenzymes; a long, multistep pathway for methane; and a number of unique membrane-bound enzyme complexes for coupling to the proton motive force (see below).

Given the complexity of this process, it is not surprising that methanogens appear to be monophyletic. Hence, all modern methanoarchaea possess an ancient ancestor within the Euryarchaeota (Fig. 1). Although the branching order of the deep branches is not certain, several lineages of nonmethanogenic archaea appear within this clade, which argues for the antiquity of the methanogen group. Thus, the phylogeny suggests that the lineages represented by *Haloferax*, *Thermoplasma* and *Archaeoglobus* were derived from methanogenic ancestors. The sulfate-reducer *Archaeoglobus* also possesses many of the unusual coenzymes found in methanogens, providing further evidence of this hypothesis.

The methanoarchaea are also the only Archaea currently cultivated that are truly cosmopolitan, being found in a wide variety of the anaerobic environments on earth. Thus, in addition to many temperate habitats, methanogens are also common at extremes of temperature and salinity. As examples, psychrophilic and psychrotolerant species isolated from a meromictic lake in Antarctica grow well at 0–5°C (Franzmann et al., 1992; Franzmann et al., 1997), and hyperthermophilic species obtained from geothermal springs and submarine vents grow well up to 110°C (e.g., Jones et al., 1983; Kurr et al., 1991). While many methanogens grow well in fresh water, the extremely halophilic *Methanohalobium evestigatus* requires 4.3 M NaCl for optimal growth (Zhilina and Zavarin, 1987). The broad distribution of the methanogens is in contrast to the other cultivated archaea, which appear to be limited to extreme environments of high temperature, low pH, or high salinity. Presumably, archaea fair poorly in direct competition with temperate bacteria and eukaryotes and are limited to either extreme habitats or niches where they do not compete with bacteria or eukaryotes. This hypothesis implies that the currently uncultivated Crenarchaeota common in temperate soil and marine environments occupy a physiological niche unavailable to the Bacteria (for a review of these organisms, see Phylogenetic and Ecological Perspectives on Uncultured Crenarchaeota and Korarchaeota in Volume 3). It also poses an interesting physiological mystery as to why Bacteria might out-compete Archaea in many temperate environments.

Ecology of Methanogenesis

While the ecology of the methanoarchaea is discussed in detail in the chapters about specific groups, some generalities are appropriate here (for a more detailed review, see Zinder et al., 1993). The methanoarchaea flourish in anaerobic environments where sulfate, oxidized metals, and nitrate are absent. In these environments, the substrates for methanogenesis are readily available as the fermentation products of Bacteria and eukaryotes, and the methanoarchaea catalyze the terminal step in the anaerobic food chain where complex polymers are converted to methane and CO_2. In this food chain, polymers are first degraded by specialized microorganisms, like the cellulolytic Bacteria, to produce simple sugars (such as glucose), disaccharides (such as cellobiose), lactate, and volatile fatty acids (VFAs; such as acetate, propionate and butyrate) and alcohols (such as ethanol). These products are further metabolized by the intermetabolic group. These microorganisms convert simple sugars to VFAs and alcohols.

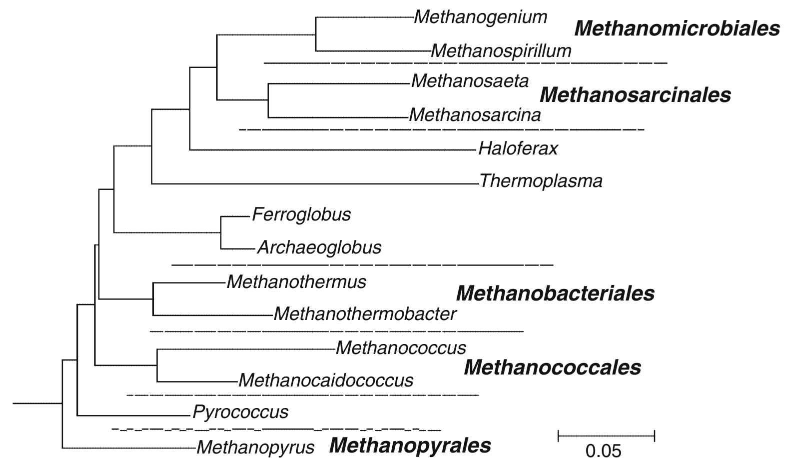


Fig. 1. Phylogeny of the methanoarchaea and related euryarchaeota. The gene tree of the 16S rRNA was calculated with the Fitch-Margoliash algorithm in PHYLIP and about 1260 bp of the small subunit rRNA sequences for representatives of each genus. The orders of methanogens are indicated on the right. The scale bar represents the Jukes-Cantor evolutionary distance.

They also convert VFAs and alcohols to acetate, H_2 and CO_2, which are major substrates for the methanoarchaea.

Molecular hydrogen (H_2) is a key intermediate in this process. Under standard conditions, when the H_2 partial pressure is 1 atmosphere, the fermentations of VFAs and alcohols to acetate and H_2 are thermodynamically unfavorable. Therefore, microorganisms that catalyze these reactions cannot grow, and toxic levels of the VFAs accumulate. However, if the methanoarchaea are present, H_2 is rapidly metabolized, and its partial pressure is maintained below 10^{-3}–10^{-4} atmospheres. Under these conditions, the fermentations of VFAs and alcohols are thermodynamically favorable, they are rapidly metabolized, and their concentrations are maintained below the toxic levels. This interaction between the H_2-producing intermetabolic organisms and the H_2-consuming methanogens is an example of interspecies hydrogen transfer (Syntrophism among Prokaryotes in this Volume). In many anaerobic environments, it is a key regulatory mechanism. Because the H_2-consuming methanogens play a critical role, they are said to "pull" the fermentation of complex organic polymers to methane and CO_2.

Interspecies hydrogen transfer is not limited to methanogenic food chains. In environments rich in sulfate, oxidized metals or nitrate, anaerobic Bacteria oxidize H_2, VFAs and alcohols. When sulfate is present, this activity is catalyzed by the sulfate-reducing bacteria. Because the oxidation of H_2 with sulfate as the electron acceptor is thermodynamically more favorable than when CO_2 is the electron acceptor (as in methanogenesis), the sulfate-reducing bacteria out-compete the methanogens for H_2. For similar reasons, the sulfate-reducing bacteria also out-compete the methanogens for other important substrates like acetate and formate. Therefore, methanogenesis is greatly limited in marine sediments that are rich in sulfate. The oxidation of H_2 with nitrate, Fe^{+3}, and Mn^{+4} as electron acceptors is also thermodynamically more favorable than methanogenesis. The denitrifying and iron- and magnesium-reducing bacteria also out-compete the methanogens when these electron acceptors are present. In conclusion, methanogenesis only dominates in habitats where CO_2 is the only abundant electron acceptor for anaerobic respiration.

Just as aerobic microorganisms rapidly deplete the O_2 in environments rich in organic matter to establish anaerobic conditions, sulfate-reducing bacteria, iron- and magnesium-reducing bacteria, and denitrifying bacteria frequently consume all the sulfate, Fe^{+3}, Mn^{+4} and nitrate in anaerobic environments and rapidly establish the conditions for methanogenesis. In these environments, CO_2 is seldom limiting because it is also a major fermentation product. Thus, methanogenesis is the dominant process in many anaerobic environments that contain large amounts of easily degradable organic matter. Especially important environments of this type include freshwater sediments found in lakes, ponds, marshes, and rice paddies.

Although methanoarchaea are most frequently found at the bottom of anaerobic food chains associated with the intermetabolic microorganisms, in some ecosystems they are the primary consumers of geochemically produced H_2 and CO_2. The submarine hydrothermal vents found on the ocean floor expel large volumes of very hot water containing H_2 and H_2S. As the water cools from several hundred degrees Celsius to the temperature of the ocean, zones suitable for the growth of thermophilic methanoarchaea are established. The methane produced escapes into the surrounding water where it is utilized by symbiotic methylotrophic bacteria living in marine invertebrates. In this ecosystem, the methanogens are at the top of the food chain.

Biogeochemistry of Methanogenesis

Methane is a major trace gas found in the earth's atmosphere, and most of the atmospheric methane is produced by the methanoarchaea (Conrad, 1996; Monson and Holland, 2001; Reeburgh, 2003). In the last several hundred years, the atmospheric concentration has more than doubled, to reach about 1.8 ppm in 1998. This increase in methane concentration is responsible for about 20% of the increased greenhouse effect observed for all radiatively important trace gases. Currently, methane emissions to the atmosphere are about 500–600 teragrams (Tg) of CH_4 year^{-1}. Once in the atmosphere, methane has a half-life of about 8.4 years, being removed primarily by reactions with hydroxyl radicals in the troposphere.

Major sources of atmospheric methane are shown in Table 1. The most important biogenic emissions are from habitats where gases formed anaerobically can exchange rapidly with the atmosphere, such as wetlands and rice paddies. Similarly, large amounts of methane are emitted from the rumen of livestock, which escapes directly to the atmosphere by eructation. However, large amounts of methane, probably about 700 Tg of CH_4 year^{-1}, are also consumed by aerobic and anaerobic methane-oxidizers without ever appearing in the atmosphere (Reeburgh et al., 1993; Valentine and Reeburgh, 2000; Reeburgh, 2003). Rice paddies are particularly active sites for methane oxidation, where oxygen is available in the root zone owing to transport through the plants. This allows for growth of the aerobic methane-oxidizing bacteria and consumption of 45–90% of the methane produced. Given that about 80% of the methane emitted to the atmosphere and nearly all of the methane oxidized prior to release to the atmosphere is microbially produced, an estimate of total microbial methane production is about 1100 Tg of CH_4 year^{-1}, which represents about 825 Tg of C year^{-1}. During the production of methane from carbohydrates, one mole of CO_2 is formed for every mole of CH_4. Thus, the total carbon processed in these systems is about 1650 Tg of C year^{-1}, or 1.65% of the C fixed by photosynthesis each year (Schlesinger, 1991). This estimate emphasizes the biogeochemical significance of this link in the carbon cycle.

Table 1. Sources of atmospheric methane.

Sources of methane	Methane evolved (Tg of CH_4/year)
Biogenic sources	
Natural wetlands	92
Rice paddies	88
Livestock	81
Manure decomposition	14
Termites	25–150
Landfills	15–81
Oceans	38–308
Tundra	42
Subtotal	395–856
Other sources	
Biomass burning	50
Coal mining	10–35
Venting and flaring	15–30
Industrial and pipeline losses	15–45
Methane hydrates	5
Subtotal	95–165
Total[a]	600 (490–1021)

Abbreviation: Tg, teragram, 10^{12} grams or 1 million metric tons.

[a]Best estimate with range in parentheses.

Modified from Tyler (1991) and Reeburgh (2003).

Systematics of the Methanoarchaea

Phylogenetic analyses of the rRNA and other genes indicate that the methanoarchaea are an ancient monophyletic lineage within the Euryarchaeota (Fig. 1). Although the branching orders of the deep groups of the Euryarchaeota are not known for certain, a number of nonmethanogenic organisms, such as the halobacteria and the sulfate-reducing *Archaeoglobus*, appear to have arisen within the methanogenic lineage. To produce descendants with very different phenotypic properties, the methanogenic lineage must be very ancient.

The current taxonomy for methanogens follows the general schema of Boone et al. (1993) and Whitman et al. (2001), which tried to form taxa of similar phylogenetic depth among groups of fairly unrelated organisms. This work attempted to deal with three major problems. First, because of their chemolithotrophic energy metabolism, it is often difficult to distinguish taxa on the basis of phenotype. Second, in spite of the phenotypic similarity, the methanogens are genetically, extremely diverse. The high genetic diversity suggests that, even though many of these organisms appear to do the same thing, the way they do these things are very different. Detailed studies of the physiology have tended to support this view, and large differences in cellular structure, metabolic pathways, and regulation have been observed. And third, on the basis of ribosomal RNA gene libraries of environmental DNA, many more taxa await to be isolated and characterized (see below). The cultured methanogens represent a very sparse sampling of the likely diversity in nature, and our knowledge

of this group is necessarily incomplete. Thus, the current taxonomy is best considered a work in progress with plenty of opportunities for improvement.

The methanoarchaea are a diverse group of organisms, containing five well-established orders and 31 genera (Table 2). For species, less than 70% DNA hybridization of the genomic DNAs is considered definitive evidence for novel species (Wayne et al., 1987). In the absence of DNA hybridization data, ribosomal RNA sequence similarity of less than 98% is considered equivalent evidence for novel species, even though this value is probably very conservative (Stackebrandt and Goebels, 1994; Keswani and Whitman, 2001). Less than 93–95% ribosomal RNA sequence similarity is evidence of novel genera, and less than 88–93% ribosomal RNA sequence similarity is evidence of novel families. The rank of order is then used to recognize deeper phylogenetic differences. In general, this taxonomy is supported by the chemotaxonomy of cellular lipids and distribution of other biological properties. Subsequently, three classes were also proposed: Methanobacteria (to include the Methanobacteriales), the Methanococci (to include the Methanococcales, Methanomicrobiales and Methanosarcinales), and the Methanopyri (to include the Methanopyrales; Boone, 2001). These classes were inferred from the deep phylogenetic relationships in the ribosomal RNA gene tree, which are imperfectly understood, and their biological significance remains to be further elucidated.

A summary of the major genera of methanoarchaea is given in Table 2. In prokaryotic nomenclature, the names of genera of methanoarchaea contain the prefix "methano-." This prefix distinguishes them from an unrelated group of aerobic Bacteria, the methylotrophic Bacteria, which consume methane and whose names contain the prefix "methylo-." More complete descriptions of the nutrition, growth properties, morphology, ecology and other general properties of these taxa are reviewed in other chapters of this volume (The Order Methanomicrobiales, The Order Methanobacteriales, and The Order Methanosarcinales in Volume 3) as well as *Bergey's Manual of Systematic Bacteriology* (Whitman et al., 2001). The descriptions of *Methanomethylovorans* and *Methanomicrococcus* and can be found in Lomans et al. (1999) and Sprenger et al. (2000).

Recent studies of the genus *Methanobrevibacter* serve to illustrate the incomplete nature of the current systematics of methanogens (Miller, 2001; The Order Methanobacteriales in Volume 3). This taxon is abundant in the gastrointestinal tracts of mammals, birds and termites as well as other habitats. It is particularly interesting because it illustrates the diversity of these organisms and some of the potential complexities of their lifestyle. In humans, methane is formed by the methanoarchaea in the anaerobic microflora of the large bowel. About one-third of healthy adults excrete methane gas. Some methane is also absorbed in the blood and excreted from the lungs. The most numerous methanogen in humans is *Methanobrevibacter smithii*. In people who excrete methane, it is found in numbers of 10^7–10^{10} cells per gram dry weight of feces, or between 0.001 and 12% of the total number of viable anaerobic prokaryotes (Miller and Wolin, 1982). Why the numbers of *M. smithii* fluctuates so greatly in apparently healthy individuals remains a mystery. Other species of *Methanobrevibacter* are encountered in the feces of other animals. *Methanobrevibacter gottschalkii* was isolated from horse and pig feces, *Methanobrevibacter thaueri* was isolated from cattle feces, *Methanobrevibacter wolinii* was isolated from sheep feces, and *Methanobrevibacter woesei* was isolated from goose feces (Miller and Lin, 2002). These characterized organisms apparently only represent a small fraction of the diversity present in nature.

The rumen is another major habitat for methanogens, and about 10–20% of the total methane emitted to the earth's atmosphere originates in the rumen of cows, sheep and other mammals. In this habitat, complex polymers from grass and other forages are degraded to acetate, volatile fatty acids, H_2 and CO_2 by the cellulolytic and intermetabolic groups of bacteria, fungi and protozoans (Miller, 1992). The acetate and VFAs are absorbed by the animal and are major energy sources. Thus, little methane is produced from acetate. The H_2 is used to reduce CO_2 to methane, which is emitted. Methanogenesis represents a significant energy loss to the animal, and up to 10% of the caloric content of the feed may be lost as methane. *Methanobrevibacter* species are also common in the rumen. For the bovine rumen, *M. ruminantium* is the predominant methanogen isolated. However, in a survey of rRNA genes in the rumen of sheep fed different diets, 62 phylotypes of *Methanobrevibacter* were recognized, many of which were not closely related to described species and were likely to represent members of at least four novel species (Wright et al., 2004). These results demonstrate the incomplete characterization of the methanoarchaea, even from a fairly well studied environment, and the substantial intraspecies diversity within these organisms. Similarly, an extensive analysis of 120 *Methanobrevibacter* sequences from cultures and clone libraries suggested the presence of at least ten deep lineages, many of which contained more than one described species (Dighe et al., 2004). Thus, this

Table 2. Taxonomy of the methane-producing Archaea.[a]

Order, family and genus	Morphology	Major energy substrates[b]	Temperature optimum (°C)	Cell wall[c]
Order Methanobacteriales				
Family Methanobacteriaceae				
Genus *Methanobacterium*	Rod	H_2, (formate, alcohols)	37–45	Pseudomurein
Methanothermobacter	Rod	H_2, (formate)	55–65	Pseudomurein
Methanobrevibacter	Short rod	H_2, (formate)	37–40	Pseudomurein
Methanosphaer[a]	Coccus	H_2 + methanol	37	Pseudomurein
Family Methanothermaceae				
Genus *Methanothermus*	Rod	H_2	80–88	Pseudomurein + protein
Order Methanococcales				
Family Methanococcaceae				
Genus *Methanococcus*	Coccus	H_2, formate	35–40	Protein
Methanothermococcus	Coccus	H_2, formate	60–65	Protein
Family Methanocaldococcaceae				
Genus *Methanocaldococcus*	Coccus	H_2	80–85	Protein
Methanotorrts	Coccus	H_2	88	Protein
Order Methanomicrobiales				
Family Methanomicrobiaceae				
Genus *Methanomicrobium*	Rod	H_2, formate	40	Protein
Methanoculleus	Irregular coccus	H_2, formate (alcohols)	20–55	Glycoprotein
Methanofollis	Irregular coccus	H_2, formate (alcohols)	37–40	Glycoprotein
Methanogenium	Irregular coccus	H_2, formate (alcohols)	15–57	Protein
Methanolactnia	Rod	H_2 (alcohols)	40	Glycoprotein
Methanoplama	Plate or disc	H_2, formate (alcohols)	32–40	Glycoprotein
Family Methanospirillaceae				
Methanospirillum	Spirillum	H_2, formate (alcohols)	30–37	Protein + sheath
Family Methanocorpusculaceae				
Genus *Methanocorpusculum*	Small coccus	H_2, formate (alcohols)	30–40	Glycoprotein
Methanocalculus[d]	Irregular coccus	H_2, formate	30–40	ND
Order methanosarcinales				
Family Methanosarcinaceae				
Genus *Methanosarcina*	Coccus, packets	Methanol, $MeNH_2$, (H_2, Ac, DMS)	35–60	Protein + HPS
Methanococcoides	Coccus	Methanol, $MeNH_2$	23–35	Protein
Methanohalophilus	Irregular coccus	Methanol, $MeNH_2$	35–40	Protein
Methanohalobium	Flat polygons	Methanol, $MeNH_2$	40–55	ND
Methanolobus	Irregular coccus	Methanol, $MeNH_2$ (DMS)	37	Glycoprotein
Methanomethylovorans	coccus, packets	Methanol, $MeNH_2$ DMS, MT	34–37	ND
Methanomicrococcus	Flat polygons	H_2 + Methanol, H_2 + $MeNH_2$	39	ND
Methanosalsum	Irregular coccus	Methanol, MeH_2, DMS	35–45	ND
Family Methanosaetaceae				
Genus *Methanosaeta (Methanothrix)*	Rod	Ac	35–60	Protein + sheath
Order Methanopyrales				
Family Methanopyraceae				
Genus *Methanopyrus*	Rod	H_2	98	Pseudormurein

Abbreviations: $MeNH_2$, methylamines (monomethylamine, dimethylamine, and trimethylamine); DMS, dimethylsulfide; MT, methanethiol; Ac is acetate; HPS, heteropolysaccharide; and ND, not determined.
[a]All of the methanoarchaea are members of the phylum Euryarchaeota.
[b]Major energy substrates for methane synthesis. Alcohols are some or all of ethanol, isopropanol, isobutanol and cyclopentanol. Parentheses means utilized by some but not all species or strains.
[c]Cell wall components.
[d]Placement in higher taxon is tentative.

genus appears to be very deep, with a large amount of interspecies variation as well. Recognizing that only a small fraction of the organisms in even a single genus are in culture leads to the conclusion that the full extent of the diversity of these organisms is largely unknown.

Pathways of Methanogenesis—An Overview

Methanoarchaea derive their metabolic energy from the conversion of a restricted number of substrates to methane (Table 3; Fig. 2). Most

Table 3. Free energies for typical methanogenic reactions.

Reaction	)G^E (kJ/mol of CH_4)
Type 1	
$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	–130
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	–120
CO_2 + 4 (isopropanol) → CH_4 + 4 (acetone) + 2 H_2O	–37
Type 2	
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	–113
$4\ CH_3OH \rightarrow 3CH_4 + CO_2 + 2\ H_2O$	–103
$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	–74
$2\ (CH_3)_2S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	–49
Type 3	
$CH_3COOH \rightarrow CH_4 + CO_2$	–33

methanoarchaea can reduce CO_2 to CH_4. The major electron donors for this reduction are H_2 and formate. In addition, some methanoarchaea can use alcohols like 2-propanol, 2-butanol, cyclopentanol and ethanol as electron donors. For the secondary alcohols, a two-electron oxidation to the ketone is performed. For instance, 2-propanol is oxidized to acetone. Ethanol is somewhat different in that a four-electron oxidation to acetate is performed. Because eight electrons are required to reduce CO_2 to methane, four molecules of H_2, formate, or 2-propanol are consumed. Even though formate is a reduced C_1 compound, it is oxidized to CO_2 before reductionto methane. The reduction of CO_2 to CH_4 proceeds via carrier-bound one-carbon intermediates. Methanofuran (MFR), tetrahydromethanopterin (H_4MPT) or its derivatives, and 2-mercaptoethanesulfonate (coenzyme M, CoM-SH) are the three carriers involved (DiMarco et al., 1990; Gorris and van der Drift, 1994). These coenzymes were until recently thought to be unique for methanoarchaea but have now also been detected in nonmethanogenic bacteria and archaea (see below). The reaction sequence starts with a two-electron reduction of CO_2 and MFR to formyl-MFR where the formyl-group is bound to the amino-group of the coenzyme. The formyl-group is then transferred to the N5 of H_4MPT, the formyl-H_4MPT thus generated cyclizes to the methenyl-H_4MPT, which is reduced in two steps to the methyl-H_4MPT.

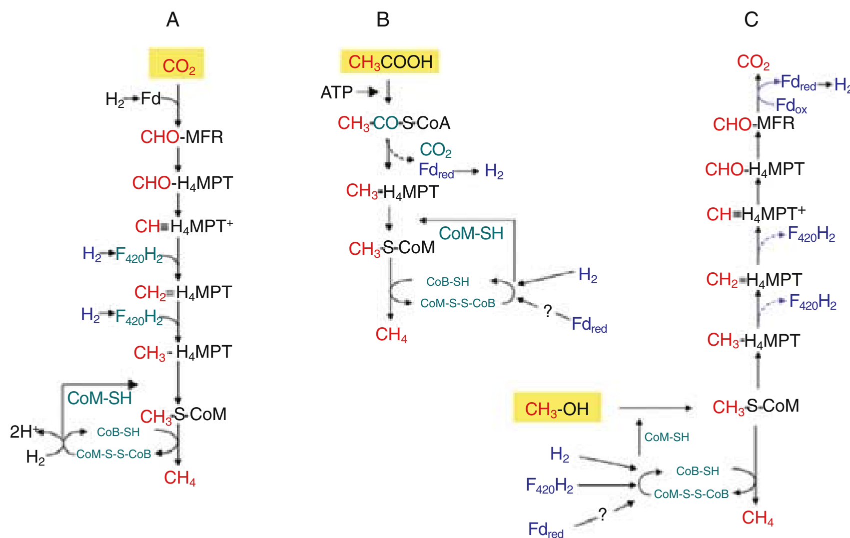


Fig. 2. Scheme of methanogenesis from H_2/CO_2 (A), acetate(B) and methanol (C). Methyl-coenzyme M (CH_3-S-CoM) is a central intermediate in all three pathways. It is converted to methane and the heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB). CoM-S-S-CoB thus generated functions as the terminal electron acceptor of different respiratory chains. H_2 and reduced coenzyme F_{420} ($F_{420}H_2$) have been identified as electron donors for the reduction of CoM-S-S-CoB. The unknown mechanism of electron transfer from the reduced ferredoxin ($Fd_{(red)}^-$) to CoM-S-S-CoB in acetate- and methanol metabolism is symbolized by a question mark. The role of H_2 as an intermediate of this reaction is discussed below (see Fig. 14). Abbreviations: CHO-FMR, *N*-formylmethanofuran; CHO-H_4MPT, N^5-formyltetrahydromethanopterin; CH=H_4MPT^+, N^5,N^{10}-methenyl-tetrahydromethanopterin; CH_2=H_4MPT, N^5,N^{10}-methylene-tetrahydromethanopterin; and CH_3-H_4MPT, N^5-methyl-tetrahydromethanopterin. For structures of the coenzymes, see Figs. 3, 7 and 9. For simplicity, only tetrahydromethanopterin (H_4MPT) is shown. For other methanopterin derivatives, see Fig. 9.

Finally, the methyl-group is transferred to the thiol group of coenzyme M. The methyl-thioether formed is reduced to CH_4 in the final step of the pathway.

The second type of substrate for methanogenesis includes C_1 compounds containing a methyl-group carbon bonded to O, N or S. Compounds of this type include methanol, monomethylamine, dimethylamine, trimethylamine, tetramethylammonium, dimethylsulfide and methane thiol. The methyl-group enters the C_1-pathway at the level of coenzyme M and is reduced to methane. The electrons for this reduction are obtained from the oxidation of an additional methyl group to CO_2 using the reverse of the steps of the reductive C_1-pathway. Because six electrons can be obtained from this oxidation and only two are required to reduce a methyl group to methane, the stoichiometry of this reaction is three molecules of methane formed for every molecule of CO_2 formed. In the presence of both a methyl-group donor and H_2, the methyl oxidation is inhibited and the methyl-groups are completely reduced to CH_4. An exception to this behavior is found in *Methanosphaera* and *Methanomicrococcus*, which lack the ability to oxidize methyl groups. These organisms only grow on methyl compounds when H_2 is also present. They are highly specialized for this activity and are unable to reduce CO_2 with H_2 or other electron donors.

The third type of substrate is acetate. In this reaction, the methyl (C-2) carbon of acetate is reduced to methane using electrons obtained from the oxidation of the carboxyl (C-1) carbon of acetate. This reaction is called the "aceticlastic reaction" because it results in the splitting of acetate into methane and CO_2. In this metabolism, the methyl group enters the C_1-pathway at the level of methyl-H_4MPT.

Key reactions of the different methanogenic pathways will be described below. For a detailed description of methanogenesis, the reader is referred to reviews (Thauer, 1998; Deppenmeier et al., 1999; Ferry, 1999; Deppenmeier, 2002a). For a historical overview on methanogenesis, see Wolfe (1991) and The Archaea: A Personal Overview of the Formative Years in Volume 3).

Key Reactions in Biological Methane Formation

The Final Step of Methanogenesis

Although every pathway starts out differently, they all end with the same step, the reaction of methyl-coenzyme M (CH_3-S-CoM) with a second thiol coenzyme, called "coenzyme B" (CoB-SH), to form methane and the mixed disulfide (also called "heterodisulfide," CoM-S-S-CoB) of coenzyme M and coenzyme B (Fig. 3). This reaction is catalyzed by methyl-coenzyme M reductase (Mcr), making Mcrthe key enzyme in methanogenesis (Thauer, 1998). In its active site, this enzyme contains a unique prosthetic group, which is a nickel (Ni) porphinoid called "coenzyme F_{430}" (Fig. 3). For the enzyme to be active, Ni has to be strongly reducing and in the Ni(I) state. From the crystal structure of various forms of the inactive Ni(II) state with the bound coenzymes, it is known that the enzyme has an active site channel which extends from the protein surface deeply into the interior of the protein complex (Ermler et al., 1997). Coenzyme F_{430} forms the bottom of this channel. Methyl-coenzyme M has to enter this channel before the channel is blocked by coenzyme B. Upon binding, coenzyme B fills the narrowest segment of the channel, with its thiol group facing coenzyme F_{430} (Fig. 4). The Mcr crystal structure reveals five modified amino acids near the active site. The side chains of specific histidine, arginine, glutamine and cysteine residues are methylated, and the carbonyl oxygen of a glycine residue is substituted by sulfur. Their high degree of con-

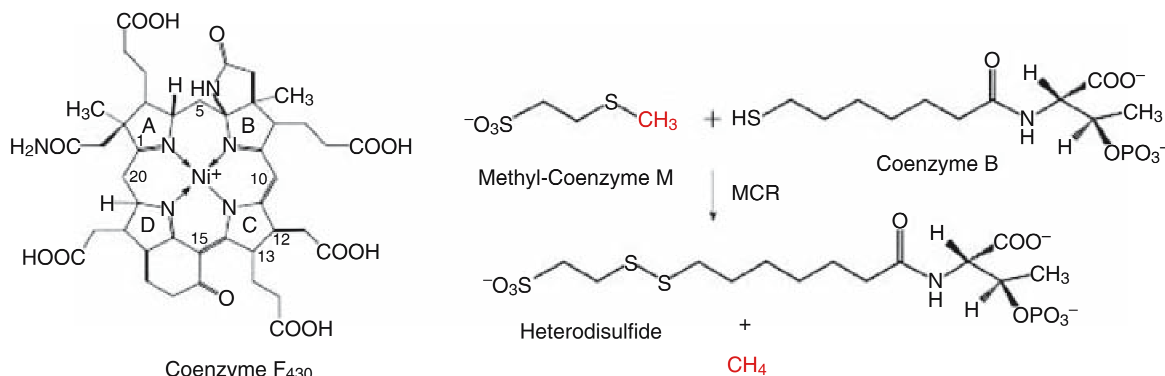


Fig. 3. Structure of coenzyme F_{430} and the reaction catalyzed by methyl-coenzyme M reductase.

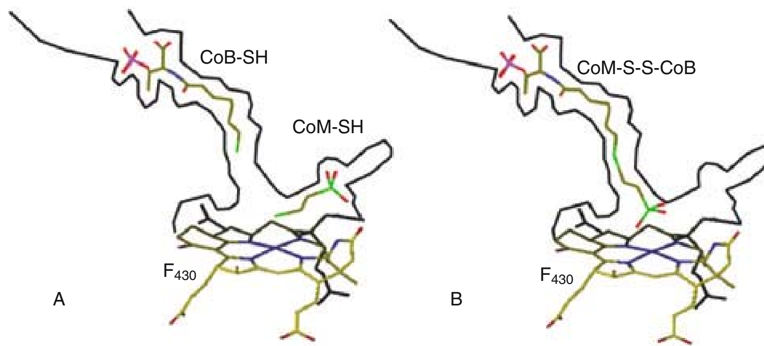


Fig. 4. Active-site of methyl-coenzyme M reductase with bound coenzymes. (A) Coenzyme B and coenzyme M are bound in the substrate channel. Note that coenzyme M is neither a substrate nor a product. In this structure, coenzyme M probably mimics the binding position of methyl-coenzyme M with respect to the binding of the sulfonate moiety but not with respect to the binding mode of the thiol group. A Ni-S-CoM species is not considered to be an intermediate in the catalytic cycle. (B) Heterodisulfide (CoM-S-S-CoB) product is bound in the active-site channel. The regions indicated with solid lines are the substrate channels near the active site.

servation and their location near the active site suggest an important function of these residues for the formation of the active site and catalysis. The reaction catalyzed by Mcr is rather unusual. Although the actual mechanism is still elusive, all of the proposed catalytic mechanisms involve radical chemistry (Goenrich et al., 2004). The closed hydrophobic environment of the substrate-binding pocket may be optimal for a mechanism employing unstable free radicals.

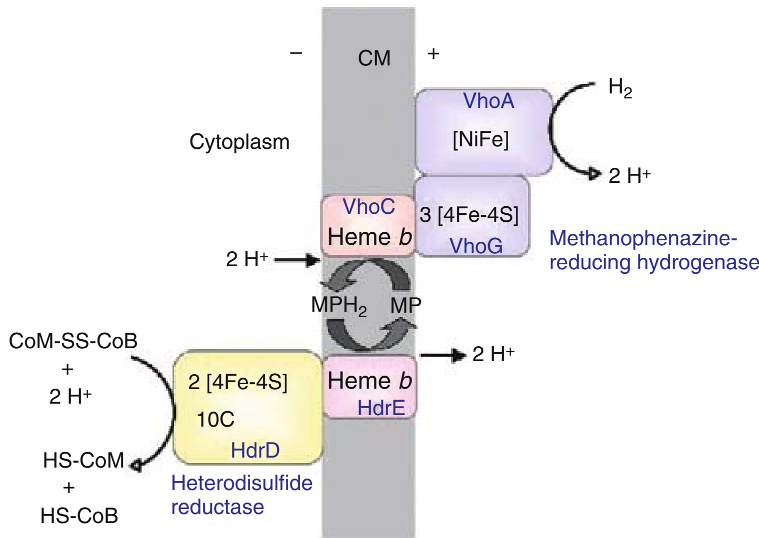
Energy Conservation via Disulfide Respiration

While the methane formed in the Mcr reaction can be regarded as a waste product, the heterodisulfide product is of central importance for the cell (Hedderich et al., 1998). Reduction of this disulfide is coupled with energy conservation (Deppenmeier et al., 1999; Deppenmeier, 2004). Hence, CoM-S-S-CoB can be regarded as the terminal electron acceptor of a respiratory chain in methanoarchaea. The electron donor can be either H_2 or coenzyme $F_{420}H_2$, depending on the growth substrate.

Evidence that heterodisulfide reduction is coupled to energy conservation comes from studies with *Methanosarcina* species. In Methanosarcina, all components of the respiratory chain are tightly membrane-bound, including a membrane-bound hydrogenase or $F_{420}H_2$ dehydrogenase, the lipophilic electron carrier methanophenazine, and a membrane-bound disulfide reductase (called "heterodisulfide reductase," Hdr). The latter enzyme functions as a terminal reductase and reduces CoM-S-S-CoB (Fig. 5). Methanophenazine is another novel coenzyme recently discovered in methanogens (Abken et al., 1998). Unlike the other methanogenic coenzymes, methanophenazine seems to be restricted to methanoarchaea belonging to the order Methanosarcinales. The function of this coenzyme is comparable with that of quinones in other respiratory chains.

Methanophenazine is reduced by one of the dehydrogenases, a membrane-bound [NiFe] hydrogenase or $F_{420}H_2$ dehydrogenase. For the [NiFe] hydrogenase, two isoenzymes are known. They are both anchored in the membrane via a β-type cytochrome (Deppenmeier et al., 1995b; Deppenmeier et al., 1999). The catalytic site is most probably facing the extracytoplasmic space, thus releasing two H^+ to the outside of the cell after H_2 oxidation. This enzyme, which in the past has been called "methylviologen-reducing hydrogenase" or "F_{420}-non-reducing hydrogenase," has now been designated "methanophenazine-reducing hydrogenase" (The H2-Metabolizing Prokaryotes in this Volume).

The $F_{420}H_2$ dehydrogenase (Fpo) is a multisubunit enzyme composed of six hydrophilic and seven integral membrane proteins (Bäumer et al., 2000; Fig. 6). The hydrophilic subunits are facing the cytoplasm. Eleven out of thirteen subunits of the enzyme reveal high sequence similarity to subunits of the energy-conserving reduced nicotinamide adenine dinucleotide (NADH):quinone oxidoreductase family (complex I), in particular to the bacterial enzyme which is formed by 14 subunits. Both enzymes differ with respect to the electron input module. In complex I, the electron input module (also called "NADH-dehydrogenase fragment") is formed by three subunits, which oxidize NADH and transfer the electrons to a central module. In Fpo, the NADH-dehydrogenase fragment is replaced by a single subunit, which oxidizes $F_{420}H_2$ (Brüggemann et al., 2000).



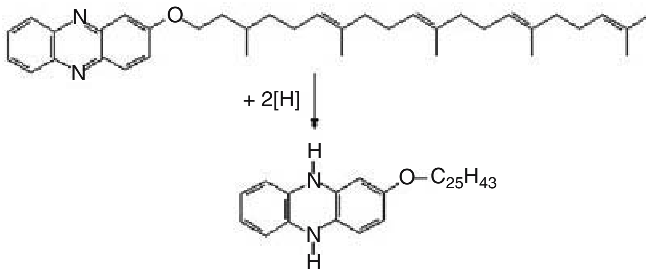


Fig. 5. Schematic representation of the respiratory chain catalyzing the reduction of CoM-S-S-CoB by H_2 in *Methanosarcina* species and structure of methanophenazine. Abbreviations: CM, cytoplasmic membrane; [NiFe], active site of the hydrogenases; [4Fe-4S], [4Fe-4S] clusters; heme b, subunits VhoC and HdrE each containing two heme β binding sites; 10C, ten highly conserved cysteinyl residues proposed to form one or two iron-sulfur clusters in the active site of Hdr; MP, oxidized methanophenazine; and MPH_2, reduced methanophenazine.

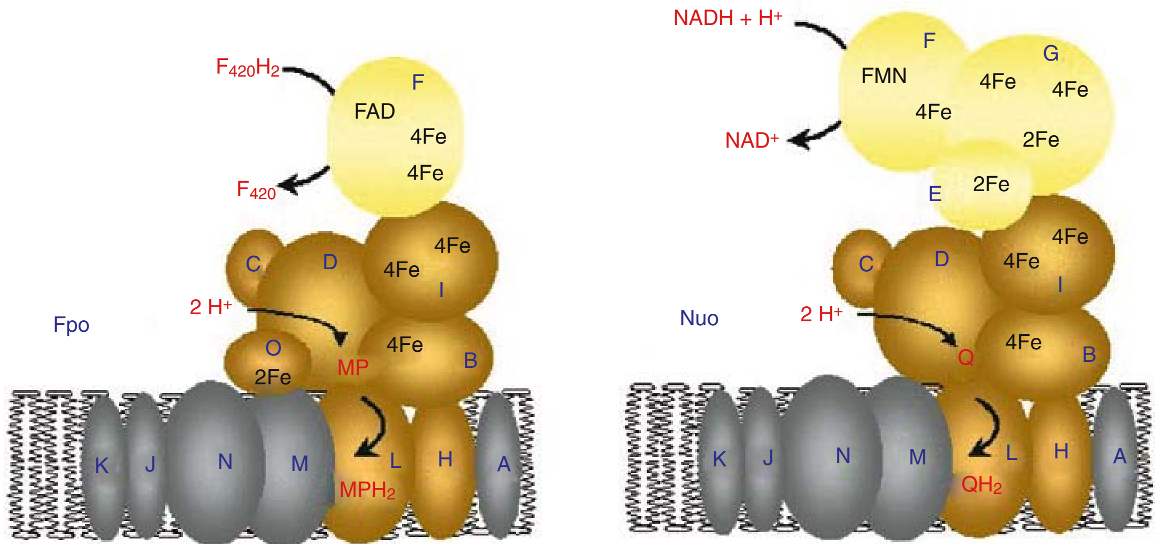


Fig. 6. Comparison of the structures of $F_{420}H_2$ dehydrogenase (Fpo) from *Methanosarcina mazei* and NADH:quinone oxidoreductase (Nuo) from *Escherichia coli*. Capital letters indicate subunits of the enzymes. Abbreviations: 4Fe: [4Fe-4S] cluster; 2Fe: [2Fe-2S] cluster; FMN: flavin mononucleotide; FAD, flavin dinucleotide; Q: ubiquinone or menaquinone; MP, oxidized methanophenazine; and MPH_2, reduced methanophenazine.FpoO has no counterpart in complex I, and its function is not known.

Reduced methanophenazine generated in the hydrogenase or $F_{420}H_2$ dehydrogenase reaction donates electrons to heterodisulfide reductase (Hdr), which catalyzes the reduction of CoM-S-S-CoB (Hedderich et al., 1998). In *Methanosarcina* species, this enzyme is formed by two subunits, a membrane-anchoring β-type cytochrome and a hydrophilic catalytic subunit, which is facing the cytoplasm. Unlike most other disulfide reductases, Hdr contains an iron-sulfur cluster in its active site which mediates the reductive cleavage of the disulfide substrate in two one-electron steps (Madadi-Kahkesh et al., 2001; Duin et al., 2003).

Using inverted membrane vesicles of *Ms. mazei*, both the H_2:CoM-S-S-CoB oxidoreductase and the $F_{420}H_2$:CoM-S-S-CoB oxidoreductase reactions were found to be coupled with the transfer of $4H^+/2e^-$ across the cytoplasmic membrane. More recent studies revealed that each partial reaction, the reduction of methanophenazine by H_2 or $F_{420}H_2$, and the reduction of CoM-S-S-CoB by reduced methanophenazine is coupled to the translocation of $2H^+/2e^-$ (Ide et al., 1999; Bäumer et al., 2000). As shown in Fig. 5, H^+-translocation in both the hydrogenase- and the heterodisulfide reductase reactions could function via a redox-loop mechanism. This mechanism cannot apply for the $F_{420}H_2$ dehydrogenase. Like complex I, this enzyme is thought to function as a proton pump (Bäumer et al., 2000).

In the hydrogenotrophic methanoarchaea belonging to the four other orders of methanoarchaea, the respiratory chain catalyzing the reduction of CoM-S-S-CoB differs significantly from that described above for *Methanosarcina*. These organisms do not contain heme. Thus β-type cytochromes can be excluded as membrane anchors and electron carriers of membrane bound dehydrogenases and reductases. Furthermore, methanophenazine has not been detected in these organisms (U. Deppenmeier, personal communication). As deduced from genome sequences, the membrane-bound $F_{420}H_2$-dehydrogenase (Fpo) is also lacking from these organisms.

Reduction of CoM-S-S-CoB in a non-*Methanosarcina* species has mainly been studied in *Methanothermobacter marburgensis*, which belongs to the order Methanobacteriales. In this organism heterodisulfide reductase, which is composed of three hydrophilic subunits, forms a tight and catalytically active complex with a [NiFe] hydrogenase (Setzke et al., 1994; Hedderich et al., 1998). This complex was designated as "H_2:heterodisulfide oxidoreductase complex." Hdr from this organism shares the catalytic subunit with the enzyme from *Methanosarcina* but is lacking a membrane anchor (Hedderich et al., 1998). The hydrogenase present in this complex is also lacking a membrane subunit and therefore is clearly different from the methanophenazine-reducing hydrogenase present in *Methanosarcina* species. Hence, the six subunits of the H_2:heterodisulfide oxidoreductase complex are all hydrophilic. The three transcriptional units encoding the different subunits of the complex do not contain additional open reading frames (ORFs) encoding potential integral membrane proteins, which might have been separated from the hydrophilic part during the purification. Hence, there is at present no conclusive answerhow this apparently cytoplasmic protein complex can couple the reduction of CoM-S-S-CoB by H_2 with the generation of a proton motive force.

Reductive Activation of CO_2 to Formylmethanofuran

The reduction of CO_2 to formylmethanofuran (CHO-MFR) is the first step of the methanogenic pathway from H_2/CO_2 (Fig. 7). This reaction is highly endergonic with H_2 as electron donor ($\Delta G^{\circ\prime}$ = +16 kJ·mol^{-1}). It becomes even more endergonic ($\Delta G'$ = +45 kJ·mol^{-1}) under the low hydrogen partial pressures prevailing in the natural habitats of methanoarchaea. In cell suspension experiments it was shown that this reduction is driven by reversed electron transport and requires an electrochemical ion gradient (Kaesler and Schönheit, 1989; Deppenmeier et al., 1996). A key enzyme involved in the catalysis of the reaction (Fig. 7) is formylmethanofuran dehydrogenase (Fmd; for a review, see Vorholt and Thauer, 2002). The enzyme catalyzes the reversible dehydrogenation of formylmethanofuran to CO_2 and methanofuran. In vitro this enzyme can be assayed with viologen dyes as artificial electron donors or acceptors. Fmd purified from *Methanosarcina barkeri* is a

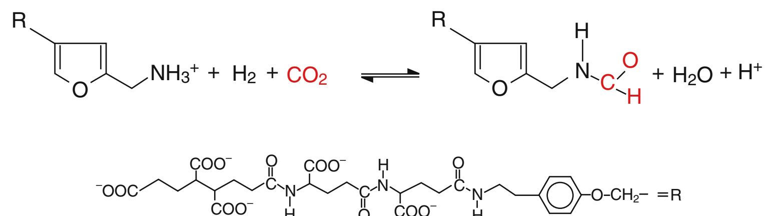


Fig. 7. Reaction catalyzed by formylmethanofuran dehydrogenase.

membrane-associated molybdenum iron-sulfur protein composed of six subunits. Other organisms, such as *Methanothermobacter* species, contain two Fmds, one enzyme containing molybdenum and the second enzyme containing tungsten bound to the molybdopterin cofactor.

The electron donor to Fmd and the hydrogenase participating in this reaction were unknown until recently. Upon identification of a novel membrane-bound [NiFe] hydrogenase in *Methanosarcina barkeri*, called the "*e*nergy-*c*onverting *h*ydrogenase" or Ech, it was suggested that this enzyme could play an essential role in driving this endergonic redox-reaction (Künkel et al., 1998; Meuer et al., 1999). Ech is a multisubunit membrane-bound [NiFe] hydrogenase. The six subunits of the enzyme exhibit high sequence similarity to subunits of the energy-conserving NADH:quinone oxidoreductase (complex I) of mitochondria and bacteria (Hedderich, 2004). In vitro, Ech catalyzes the reversible reduction of a special ferredoxin containing two [4Fe-4S] clusters by H_2 (Meuer et al., 1999). The oxidation of formylmethanofuran to CO_2 by the *M. barkeri* membrane fraction is also ferredoxin-dependent, and ferredoxin was therefore proposed to function as an electron carrier between Ech and Fmd in vivo. Support for this hypothesis came from the physiological characterization of a Δ*ech* mutant. This mutant was unable to make Ech and unable to form formylmethanofuran and reduce CO_2 to methane (Meuer et al., 2002). In addition, the Δ*ech* mutant was unable to biosynthesize acetyl-CoA and pyruvate via the acetyl-CoA synthase and pyruvate oxidoreductase reactions, respectively, using H_2 as the electron donor. Like the reaction catalyzed by Fmd, these reactions requirea strong reductant.

These data support the model depicted in Fig. 8. According to this scheme, Ech catalyzes the reduction of a low potential ferredoxin by H_2. Reduced ferredoxin can then function as the electron donor of Fmd but also of other oxidoreductases which require a low-potential electron donor. Since Ech is tightly membrane-bound via two integral membrane subunits and resembles the central part of complex I, it was suggested that ferredoxin reduction is driven by reversed electron transport. In contrast, the ferredoxin-dependent reduction of CO_2 to formylmethanofuran is not directly linked to an electrochemical ion gradient. This conclusion is supported by further experiments with the Δ*ech* mutant. In this mutant, CH_4 formation from H_2/CO_2 can be restored by CO or pyruvate, two strong electron donors which can couple to Fmd (Stojanowic and Hedderich, 2004). Moreover, when H_2 was replaced by CO as electron donor for the first step of methanogenesis, CH_4 formation was no longer dependent on an energized

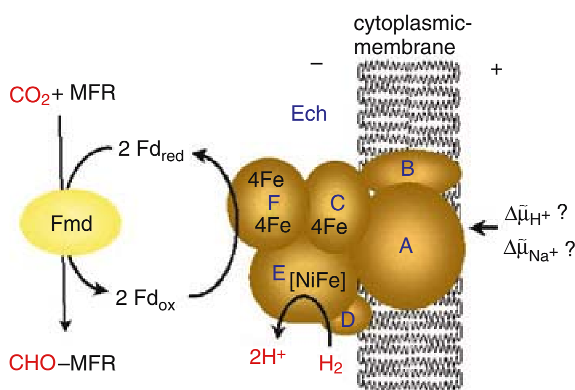


Fig. 8. Proposed function of Ech hydrogenase in the first step of methanogenesis from H_2/CO_2 in *Methanosarcina barkeri*. Whether Ech hydrogenase uses a H^+- or Na^+-motive force to drive the reduction of the ferredoxin by H_2 remains to be demonstrated. Abbreviations: Fmd, formylmethanofuran dehydrogenase; Fd, 2[4Fe-4S] ferredoxin; MFR, methanofuran; and CHO-MFR, formylmethanofuran.

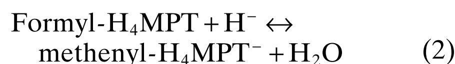
membrane. Thus, reduction of the electron carrier by H_2, which is catalyzed by Ech, and not the Fmd reaction per se, is dependent on an energized membrane.

Tetrahydromethanopterin Replaces Tetrahydrofolate in the C_1-Pathway of Methanoarchaea

Tetrahydromethanopterin (H_4MPT) carries the one-carbon unit at the oxidation level of formate, formaldehyde and methanol. In Methanococcales, Methanosarcinales and Methanomicrobiales, derivatives of H_4MPT such as tetrahydrosarcinapterin (H_4SPT) are used instead of H_4MPT (DiMarco et al., 1990; Gorris and van der Drift, 1994). H_4MPT is an analogue of tetrahydrofolate (H_4F), which is the C_1 carrier used by most other organisms (Fig. 9). The most important structural differences between the coenzymes are that H_4F has an electron-withdrawing carbonyl-group conjugated to the N10 via the aromatic ring, and H_4MPT has methyl groups at the ring carbons C7 and C11 (Maden, 2000). The entry into the H_4MPT-dependent reaction cascade is catalyzed by formyl-MFR:H_4MPT formyltransferase (Ftr), forming N^5-formyl-H_4MPT (reaction 1; Shima et al., 2002).

$$\text{Formyl-MFR} + H_4MPT \leftrightarrow \text{MFR} + \text{formyl-}H_4MPT \quad (1)$$

This is different from folate biochemistry where only N^{10}-formyl-H_4F is known. A cyclohydrolase then converts N^5-formyl-H_4MPT to N^5,N^{10}-methenyl-H_4MPT (reaction 2; Shima et al., 2002).



$$\text{Formyl-}H_4MPT + H^- \leftrightarrow \text{methenyl-}H_4MPT^- + H_2O \quad (2)$$

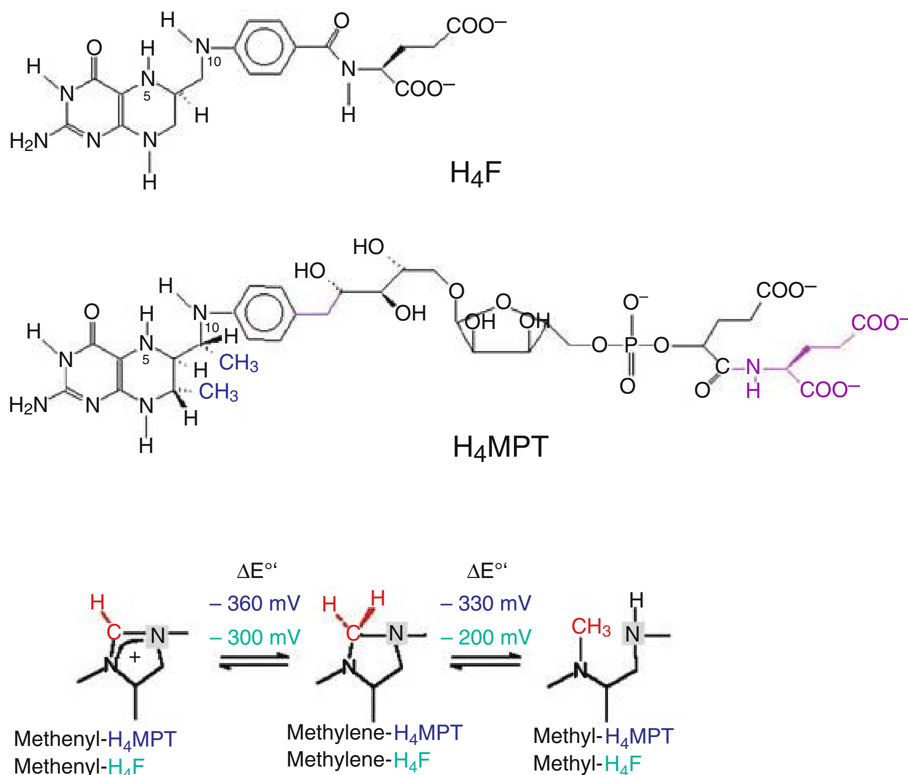
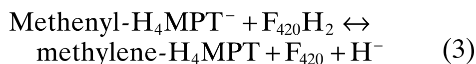


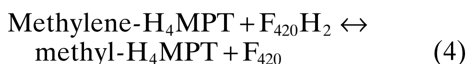
Fig. 9. Structure of tetrahydromethanopterin (H_4MPT), tetrahydrofolate (H_4F) and their C_1-derivatives. *Methanosarcina* species contain a H_4MPT analog called "tetrahydrosarcinapterin" (H_4SPT) in which the α carboxyl-group of the side chain is linked to a glutamate residue (highlighted in magenta).

The following reduction to methylene-H_4MPT is catalyzed by N^5,N^{10}-methylene-H_4MPT-dehydrogenase using reduced coenzyme F_{420} ($F_{420}H_2$) as electron donor (reaction 3; Hagemeier et al., 2003).



$$\text{Methenyl-}H_4MPT^- + F_{420}H_2 \leftrightarrow \text{methylene-}H_4MPT + F_{420} + H^- \quad (3)$$

This reaction is analogous to the N^5, N^{10}-methylene-H_4F-dehydrogenase reaction, although the electron donor is different. As will be described below, some methanoarchaea have an alternative enzyme which catalyzes methenyl-H_4MPT reduction with H_2 as electron donor. N^5,N^{10}-methylene-H_4MPT is then reduced to N^5-methyl-H_4MPT by N^5,N^{10}-methylene-H_4MPT reductase using $F_{420}H_2$ as electron donor (reaction 4; Shima et al., 2002).



$$\text{Methylene-}H_4MPT + F_{420}H_2 \leftrightarrow \text{methyl-}H_4MPT + F_{420} \quad (4)$$

In contrast to the corresponding enzyme of the H_4F-dependent pathway, the reductase from methanoarchaea lacks a flavin.

In the H_4MPT pathway, the methylene-H_4MPT and methenyl-H_4MPT redox couples are substantially more negative than in the H_4F pathway (Fig. 9). This enables reversible coupling to

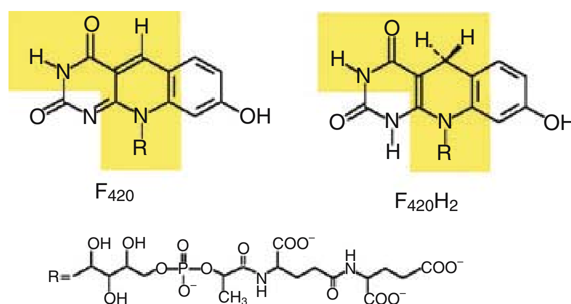


Fig. 10. Structure of coenzyme F_{420} in the oxidized and the reduced state. The structural similarity to pyridine nucleotides is indicated. *Methanothermobacter thermautotrophicus* and *Methanococcus voltae* F_{420} contain two glutamyl residues (F_{420}-2), and *Methanosarcina barkeri* contains F_{420} with four and five glutamyl residues (F_{420}-4,5).

the low redox-potential cofactor F_{420} (Fig. 10). This could be one reason why methanoarchaea utilize methanopterin instead of tetrahydrofolate in their C_1-pathway (Maden, 2000). F_{420} is a 5-deazaflavin, which is responsible for the blue-green fluorescence of methanoarchaea because of its high abundance in the cell. Unlike flavins, F_{420} only catalyzes hydride transfer reactions. Thus, it functions as a two-electron donor like $NAD(P)^+$. The $E^{\circ\prime}$ of the $F_{420}/F_{420}H_2$ couple is, however, about –360 mV, compared with

–320 mV for the $NAD(P)^+/NAD(P)H$ couple (Warkentin et al., 2001).

A Sodium Ion Pumping Methyltransferase

The last H_4MPT-dependent reaction in the methanogenic C_1-pathway is the transfer of the methyl-group from N^5-methyl-H_4MPT to coenzyme M (Fig. 11). This exergonic reaction ($\Delta G^{\circ\prime}$ = –30 kJ·mol^{-1}) is catalyzed by N^5-methyl-H_4MPT:CoM-SH methyltransferase (Mtr), a membrane-integral multienzyme complex composed of eight different subunits (MtrA-H; for a review, see Gottschalk and Thauer, 2001; Fig. 11). The enzyme is strictly dependent on sodium ions (Weiss et al., 1994). Subunit MtrA harbors a cob(I)amide prosthetic group (Gärtner et al., 1993), which is methylated and demethylated during the catalytic cycle. The demethylation reaction is Na^+-dependent. After reconstitution into proteoliposomes, the enzyme was shown to pump Na^+ (Lienard et al., 1996). A ratio of 1.7 mol of Na^+ translocated per mol of methyl-group transferred was determined. Thus, the enzyme appears to be a sodium pump that couples the methyl transfer to coenzyme M with formation of a sodium motive force.

The bound cobamide may play an important role in the enzyme mechanism. In aqueous solution, cob(II)amide and methylcob(III)amide contain axial ligands to the cobalt, whereas cob(I)amide does not (Kräutler, 1998). In unmethylated MtrA, the bound cob(I)amide should therefore have no axial ligand. Upon methylation of cob(I)amide to methylcob(III)amide, the methylcob(III)amide is expected to bind a histidine residue of the protein as an axial ligand (Harms and Thauer, 1997; Fig. 12). Binding of this axial ligand could therefore be associated with a conformational change of the protein. Upon demethylation of the cobamide, the axial ligand would be lost, and the conformational change would be reversed. Since demethylation is Na^+-dependent, the conformational change associated with this step can be coupled with the vectorial translocation of Na^+. The MtrH subunit can be separated from the MtrA-H-complex. The isolated MtrH subunit can catalyze the methylation of free cob(I)amide with methyl-H_4MPT (Hippler and Thauer, 1999).

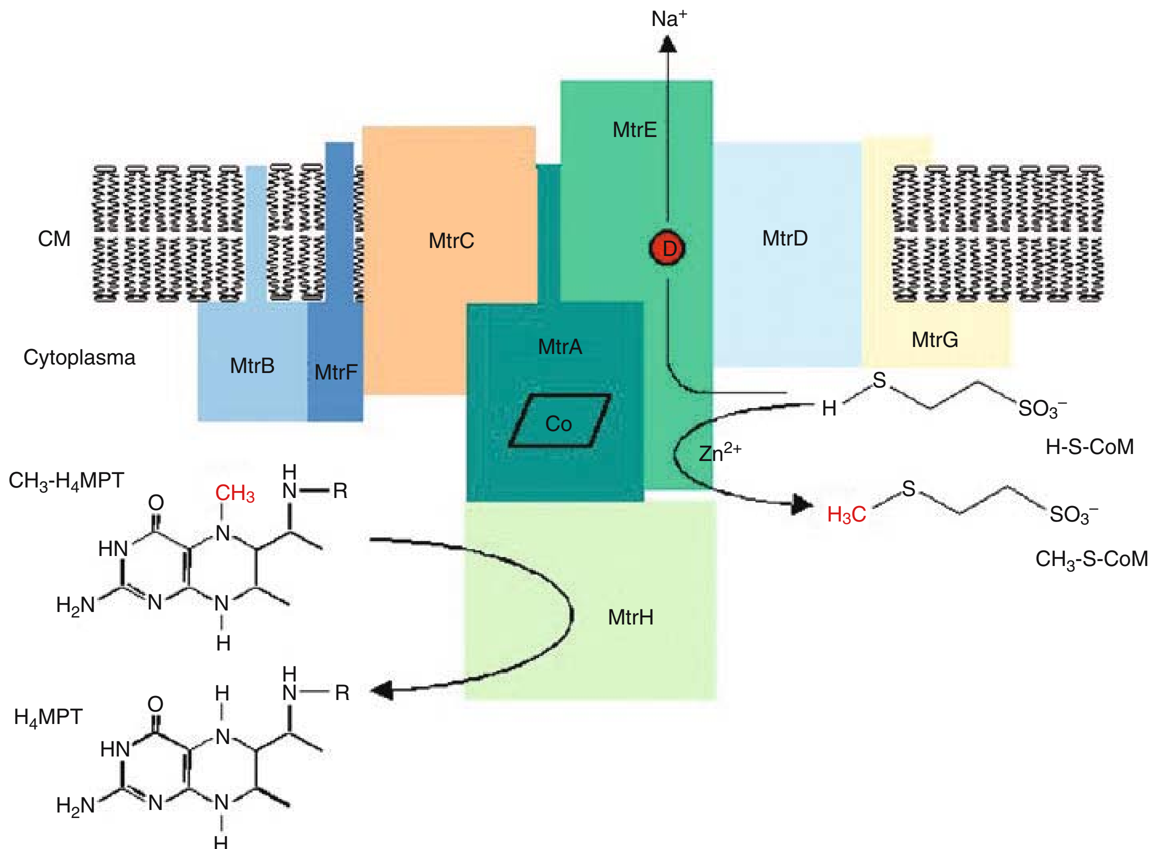


Fig. 11. Model of the methyl-H_4MPT:CoM-SH methyltransferase complex. A conserved aspartate residue (D) predicted to be located in a transmembrane helix of subunit MtrE is highlighted. This residue could be essential for sodium ion translocation. Modified from Gottschalk and Thauer (2001).

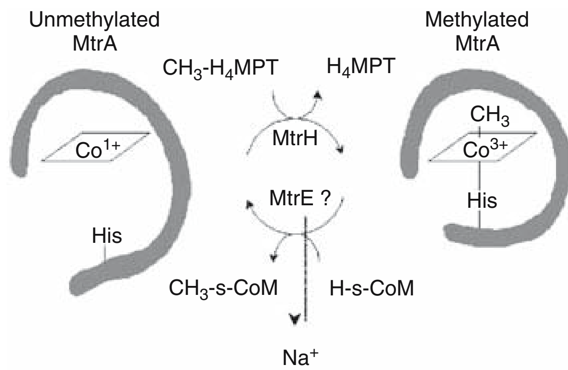


Fig. 12. Proposed conformational change of subunit MtrA of the methyl-H_4MPT:CoM-SH methyltransferase complex upon methylation and demethylation of its corrinoid prosthetic group. The MtrH subunit is proposed to catalyze the methylation of the cobamide bound to MtrA. The demethylation reaction is thought to be catalyzed by MtrE and coupled with vectorial sodium ion translocation since this reaction is sodium ion dependent. Modified from Gottschalk and Thauer (2001).

Therefore, MtrH is thought to catalyze the methylation of the corrinoid prosthetic group, which is bound to MtrA. Subunit MtrE is thought to transfer the methyl-group from the corrinoid prosthetic group of MtrA to coenzyme M, which is the Na^+-dependent reaction (Gottschalk and Thauer, 2001). MtrE is predicted to form six transmembrane spanning helixes and to have a large cytoplasmic domain containing a typical zinc-binding motif. All enzymes known to date that catalyze the alkylation of a thiol group are zinc proteins.

The reaction catalyzed by N^5-methyl-H_4MPT:CoM-SH methyltransferase is analogous to the formation of methionine from N^5-methyl-H_4F and homocysteine, which is catalyzed by methionine synthase (Banerjee et al., 1989). However, methionine synthase is a soluble enzyme containing only one type of subunit, reflecting the fact that the methyl transfer to homocysteine is not coupled to energy conservation.

Activation of Methanol and Methylamines

As shown in Fig. 13, methylotrophic methanogenesis begins with the transfer of the methyl-group from a variety of substrates to coenzyme M. For each substrate, there is a different methyltransferase system, specific for methanol (Mta), monomethylamine (Mtm), dimethylamine (Mtb), trimethylamine (Mtt), tetramethylammonium (Mtq), and methylthiols (Mts; Thauer and Sauer, 1999; Ferguson et al., 2000). Each system is composed of two methyltransferases, designated “MT1” (MtaB, MtmB, MtbB, MttB, and MtqB) and “MT2” (MtaA, MtbA, and MtqA), and a substrate-specific methylotrophic corrinoid protein (MtaC, MtmC, MtbC, MttC, and MtqC) containing a modified cobamide. MT1 in each system catalyzes the methylation of the reduced corrinoid protein, and MT2 catalyzes the transfer of the methyl group from the corrinoid protein to coenzyme M. Only in dimethylsulfide:coenzyme M methyltransferase are both methyl transfer reactions catalyzed by the same subunit (MtsA; Tallant et al., 2001). The MT2 proteins have high sequence similarity and contain zinc in the active site. Likewise, the sequences of the corrinoid proteins are related, all exhibiting a corrinoid-binding motif. In contrast, the substrate-activating MT1 enzymes are not phylogenetically related. For instance, MtaB, which activates methanol, is a zinc protein, but the other methylamine methyltransferases are not (Sauer and Thauer, 1997).

The genes encoding MtmB, MtbB, and MttB contain a single conserved in-frame amber codon (UAG) that is read through during translation (James et al., 2001). In the structure of MtmB, the UAG-encoded residue was identified as a lysine in amide-linkage to

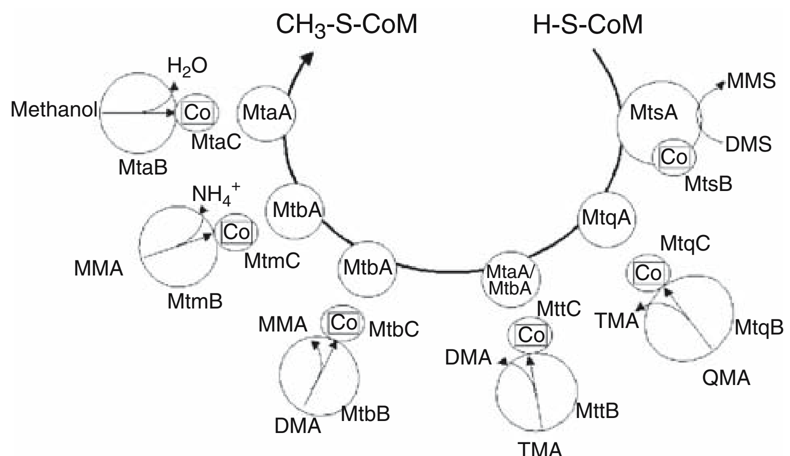
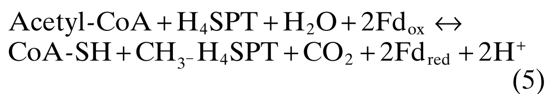


Fig. 13. Enzymes involved in the formation of methyl-coenzyme M from methanol, monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), tetramethylammonium (QMA) and dimethylsulfide (DMS). Except for DMS, the B subunits transfer the methyl groups from the substrates to the corrinoid prosthetic groups of the C subunits. The A subunits then transfer the methyl-groups from the corrinoid to CoM-SH. For DMS, the A subunit catalyzes both transfers. Abbreviations: MMS, methanethiol; and Co, corrinoid prosthetic group.

(*4R, 5R*)-4-substituted-pyrroline-5-carboxylate (called "pyrrolysine"; Hao et al., 2002). Furthermore, an amber decoding tRNA was identified (Srinivasan et al., 2002). Pyrrolysine can therefore be regarded as the twenty-second genetically encoded amino acid. Pyrrolysine is thought to position the methyl-group of methylamine for attack by the corrinoid protein (Hao et al., 2002).

The Aceticlastic Reaction

Species of *Methanosarcina*, as well as those of *Methanosaeta*, grow during the catabolism of acetate to CO_2 and CH_4 (Ferry, 1997). This is the acetate cleavage or aceticlastic reaction, where methane is formed without oxidation of the methyl group of acetate. Instead, after activation to acetyl-CoA, the acetyl C-C bond is cleaved by the multienzyme complex of acetyl-CoA synthase and carbon monoxide dehydrogenase (Acs/CODH), which in *Methanosarcina barkeri* and *Methanosarcina thermophila* is composed of five different subunits (α subunit, CdhA; β subunit, CdhC; γ subunit, CdhE; δ subunit, CdhD; and subunit, CdhB). The overall reaction catalyzed by the complex is the conversion of acetyl-CoA and tetrahydrosarcinapterin (H_4SPT) to CO_2, N^5-methyltetrahydrosarcinapterin (CH_3-H_4SPT), CoA-SH, and reducing equivalents (reaction 5). Tetrahydrosarcinapterin is similar in structure and function to tetrahydromethanopterin, which is common in the hydrogenotrophic methanogens.



$$\text{Acetyl-CoA} + H_4SPT + H_2O + 2Fd_{ox} \leftrightarrow \text{CoA-SH} + CH_3\text{-}H_4SPT + CO_2 + 2Fd_{red} + 2H^+ \quad (5)$$

A ferredoxin was identified as the physiological electron acceptor. In autotrophic methanoarchaea and the homoacetogenic bacteria like *Moorella thermoacetica*, a homologous enzyme system functions in the reverse direction for the biosynthesis of acetyl-CoA.

This overall reaction is made up of a series of partial reactions catalyzed by different protein subcomponents of the complex (Abbanat and Ferry, 1991; Grahame and DeMoll, 1996). The β subunit, the recombinant form of which can be produced in *Escherichia coli*, reacts with acetyl-CoA to form an acetyl-enzyme intermediate. Furthermore, this subunit catalyzes the formation of acetyl-CoA from CoA-SH, CO and methylcobalamin in the absence of other Acs/CODH subunits, demonstrating that this subunit catalyzes the reversible C-C bond activation (Gencic and Grahame, 2003). The β subunit also harbors the "A-cluster," which contains a Ni-Ni-[4Fe-4S] site, as deduced from the crystal structures of Acs/CODH from *Moorella thermoacetica* (Darnault et al., 2003; Seravalli et al., 2004) and *Carboxydothermus hydrogenoformans* (Svetlitchnyi et al., 2004).

The CO generated in the C-C cleavage reaction is transferred via a gas channel to the site of the CO dehydrogenase activity, which is on the α subcomplex. The isolated α subcomplex catalyzes the oxidation of CO to CO_2. Furthermore, the sequence of the α subunit is related to the sequences of the much simpler CO dehydrogenases from *Rhodospirillum rubrum* and *Carboxydothermus hydrogenoformans*. The active site of CO-dehydrogenase also contains a Ni-Fe/S center, which could be either a [Ni-Fe_4-S_4] or a [Ni-Fe_4-S_5] center, as deduced from the crystal structure of these enzymes (Dobbek et al., 2001; Drennan et al., 2001).

The methyl group generated in the β subunit is transferred to the corrinoid cofactor present in the γδ subcomplex, which catalyzes the subsequent methyl-transfer to the substrate H_4SPT. Here the methyl-group enters the general methanogenic pathway, which leads to the formation of CH_4 (Fig. 2). Reducing equivalents required for the reduction of the heterodisulfide are provided by reduced ferredoxin formed in the CO dehydrogenase reaction.

There might be alternative electron transport chains to couple ferredoxin oxidation to heterodisulfide reduction. In *Methanosarcina barkeri*, H_2 is thought to be an intermediate in this electron transfer reaction. This conclusion is based on several observations. First, H_2 accumulates during growth on acetate. Second, acetate-grown cells have high levels of the Ech hydrogenase and methanophenazine-reducing hydrogenase. Third, Ech hydrogenase is essential for growth of *M. barkeri* on acetate. It has therefore been proposed that this enzyme catalyzes H_2-formation from reduced ferredoxin (Meuer et al., 2002). H_2 thus formed could then diffuse to the extracytoplasmic side of the membrane, where it becomes oxidized by the methanophenazine-reducing hydrogenase. Reduced methanophenazine is then the electron donor for the heterodisulfide reductase (Fig. 14). On the other hand, *M. acetivorans* forms methane from acetate but lacks a functional Ech hydrogenase (Galagan et al., 2002). Hence, there must exist an alternative route to channel electrons from reduced ferredoxin into a membrane-bound electron transport chain that leads to heterodisulfide reduction.

The Hydrogenases of Methanoarchaea—A Summary

For most methanoarchaea, methanogenesis from H_2 and CO_2 is the only way to obtain energy for growth. Also growth on acetate could involve

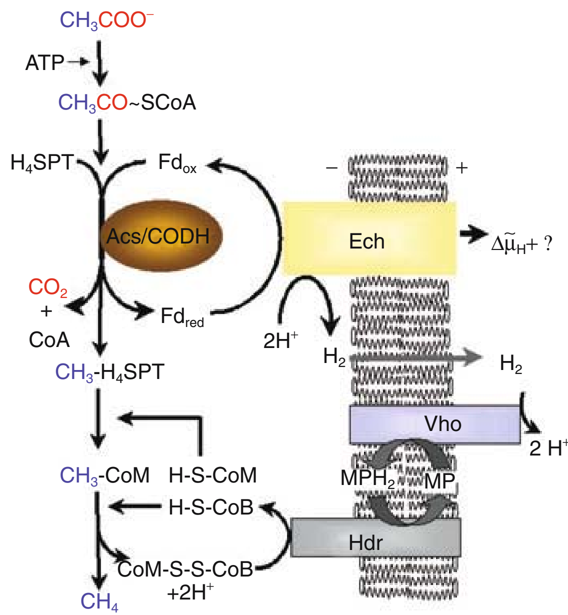


Fig. 14. Pathway of methanogenesis from acetate in *Methanosarcina barkeri*. Recent data indicate that the 2 [4Fe-4S] ferredoxin (Fd) from *M. barkeri* mediates electron transfer between acetyl-CoA synthase/CO dehydrogenase (Acs/CODH) and Ech hydrogenase. Abbreviations: CH_3-H_4SPT, methyl-tetrahydrosarcinapterin; MP, methanophenazine and MPH_2, reduced methanophenazine.

H_2-formation and H_2-consumption as discussed above. Therefore hydrogenases are essential enzymes for methanoarchaea, which is reflected by the presence of five different types of hydrogenases in these organisms. Four of these enzymes are [NiFe]-hydrogenases, and one enzyme is an iron-sulfur cluster-free hydrogenase that has only been found in methanoarchaea. Methanoarchaea seem to be lacking [FeFe] hydrogenases. For a more detailed description of hydrogenases including those from methanoarchaea, see The H2-Metabolizing Prokaryotes in this Volume.

F_{420}-Reducing Hydrogenase

This enzyme (Frh) is conserved in all methanoarchaea studied. Some organisms contain two closely related isoenzymes. The enzyme catalyzes the reduction of the deazaflavin coenzyme F_{420} and thus provides the reducing equivalents for the two intermediate reduction steps of the C_1-pathway. Frh is a soluble [NiFe] hydrogenase composed of three subunits, including the “hydrogenase large subunit” and the “hydrogenase small subunit” that form the basic module of all [NiFe] hydrogenases. The third subunit contains iron-sulfur clusters and FAD. It is assumed to harbor the F_{420}-binding site (Sorgenfrei et al., 1997).

H_2-Forming Methylene-H_4MPT Dehydrogenase

As outlined above, all hydrogenotrophic methanogens possess an F_{420}-dependent dehydrogenase for the reduction of methenyl-H_4MPT. Reduction of F_{420} to $F_{420}H_2$ by H_2 is catalyzed by Frh. Methanoarchaea belonging to the orders Methanobacteriales, Methanococcales and Methanopyrales also possess an enzyme that directly reduces methenyl-H_4MPT to methylene-H_4MPT using H_2 as the electron donor (Thauer et al., 1996; Fig. 15). Because this enzyme oxidizes H_2, it is a hydrogenase by definition. However, because this reaction is so unusual, it has been called the “H_2-forming methylene-H_4MPT dehydrogenase” (Hmd). In contrast to the well characterized [NiFe] hydrogenases and [FeFe] hydrogenases, Hmd does not contain Ni or iron-sulfur clusters. The primary sequence of Hmd does not possess similarity to known proteins. Furthermore, the enzyme is not inhibited by CO at concentrations known to inhibit other hydrogenases, and it does not catalyze the reduction of redox-dyes such as benzyl- or methylviologen. It does catalyze the exchange between H_2 and protons and the conversion of *para* H_2 to *ortho* H_2 but only in the presence of methenyl-H_4MPT. More detailed mechanistic studies have shown that the enzyme catalyzes the reversible reduction of methenyl-H_4MPT to methylene-H_4MPT in a ternary complex catalytic mechanism. In this reaction, a hydride is transferred from H_2 into the *pro*-R position at C^{14} of methenyl-H_4MPT.

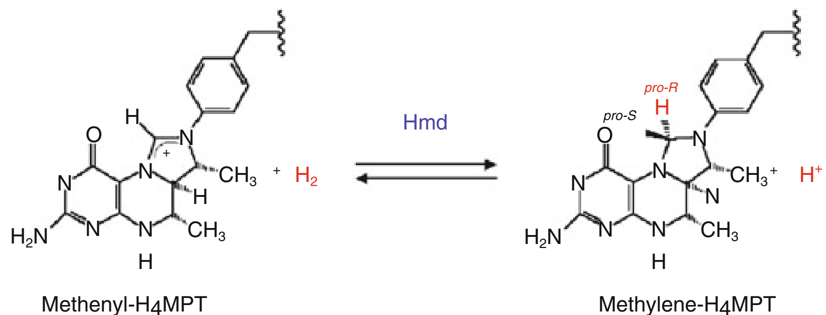


Fig. 15. Reaction catalyzed by H_2-forming methylene-H_4MPT dehydrogenase (Hmd). In the presence of H_2, methenyl-H_4MPT is reduced to methylene-H_4MPT.

Iron at concentrations up to 1 mol of Fe per mol of enzyme is the only metal that has been detected in Hmd. This iron was not redox-active and not considered to be functional. The enzyme was therefore called "metal-free" hydrogenase. Recently active enzyme was shown to contain a cofactor (Buurman et al., 2000). Addition of the purified cofactor to the apoprotein, which can be produced in *E. coli*, resulted in active enzyme. The structure of the active cofactor is not yet known. But upon illumination with ultraviolet (UV)-A/blue light, the cofactor is inactivated and Fe and CO are released (Lyon et al., 2004b). The remaining organic component could be cleaved by phosphodiesterase to GMP and a pyridone moiety, which is a new structure in biology (Shima et al., 2004). How this organic compound is involved in iron complexation in the active Hmd cofactor remains to be shown. There is experimental evidence that two CO are bound to the iron-center (Lyon et al., 2004a). Interestingly, CO is also a ligand to the iron center in [NiFe]- and [FeFe]-hydrogenases.

In cells cultivated under Ni-limiting conditions, the [NiFe] hydrogenase Frh is barely detectable, while the concentration of Hmd in the cell increases (Afting et al., 1998). Hmd in combination with F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) mediates the reduction of coenzyme F_{420} by H_2 and thus provides an alternative source for reduced coenzyme F_{420}. This allows the cell to spare Ni. In contrast to the [NiFe] hydrogenase Frh, Hmd and Mtd are not oxygen sensitive. This becomes important in the context of the recent finding that methanoarchaea contain an $F_{420}H_2$ oxidase, which catalyzes the reduction of O_2 to H_2O with $F_{420}H_2$ as the electron donor (Seedorf et al., 2004). The reduction of O_2 with H_2 in methanoarchaea is not coupled with energy conservation. The function of this oxidase is most probably to reduce the intracellular O_2 concentration to a level that allows growth and methanogenesis. There is evidence that the O_2 concentration has to be lowered well below 5 μM in order for a "nanaerobe" to grow (Baughn and Malamy, 2004). The function of $F_{420}H_2$ oxidase is, therefore, O_2 detoxification.

F_{420}-Non-Reducing Hydrogenase

F_{420}-non-reducing hydrogenase (Mvh) is a soluble [NiFe] hydrogenase. In addition to the basic hydrogenase module of two subunits, the enzyme contains a third subunit, a 17-kDa protein that carries a [2Fe-2S] cluster. In *M. marburgensis*, Mvh forms an enzyme complex with heterodisulfide reductase (Hdr). There is indirect evidence that the hydrogenase interacts via its 17-kDa subunit with Hdr (Stojanowic et al., 2003). This type of hydrogenase is not found in *Methanosarcina* species.

Methanophenazine-Reducing Hydrogenases

Methanosarcina species form two closely related [NiFe] hydrogenases, encoded by the *vho* and the *vht* transcriptional units. In addition to the basic hydrogenase module, these enzymes contain a membrane-anchoring β-type cytochrome, which easily becomes separated from the hydrogenase module during purification. These enzymes possess the highest similarity to the membrane-bound, periplasmically oriented uptake hydrogenases of bacteria (Vignais et al., 2001). Vho and Vht also contain a twin-arginine leader peptide in their hydrogenase small subunit, indicating that the hydrophilic subunits of these enzymes are translocated across the membrane by twin arginine translocation (TAT) machinery. This type of hydrogenase has only been found in *Methanosarcina* species where it is part of the H_2:CoM-S-S-CoB oxidoreductase system (Deppenmeier et al., 1999; Fig. 5). The *vhoGAC* operon is expressed during growth on H_2/CO_2, methanol or acetate. The *vhtGAC* operon is only expressed during growth on H_2/CO_2 and methanol but not during growth on acetate (Deppenmeier, 1995a). Whether this pattern of expression reflects a different metabolic function is not known.

Energy-Converting [NiFe] Hydrogenases

Energy-converting [NiFe] hydrogenase (Ech) is an integral membrane protein, which, when purified, is composed of six subunits, corresponding to the products of the *echABCDEF* operon (Künkel et al., 1998; Meuer et al., 1999). Ech hydrogenase is only distantly related to the other [NiFe] hydrogenases found in methanoarchaea. The subunits of this enzyme are closely related to members of a small group of membrane-bound [NiFe] hydrogenases, such as hydrogenase 3 from *E. coli* and the CO-induced hydrogenase from *Rhodospirillum rubrum*. The sequences of the six subunits conserved in these enzymes are closely related to subunits present in the central part of complex I from mitochondria and bacteria (Hedderich, 2004). The EchA and EchB subunits of the enzyme are predicted to be membrane-spanning proteins, while the other four subunits are expected to extrude into the cytoplasm. A low-potential, soluble two [4Fe-4S] ferredoxin ($E_0' = -420$ mV) isolated from *M. barkeri* was identified as the electron donor/acceptor of Ech. As outlined above, this enzyme provides the cell with reduced ferredoxin required for the first step of methanogenesis and for certain anabolic reactions. In vivo the reduc-

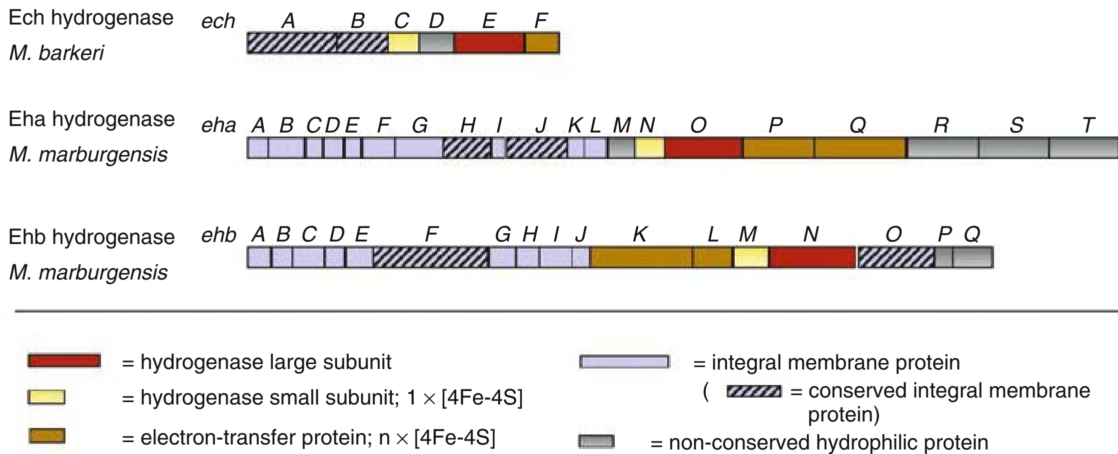


Fig. 16. Organization of the *Methanosarcina barkeri ech* operon and the *Methanothermobacter marburgensis eha* and *ehb* operons. Abbreviations: [4Fe-4S] iron-sulfur cluster; and n x [4Fe-4S], polyferredoxin encoded by the operon.

tion of the ferredoxin by H_2 is thought to be driven by reversed electron transport.

In aceticlastic methanogenesis, Ech was proposed to catalyze the reverse reaction (i.e., the production of H_2 with reduced ferredoxin as electron donor). This has been concluded from experiments with intact cells. Cell suspensions of wild-type *M. barkeri* convert CO quantitatively to CO_2 and H_2. Cell suspensions of the Δ*ech* mutant catalyzed the oxidative half of the aceticlastic pathway (conversion of CO to CO_2 and H_2) at a significantly lower rate than the wild type, indicating that Ech is the hydrogenase involved in this reaction (Meuer et al., 2002). Importantly, the conversion of CO to CO_2 and H_2 in wild-type *M. barkeri* was found to be coupled to the generation of a proton motive force. This is consistent with the putative ion-translocating activity of Ech.

Ech hydrogenase thus far has only been purified from *Methanosarcina* species. The genomes of *Methanothermobacter thermoautotrophicus*, *Methanococcus jannaschii* and *Methanopyrus kandleri* do not encode a homologue of the six-subunit Ech-hydrogenase present in *Methanosarcina*. However, these organisms encode related enzymes, which are predicted to have a much more complex subunit architecture (Fig. 16). *Methanothermobacter thermoautotrophicus*, *M. marburgensis* and *M. jannaschii* each encode two hydrogenases of this type, designated "Eha" and "Ehb" (Tersteegen and Hedderich, 1999). *Methanopyrus kandleri* only encodes for one of these hydrogenases (Slesarev et al., 2002). In *M. marburgensis* the length of the transcription units was determined. The *eha* operon (12.5 kb) and the *ehb* operon (9.6 kb) were found to be composed of 20 and 17 ORFs, respectively. Sequence analysis of the deduced proteins indicated that the *eha* and *ehb* operons each encode a [NiFe] hydrogenase large subunit, a [NiFe] hydrogenase small subunit, and two conserved integral membrane proteins. These proteins show high sequence similarity to subunits of Ech hydrogenase from *Methanosarcina barkeri*. In addition to these four subunits, the *eha* operon encodes a 6[4Fe-4S] polyferredoxin, a 10[4F-4S] polyferredoxin, four nonconserved hydrophilic subunits, and ten nonconserved integral membrane proteins; the *ehb* operon encodes a 2[4Fe-4S] ferredoxin, a 14[4Fe-4S] polyferredoxin, two nonconserved hydrophilic subunits, and nine nonconserved integral membrane proteins. Since *Methanothermobacter* species only grow with H_2/CO_2 as energy substrates, it has been proposed that these membrane-bound [NiFe] hydrogenases catalyze the reduction of a low-potential ferredoxin or polyferredoxins by H_2 in a reaction driven by reversed electron transport, in analogy to the function of Ech hydrogenase in *M. barkeri* when the organism is cultivated on H_2/CO_2. A purification of these enzymes has not been achieved thus far.

Methanogenic Coenzymes and Enzymes in Nonmethanogenic Archaea and Bacteria

Sulfate-Reducing Archaea Use Three Methanogenic Coenzymes for the Oxidation of Reduced C_1-Compounds to CO_2

So far, all isolated archaeal sulfate reducers belong to the genus *Archaeoglobus*. The best-studied species is *A. fulgidus*, for which the genome sequence is also known (Klenk et al.,

1997). *Archaeoglobus fulgidus* couples the oxidation of lactate to CO_2 with the reduction of sulfate to H_2S. Lactate is first oxidized to pyruvate, which is subsequently converted to acetyl-CoA, CO_2 and 2[H]. Cleavage of the C-C-bond of acetyl-CoA is catalyzed by the Acs/CODH complex, which has the same subunit architecture and high sequence similarity to the enzyme from methanoarchaea (Dai et al., 1998). This reaction generates enzyme-bound CO, which is oxidized to CO_2, and an enzyme-bound methyl group. For the oxidation of the methyl-group to CO_2, *A. fulgidus* uses three coenzymes characteristic of the methanoarchaea: tetrahydromethanopterin, methanofuran and coenzyme F_{420} (Möller-Zinkhan et al., 1989; Gorris et al., 1991). The methyl-group is first transferred to H_4MPT and then stepwise oxidized to CO_2 by the same reactions and enzymes found in methanoarchaea (Fig. 2). The $F_{420}H_2$ formed in this oxidative pathway is reoxidized by a membrane-bound $F_{420}H_2$ dehydrogenase, which closely resembles the enzyme from *Methanosarcina* species (Kunow et al., 1994; Klenk et al., 1997). *Archaeoglobus fulgidus* contains a modified menaquinone, which probably functions as the electron acceptor of this dehydrogenase. It is not yet clear how electrons are transferred from the menaquinone pool to the enzymes of sulfate reduction. Recently a membrane-bound menaquinol-acceptor oxidoreductase that might mediate the electron transfer from the menaquinone pool to an as yet unidentified electron carrier in the cytoplasm has been isolated (Mander et al., 2002). The sequences of two of the subunits of this enzyme are related to those of the heterodisulfide reductase from *Methanosarcina* species, including the catalytic subunit of Hdr. However, *Archaeoglobus* lacks coenzymes M and B. Therefore this heterodisulfide-reductase-like enzyme has been proposed to catalyze the reduction of an unidentified disulfide substrate, which in turn could function as an electron donor of the enzymes of sulfate reduction, such as APS reductase and sulfite reductase.

Tetrahydromethanopterin-Dependent Formaldehyde Oxidation in Methylotrophic Bacteria

In the metabolism of aerobic methylotrophic bacteria, formaldehyde is formed as a central intermediate from various C_1-substrates. Different pathways of formaldehyde oxidation to CO_2 are known, one being tetrahydromethanopterin-dependent. The H_4MPT-dependent pathway was first discovered in *Methylobacterium extorquens*. This organism, in addition to the tetrahydrofolate-dependent pathway, has an H_4MPT-dependent route for formaldehyde oxidation, which is now believed to be the main catabolic route in this organism (Chistoserdova et al., 1998). The pathway involves three H_4MPT-dependent steps, which are catalyzed by an NADH-dependent methylene-H_4MPT dehydrogenase, a methenyl-H_4MPT cyclohydrolase, and a formyltransferase/hydrolase complex. H_4MPT-dependent enzymes have also been detected in many other methylotrophic proteobacteria. For a more detailed review, see Aerobic Methylotrophic Prokaryotes in this Volume.

F_{420} in Nonmethanogenic Organisms

Coenzyme F_{420} was first discovered in methanogenic archaea. Later, coenzyme F_{420} was also identified in *Archaeoglobus*, *Mycobacterium*, *Nocardia*, *Streptomyces*, cyanobacteria and some eukaryotes (Choi et al. [2001] and literature cited therein). The role of F_{420} in *Archaeoglobus* is similar to that in methanogens. Coenzyme F_{420} is used by *Streptomyces* species for tetracycline and lincomycin biosynthesis and may be used in mitomycin C biosynthesis. In *Mycobacterium* and *Nocardia* species, coenzyme F_{420} is used by a coenzyme F_{420}-dependent glucose-6-phosphate dehydrogenase. Enzymes belonging to the deazaflavin class of photolyases, which are found in the green alga *Scenedesmus* and the cyanobacterium *Synechocystis*, contain 8-hydroxyazoriboflavin (also called "coenzyme F_0"). Coenzyme F_{420} is a derivative of coenzyme F_0.

CoM-SH in Bacterial Aliphatic Epoxide Carboxylationtc "CoM-SH in Bacterial Aliphatic Epoxide Carboxylation"

Until 1999, methanoarchaea were the only organisms known to possess coenzyme M, which is the smallest organic cofactor found in nature. It was then discovered that coenzyme M also plays an essential role in the bacterial metabolism of short chain epoxyalkanes, as revealed by initial studies with *Xanthobacter autotrophicus* and *Rhodococcus rhodochrous* (Allen et al., 1999). These organisms use coenzyme M as the nucleophile for the epoxide ring opening, which results in the formation of the thioether bond between CoM-SH and a 2-hydroxyalkyl residue. After oxidation to the corresponding 2-ketoalkyl-CoM intermediate, the thioether bond is attacked by a cysteine residue present in the active site of one of the key enzymes of the pathway. This results in the formation of a mixed disulfide between CoM-SH and the active-site cysteine and a carbanion, which becomes carboxylated. Reduction of the mixed disulfide in a NADH-dependent step regenerates coenzyme M. Coenzyme M seems to be ideally suited as a

nucleophile and carrier molecule in this pathway (reviewed in Ensign and Allen, 2003).

Do Anaerobic Methane Oxidizers Use the Methanogenic Pathway in Reverse?

Although the elucidation of the pathway of CO_2 reduction in methanogens required the discovery of a large number of novel coenzymes and enzymatic reactions, many of these catalysts were subsequently found in other organisms. For many years, the reaction catalyzed by methyl-coenzyme M reductase seemed to be the only step of the pathway that was truly unique to the methanoarchaea. However, very recently genes encoding a methyl-coenzyme M reductase-like enzyme were identified in habitats where methane-oxidizing microbial communities are abundant (Hallam et al., 2003). From the biomass of one of these habitats, a methyl-coenzyme M reductase-like enzyme was isolated (Krüger et al., 2003). This protein harbored a nickel-containing prosthetic group that was identified as a heavier (mass of 951 Da) variant of coenzyme F_{430} (mass of 905 Da), the unique nickel porphinoid in Mcr. These studies led to the proposal that anaerobic methane oxidation biochemically, in principle, is a reversal of methanogenesis. For more details on anaerobic methane oxidation, see the chapter Anaerobic Biodegradation of Hydrocarbons Including Methane in this Volume.

Regulation of Gene Expression

Regulation of Catabolic Enzymes by Substrate Availability

Many methanoarchaea use only one or two energy substrates, so that one may not expect extensive metabolic regulation. Nevertheless, it was found that even organisms using H_2/CO_2 as the sole growth substrate regulate the formation of some key catabolic enzymes in response to the availability of H_2. One example is the differential expression of two methyl-coenzyme M reductase isoenzymes in the Methanobacteriales and the Methanococcales (Thauer, 1998). In *Methanothermobacter* species isoenzyme I is encoded by the *mcrBDCGA* operon, and isoenzyme II is encoded by the *mrtBDGA* operon. The two isoenzymes differ in their catalytic properties. Isoenzyme I has a lower V_{max} as compared to isoenzyme II but displays lower K_M values for its substrates, CoB-SH and methyl-coenzyme M (Bonacker et al., 1993). Expression of the two isoenzymes is differently regulated by the availability of hydrogen. Isoenzyme I is predominantly formed when growth is limited by the H_2 supply whereas isoenzyme II predominates when the H_2 supply is not growth-rate limiting (Bonacker et al., 1992; Morgan et al., 1997). In the latter case, the methylcoenzyme M reductase reaction might be a bottleneck of the pathway. Therefore, it could be of physiological relevance to synthesize an enzyme with a higher V_{max}.

There are conflicting results with respect to the regulation of other methanogenic enzymes in response to the H_2 availability. Two groups found that the formation of Hmd in *Methanothermobacter* species parallels that of isoenzyme II of Mcr (encoded by the *mrt* operon), while the formation of Frh and Mtd parallel that of isoenzyme I of Mcr (encoded by the *mcr* operon; Morgan et al., 1997; Vermeij et al., 1997). Two other groups did not observe a formation of these enzymes in response to H_2 availability with their systems (Afting et al., 2000; Luo et al., 2002). But all groups observed the same pattern of formation of McrI and McrII.

The formation of flagella in *Methanocaldococcus jannaschii* is another example of regulation in response to H_2-availibility. Although flagella are not directly involved in catabolic processes, they are essential for finding optimal substrate conditions. Under H_2-excess conditions, *M. jannaschii* cells are devoid of flagella and have almost undetectable levels of four flagella-related proteins. Flagella synthesis occurs when H_2 becomes limiting (Mukhopadhyay et al., 2000).

Many species of hydrogenotrophic methanogens use formate in place of H_2 as the electron donor for CO_2 reduction. The ability to use formate is attributed to formate dehydrogenase (Fdh), which in methanoarchaea catalyzes the formate-dependent reduction of coenzyme F_{420}. The *Methanococcus maripaludis* genome contains two formate dehydrogenase gene clusters. The transcription of both gene clusters was found to be controlled by the availability of H_2. Only in the absence of H_2 was maximal expression of both *fdh* gene clusters observed. In contrast, formate had no marked effect on the expression (Wood et al., 2003). In contrast, expression of formate dehydrogenase in *Methanobacterium formicicum* seems not to be regulated (Schauer and Ferry, 1980).

Methanogenium thermophillum can use 2-propanol as sole electron donor for CO_2 reduction. The secondary alcohol dehydrogenase responsible for 2-propanol oxidation was only formed when H_2 became limiting, irrespective of the presence of the alcohol. In other methanoarchaea able to grow with secondary alcohols, formation of alcohol dehydrogenase was dependent on the availability of an alcohol irrespective of the presence of H_2 (Widdel and Wolfe, 1989).

Another response to H_2-limitation is the synthesis of an autolytic enzyme by *Methanobacterium wolfei* (Kiener et al., 1987). The physiological role of this suicidal process is not known. It may be related to the induction of a defective bacteriophage (Stettler et al., 1995).

The regulation of the genes encoding methanogenesis from acetate in *Methanosarcina* species is also well studied. Early work had already shown that acetate is only used as energy substrate when none of the higher energy-yielding substrates methanol, methylamines or H_2/CO_2 are available, indicating that acetate catabolism is repressed by these other substrates (reviewed in Zinder, 1993). This is consistent with the observation that the key enzymes of acetate metabolism (i.e., acetate kinase, phosphotransacetylase, acetyl-CoA synthase/carbon monoxide dehydrogenase complex, and carbonic anhydrase) are formed at a lower level in cells grown on methanol as compared to acetate-grown cells (Jablonski et al., 1990). Regulation was shown to be at the mRNA level (Sowers et al., 1993; Singh-Wissmann and Ferry, 1995). On the other hand, most of the enzymes necessary for the reversible reduction of CO_2 to the level of methyl-tetrahydromethanopterin are present at a much lower level in acetate-grown cells (Jablonski et al., 1990; Mukhopadhyay et al., 1993). When *Methanosarcina* spp. are cultivated on methanol in the presence of H_2/CO_2, the oxidative branch of the methylotrophic pathway is repressed. This result is consistent with the observation that several enzymes of this pathway are formed at a lower level under these conditions (Mukhopadhyay et al., 1993). In conclusion, catabolic gene expression in *Methanosarcina* appears similar to systems in bacteria, which are regulated for preferential utilization of the most energetically favorable substrate.

For none of the regulatory systems described above has the primary sensor and the signal transduction cascade been elucidated. However, in *Methanothermobacter thermoautotrophicus*, studies have been performed which led to the proposal that coenzyme F_{390} could function as a reporter compound for H_2 limitation. Coenzyme F_{390} is formed from coenzyme F_{420} by adenylation or guanylation at its 8-hydroxy-group. This reaction is catalyzed by coenzyme F_{390} synthetase (Vermeij et al., 1994). This enzyme specifically uses oxidized coenzyme F_{420} as substrate, while reduced coenzyme F_{420} ($F_{420}H_2$) acts as a competitive inhibitor. Coenzyme F_{390} can be hydrolyzed to coenzyme F_{420} and AMP or GMP in a reaction catalyzed by coenzyme F_{390} hydrolase (Vermeij et al., 1995). This latter enzyme is redox-sensitive and is inactivated by O_2. Furthermore, this enzyme is activated by CoM-SH but inactivated by CoM-S-S-CoB. On the basis of the biochemical properties of these two enzymes, it has been predicted that the level of coenzyme F_{390} should be low when cells receive sufficient H_2 (which leads to a high coenzyme $F_{420}H_2$ to coenzyme F_{420} ratio and high CoM-SH to CoM-S-S-CoB ratio). Conversely, the coenzyme F_{390} concentration in the cell should increase when H_2 becomes limiting. This prediction was confirmed experimentally (Vermeij et al., 1997). In further studies a *Methanothermobacter thermoautotrophicus* mutant was isolated that was unable to grow under H_2-deprived conditions. This mutant was also unable to form coenzyme F_{390}. It also lacked the ability to synthesize isoenzyme I of Mcr, which is the enzyme preferentially synthesized under H_2-limiting conditions (Pennings et al., 1998). This gives further evidence for an important role of coenzyme F_{390} in the response of the cell to varying H_2-concentrations.

Regulation of Catabolic Enzymes by Trace Element Availability

In the methanogenic pathways, enzymes containing transition metals in their active site play an essential role. Therefore, not surprisingly, these organisms have developed strategies to cope with limitations on the availability of these metal ions. One example is the synthesis of different isoenzymes of formylmethanofuran dehydrogenase (Fmd; reviewed in Vorholt and Thauer, 2002). *Methanothermobacter marburgensis* and *Methanothermobacter wolfei* form two different isoenzymes, one containing tungsten bound to the molybdopterin cofactor (Fmd-W) and a second containing molybdenum bound to the molybdopterin cofactor (Fmd-M). Whereas Fmd-W is formed constitutively, Fmd-M is only formed when molybdenum is available (Hochheimer et al., 1996). A DNA binding protein, called "Tfx," was found to specifically bind to a DNA region downstream of the promoter of the *fmdECB* operon, which encodes Fmd-M. Therefore, Tfx may be a transcriptional regulator of the *fmdECB* operon (Hochheimer et al., 1999). A different set of Fmd enzymes is found in *Methanopyrus kandleri*. This organism forms two tungsten-containing Fmd isoenzymes (Vorholt et al., 1997). One isoenzyme (called "Fwu") contains selenium, whereas the second (called "Fwc") does not. In general, Fmd contains a conserved cysteine residue, which is also conserved in other molybdopterin-containing enzymes. From the crystal structure of other molybdopterin-containing enzymes, for example dimethylsulfoxide reductase, this residue is known to provide a ligand to the molybdenum center. In Fwu, this cysteine residue is replaced by selenocysteine. The gene encoding the catalytic subunit FwuB is in the polycistronic operon *fwuGDB*. The gene

encoding FwcB, the catalytic subunit of Fwc, is transcribed monocistronically. During growth of the organism on medium supplemented with selenium, only the *fwuGDB* operon is transcribed. During growth under selenium limitation, both *fwuGDB* and *fwcB* are transcribed.

Selenium-dependent gene expression has also been observed in *Methanococcus voltae*. In this organism, two isoenzymes of the coenzyme F_{420}-reducing hydrogenase (called "Fru" and "Frc"), and two isoenzymes of the coenzyme F_{420}-nonreducing hydrogenase (called "Vhu" and "Vhc") are encoded in the genome (Sorgenfrei et al., 1997). One enzyme of each type, Fru and Vhu, contains selenocysteine in the hydrogen activating reactive site. The corresponding isoenzymes, Frc and Vhc, have a cysteinyl residue in the homologous positions. The two selenium-containing hydrogenases are constitutively expressed. The operons *vhc* and *frc* encoding the selenium-free enzymes are only transcribed under selenium limitation. They are connected by a common intergenic region comprising both promoters and positive and negative regulatory sequence elements, which were defined by mutational analyses employing a reporter gene system (Noll et al., 1999). A putative activator protein has been identified but not yet further characterized (Müller and Klein, 2001). A protein binding to a negative regulatory element involved in the regulation of the two operons was purified. Through the identification of the corresponding gene, the protein was found to be a LysR-type regulator. It was named "HrsM" (*h*ydrogenase gene *r*egulator, *s*elenium dependent in *M. voltae*). Also, *hrsM* knockout mutants constitutively transcribed the *vhc* and *frc* operons in the presence of selenium (Sun and Klein, 2004).

Nickel is an essential trace element for methanoarchaea. Studies with *Methanothermobacter marburgensis* have shown that this organism has developed a strategy to spare nickel under nickel-limitation. As outlined above, coenzyme F_{420}-reducing hydrogenase (Frh), which is a [NiFe] hydrogenase, can be functionally replaced by the combined action of Hmd and Mtd. These two latter enzymes do not contain Ni. When *M. marburgensis* was cultivated under nickel-limited conditions, the specific activity of Hmd and Mtd was 6- and 4-fold higher and that of Frh up to 180-fold lower than in cells grown on nickel-sufficient medium. The *frh* transcripts were no longer detectable in cells grown under Ni-limitation, whereas the relative abundance of the *hmd* and *mtd* transcripts increased (Afting et al., 1998; Afting et al., 2000).

Regulation of Nitrogen Assimilation

Nitrogen assimilation by *Methanococcus maripaludis* is highly regulated. This organism fixes N_2 but can also use ammonia or alanine as sole nitrogen sources. In the presence of ammonia or alanine, N_2 fixation is highly repressed (Cohen-Kupiec et al., 1997; Lie and Leigh, 2002). The repressor has been isolated and is very unusual for this class of proteins. Called "NrpR," it possesses very low sequence similarity to previously described DNA-binding proteins in the prokaryotes (Lie and Leigh, 2003). NrpR also regulates the expression of *glnA* in *M. maripaludis*. In addition to transcriptional regulation, N_2 fixation is also regulated by a switch-off mechanism. Upon the addition of ammonia or alanine, nitrogen fixation ceases immediately (Kessler et al., 2001; Lie and Leigh, 2002). This regulation requires the participation of two GlnB homologs encoded by *nifI*$_1$ and *nifI*$_2$. Although this system acts very similarly to the bacterial system for the posttranslational ADP-ribosylation of the nitrogenase reductase, its mechanism of action is not currently known.

Bioenergetics of Growth

Coupling Sites in Methanogenesis

Energy-conservation by methanoarchaea is via electron transport phosphorylation as outlined above. The H_2/CO_2 pathway contains two energy-coupling sites: the H_4MPT:coenzyme M methyltransferase reaction and the reduction of the heterodisulfide. While the methyl-transferase reaction is coupled to the primary extrusion of Na^+, the heterodisulfide reductase reaction is coupled to the extrusion of H^+. Experimental proof that the latter reaction is coupled to energy-conservation is, however, only available for *Methanosarcina* species. Via a Na^+/H^+ antiporter, $\Delta{:}\mu_{Na+}$ and $\Delta{:}\mu_{H+}$ are interconvertible (Kaesler and Schönheit, 1989). Part of the energy conserved in these ion gradients is used to drive the reduction of CO_2 to formylmethanofuran by reversed electron transport, while the remaining part of the energy is used for the synthesis of ATP via ATP synthase. Methanoarchaea contain A_1A_0 ATP synthases characteristic for archaea (Müller, 2004). In *M. thermoautotrophicus* and *M. mazei*, this is the only ATP synthase encoded in the genome sequences. In contrast, the genomes of *M. barkeri* and *M. acetivorans* encode both (an A_1A_0 ATP synthase and a F_1F_0 ATP synthase). Expression of the latter enzyme in *M. barkeri* could, however, not be demonstrated (Müller, 2004). The ion specificity of A_1A_0 ATP synthases is not yet established. In silico analysis of the proteolipid of some A_1A_0 ATP synthases reveal the presence of a Na^+ binding motif and suggest that these enzymes use Na^+ as coupling ion (Müller, 2004).

In aceticlastic methanogenesis, the methyltransferase and the heterodisulfide reductase reactions are also sites of energy conservation. Formation of H_2 from reduced ferredoxin, catalyzed by Ech hydrogenase, might represent an additional energy-coupling site (Fig. 14). On the other hand, activation of acetate to acetyl-CoA requires at least one ATP in *Methanosarcina* spp. and two ATP in *Methanosaeta* spp. Thus, cells must recover the high cost of acetate activation.

When methanol or methylamines are used as energy substrates, the heterodisulfide reductase reaction is also a site of energy conservation. However, the H_4MPT:coenzyme M methyltransferase and the formylmethanofuran dehydrogenase reactions now operate in reverse. Thus, the methyltransferase reaction becomes energy consuming while the oxidation of formylmethanofuran to CO_2 and methanofuran is coupled to energy conservation.

Table 4. Bioenergetic requirements for monomer biosynthesis during growth of methanogens in mineral and rich media.

Growth conditions	Requirement (mmol/g of cell dry wt.) ~P[a]	[2H][b]	Total [2H][c]
Autotrophic growth in mineral	89	97	451
medium + acetate	89	34	388
Rich medium[d]	63	25	276
E. coli minimal medium	21	18	—

[a]ATP equivalents required.
[b]Reductant as NADH or H_2 equivalents required for anabolism.
[c]Includes the H_2 necessary for methanogenesis to make ATP with a stoichiometry of 1 ATP/CH_4.
[d]Includes acetate + the volatile fatty acids for branched chain amino acid biosynthesis + aryl acids for aromatic amino acid biosynthesis + the nucleobases (guanine, adenine and uracil) commonly taken up by the salvage pathway.

Growth Yields

Methanoarchaea possess specialized systems to generate the energy needed for growth from the process of methanogenesis, and they have only a limited capacity to metabolize complex carbon compounds. Even the secondary alcohols, which can serve as electron donors for CO_2 reduction in some species, are only partially oxidized to ketones. About half of the described species of methanogens are capable of autotrophic growth and obtain all of their cellular carbon from CO_2. While the remainder may require organic compounds for growth, these compounds are assimilated into cellular carbon and not extensively metabolized. Compounds typically assimilated include acetate and the volatile fatty acids like isovalerate, 2-methylbutyrate, isobutyrate, and propionate, which are common in anaerobic environments, as well as amino acids.

The inability to assimilate complex organic compounds has profound effects on the energy requirements for growth. On the basis of biosynthetic pathways known and inferred from the genomic sequence, *Methanococcus maripaludis*, a typical hydrogenotrophic methanogen, must expend 89 mmol of ATP equivalents and 97 mmol of [2H] for the biosynthesis of a gram of cells from CO_2 (Table 4). Given that 50% of the cell is carbon, the amount of reductant required is close to 84 mmol of [2H], or the theoretical amount necessary to reduce 42 mmol of CO_2 to the oxidation state of carbon in the cell. Presumably, the difference is due to oxidations that occur during biosynthesis and the approximation of the cell composition. The ATP requirement greatly exceeds that of a typical heterotroph such as *E. coli* growing in a minimal medium. It is also much larger than the approximately 36 mmol ATP (gram of cells)$^{-1}$ required for polymerization reactions, which includes protein, DNA and RNA biosynthesis (Forrest and Walker, 1971; Ingraham et al., 1983). Thus, monomer biosynthesis is the major energy demand for growth of a hydrogenotrophic methanogen, and the assimilation of organic carbon sources may have large effects on their growth.

Many methanogens assimilate exogenous acetate, which is frequently abundant in anaerobic habitats. From the biosynthetic pathways, about 16 mmol of acetyl-CoA are utilized in the biosynthesis of one gram of cells; hence acetate has the potential of providing about 75% of the cellular carbon. Assuming that two ATPs are consumed to active acetate via the high affinity acetyl-CoA synthetase reaction, there is no savings in the ATP requirement for growth when compared with CO_2 fixation (Table 4). If only one ATP is utilized to activate acetate via the low affinity acetate kinase reaction, about 16 mmol of ATP is spared, which is about 18% of the total ATP requirement for monomer biosynthesis. Similarly, methanogens frequently assimilate the branched-chain volatile fatty acids as sources of branched-chain amino acids and aryl acids as a source of aromatic amino acids. Together, these amino acids account for about 25% of the cellular carbon. Assuming that the carboxylic acids are assimilated by an acyl-CoA synthetase reaction requiring two ATP equivalents, followed by ferredoxin-dependent oxidoreductase requiring one ATP equivalent to activate the reductant and one [2H], and an aminotransferase (which requires one ATP and one [2H] to make glutamate), four ATP equivalents and two [2H] are required for each amino acid biosynthesized. Even then, this pathway results in a large reduction in the energy requirements for growth (Table 4).

The maximum cell yields can be estimated. For a hydrogenotrophic methanogen fixing CO_2 as its major carbon source, about 89 and 36 mmol of ATP per gram of cells are required for monomer biosynthesis and polymerization reactions, respectively. Thus, the maximal cell yield is expected to be about 8.0 g of cell dry weight per mol of ATP. For a hydrogenotrophic methanogen obtaining carbon from acetate, the volatile fatty acids and aryl acids, the yield is about 10 g of cell dry weight per mol of ATP. In contrast, for a heterotroph, the maximal cell yield is 28 g of cell dry weight per mol of ATP. For an autotroph using the Calvin cycle of CO_2 fixation, the maximal cell yield is 4.75 g of cell dry weight per mol of ATP (Forrest and Walker, 1971). Thus, while the cell yield of an autotrophic methanogen is considerably less than that of a heterotroph, it theoretically could be nearly twice that of a chemolithotroph using the Calvin cycle.

For comparison, the observed cell yield for methanogens are usually in the range of 1–6 grams of cells per mol of methane, and the measured maximal cell yields are 3–6 grams of cells per mol of methane (Vogels et al., 1988; Tsao et al., 1994; Schill et al., 1996). In the literature, the *Methanosarcina* species appear to have higher growth yields on H_2/CO_2 than *Methanothermobacter* species and others, but these results are from different laboratories and observed under different growth conditions. Following cultivation of two mesophiles, *Methanosarcina barkeri* and *Methanobrevibacter aboriphilus*, under the similar conditions on H_2/CO_2, the cell yields were 4.2 g and 1.4 g of dry cells per mol of CH_4, respectively (R. Hedderich, unpublished data). These results confirmed the lower cell yield among the Methanobacteriales. Possibly, the lower cell yield might result from a different mechanism of coupling methanogenesis to the proton motive force or from higher maintenance energy during growth.

Genomes of the Methanoarchaea

The complete genomes have been sequenced in a representative of every order of the methanoarchaea except the Methanomicrobiales, where only a partial sequence is available (Table 5). The sizes of the genomes vary from 1.6–5.8 Mbp, reflecting the great diversity in this group of organisms (Table 5). In general, the genomes of the hydrogenotrophic methanogens are smaller, in the range of 1.6–1.8 Mbp. Even among these small genomes, the gene content is not highly conserved, and only about two-thirds of the genes in any one organism are likely to be conserved within the methanoarchaea (W. B. Whitman, unpublished observation). The genomes of the methylotrophic methanogens are much larger, in the range of 2.7–5.8 Mbp. In the *Methanosarcina* spp., the large genome seems to have followed the acquisition of a large number of genes from the anaerobic *Firmicutes* (Deppenmeier et al., 2002b) and may be responsible in part for the wide substrate specificity of these organisms (Galagan et al., 2002).

The growth temperature optima of those methanoarchaea whose genomic sequences have been determined are 15–98°C. Thus, it has been possible to make detailed correlations of certain structural features in proteins and nucleic acids with growth temperature (Saunders et al., 2003). The amino acid leucine was highly enriched in

Table 5. Genomic sequences of methanoarchaea.

Organism	Genome size (kbp)	Number of ORFs	Comments	Reference(s)
Methanothermobacter thermautotrophicus)H	1751	1855	Thermophilic hydrogenotroph	Smith et al., 1997
Methanocaldococcus jannaschii JAL-1	1723	1726	Hyperthermophilic hydrogenotroph	Bult et al., 1996
Methanococcoides burtonii	2668	2676	Partial sequence, psychrotolerant methylotroph	Saunders et al., 2003
Methanococcus maripaludis S2	1661	1722	Mesophilic hydrogenotroph	Hendrickson et al., 2004
Methanogenium fridigum	1598	1815	Partial sequence, psychrophilic hydrogenotroph	Saunders et al., 2003
Methanopyrus kandleri AV19	1695	1692	Hyperthermophilic hydrogenotroph	Slesarev et al., 2002
Methanosarcina acetivorans C2A	5751	4524	Mesophilic acetotroph and methylotroph	Galagan et al., 2002
Methanosarcina barkeri Fusaro	4830	5066	Partial, mesophilic acetotroph and methylotroph	Joint Genome Institute, unpublished
Methanosarcina mazei Gö1	4096	3371	Mesophilic acetotroph and methylotroph	Deppenmeier et al., 2002b

the proteins from organisms with high growth temperatures, while the amino acids glutamine and threonine were highly enriched at low growth temperatures. In addition, the proteins of organisms with high growth temperatures were enriched in the mean fraction of charged residues in the solvent accessible as well as solvent inaccessible areas. Likewise, the contribution of hydrophobic residues to the solvent accessible area decreased with growth temperature. The tRNAs of organisms with high growth temperatures also possessed higher mol% G+C contents, especially in the stem regions (Saunders et al., 2003).

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