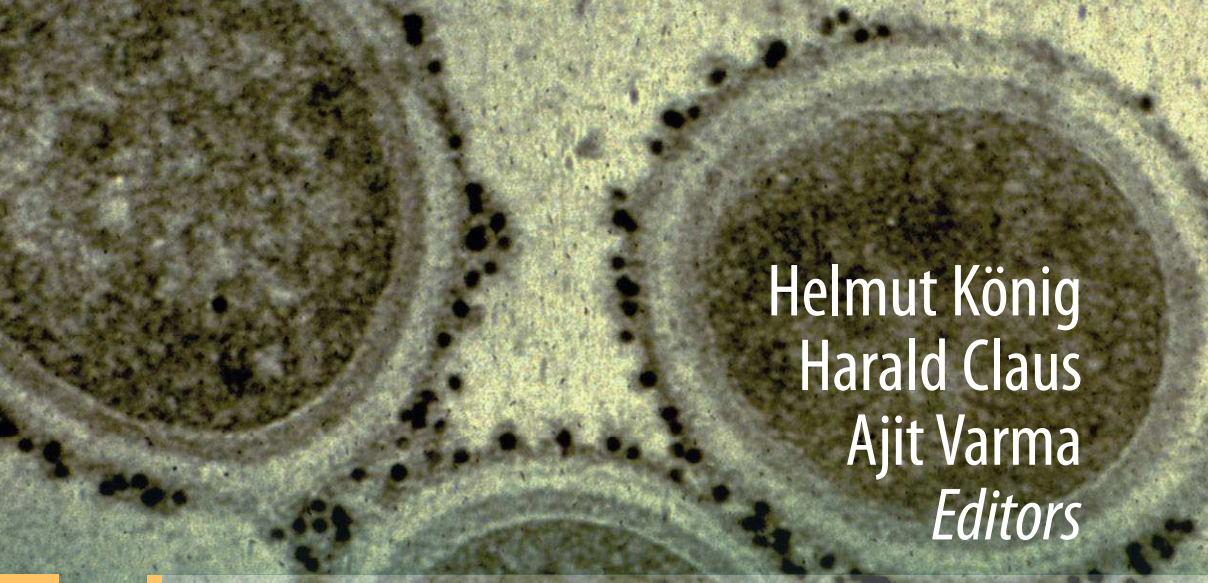

Helmut König
Harald Claus
Ajit Varma
Editors

Prokaryotic Cell Wall Compounds

Structure and Biochemistry

Springer

Prokaryotic Cell Wall Compounds

Helmut König • Harald Claus • Ajit Varma
Editors

Prokaryotic Cell Wall Compounds

Structure and Biochemistry

Springer

Editors
Prof. Dr. Helmut König
Institute of Microbiology and Wine Research
Johannes Gutenberg-University
Becherweg 15
55099 Mainz, Germany
hkoenig@uni-mainz.de

Dr. Harald Claus
Institute of Microbiology and Wine Research
Johannes Gutenberg-University
Becherweg 15
55099 Mainz, Germany
hclaus@uni-mainz.de

Prof. Dr. Ajit Varma
Director General
Amity Institute of Microbial Technology
Amity University Uttar Pradesh
& Vice Chairman
Amity Science, Technology & Innovation Foundation
Block A, Amity Campus
Sector 125
Noida, UP 201303, India
ajitvarma@aihmr.amity.edu

ISBN 978-3-642-05061-9 e-ISBN 978-3-642-05062-6
DOI 10.1007/978-3-642-05062-6
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2010922367

Cover design: WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Terry passed away shortly after writing the foreword. The death of Terry is not only a great loss for his family but also for his colleagues and the microbiological community. We offer our condolences and retain him in memory.

Foreword

Prokaryote cells are uncomplicated cells possessing a simple design. All prokaryotes, whether they are archaea or bacteria, rely on their surfaces for multiple functions, including being a barrier with their external environment; most do this via a cell envelope of which the most important structure is the cell wall. There are two fundamental structural varieties of walls, that is, the Gram-positive or Gram-negative formats, and many bacteria also possess associated structural layers on top of the wall, for example, capsules, sheaths, or S-layers. Since prokaryotes are the most diverse and ubiquitous life forms on Earth, involved in almost all natural cycles, including elemental cycling and many forms of disease, their surfaces are the required primary interface between the cell and its surroundings, often mediating or catalyzing important interactions. This book provides an up-to-date knowledge of the prokaryotic cell wall, including its structure, composition, synthesis, assembly, and some surface interactions.

Because of the importance of cell walls, many researchers have spent their whole lives attempting to enlighten the scientific community about a single aspect of a bacterial surface. One such researcher is U.B. Sleytr of the Center for NanoBiotechnology, BOKU – University of Natural Resources and Applied Life Sciences in Vienna, Austria. Throughout his career, Uwe has had one central focus; to determine the structure and function of bacterial S-layers. He began his S-layer studies in the mid-1960s, working primarily on *Bacillus stearothermophilus* (now *Geobacillus*), *B. sphaericus*, and several thermophilic clostridia. During this time, he concentrated on the detection and high-resolution structure of their constituent S (surface)-layers using transmission electron microscopy, quickly becoming a highly respected microscopist who excelled in the cryo-technique of freeze-etching. In his hands, S-layers provided breathtaking images since they are composed of regular arrays of rather large MW (glyco)proteins that could be readily viewed by electron microscopy. Linear (*p2*), tetragonal (*p4*), and hexagonal (*p3* and *p6*) lattices were discovered, which proved to be built through self-assembly processes. Uwe was quick to recognize that S-layers provided a natural nano-scale construction of regularly arranged proteins (and holes) that, under the correct conditions,

could be assembled or disassembled. This was surely a system that could be moulded into many applications, and under the guidance of Uwe's deft hands, became a promising nanobiotechnological platform for a wide range of uses (e.g. biomimetic membranes, diagnostics, biochips, supramolecular medicine, nanoglycobiology, and bioprocess technology). He now heads the Center for NanoBiotechnology in Vienna with three groups (i.e. Nanoengineered Biomaterials, Nanoglycobiology, and Molecular Biotechnology and Biomimetic Membranes) dedicated to finding applications for S-layers.

I have known Uwe since the early 1970s when we both worked on S-layers and we have been friends ever since. We both remain dedicated "cell wallogists" and share a multidisciplinary approach to our science. Uwe and several of his researchers provide chapters in this book, which reflects their expertise (along with all other authors) and their impact on the cell wall field. It is our great pleasure to dedicate this book to our good friend Uwe Sletyr on the occasion of his 65th birthday.

Guelph, July 2007 Terry J. Beveridge†

Preface

For the further evolution of single-celled organisms, the development of cell wall structures was advantageous for the successful colonization of different moderate and extreme habitats. Therefore, cell wall polymers are important cell components of prokaryotic microbes. They are involved in different manifestations of life. Cell walls play a role in shape maintenance, protection against harmful agents, cell adhesion, and positive and negative biological activities against host cells.

Studies since the 1950s revealed that quite different cell wall polymers could be part of the cell wall profiles. The structure and functions of the main cell wall polymers, such as peptidoglycan (murein, pseudomurein), outer membrane proteins, lipopolysaccharides, teichoic acids, teichuronic acids, lipoteichoic acids, S-layer subunit and cell-wall associated proteins, have been elucidated in the last 60 years.

The prokaryotes are divided into two domains, bacteria and archaea. Each of these two domains possesses its own cell wall structures. Most bacteria are characterized by peptidoglycan sacculi composed of murein, while this polymer is lacking in the archaea. Some archaeal methanogens could possess pseudomurein instead. The common cell wall compound of Archaea is a single layer of crystalline protein subunits (S-layer) covering the cytoplasmic membrane directly. S-layers are found in bacteria on the outside of the murein sacculi of Gram-positive cells or the outer membrane in Gram-negative organisms. In addition, pseudomurein, heteropolysaccarides, lipoglycans, and glutaminylglucan are found in archaea, leading to a positive Gram staining.

This book provides a comprehensive analysis of some selected current topics on the recent advances made in the structures and biochemistry of cell wall polymers, and caters to the needs of students and scientists of live sciences.

We want to thank all the authors for putting down their experiences on paper, which facilitated this comprehensive survey on the different novel aspects of microbial cell walls. Finally, we are grateful that under the responsibility of

Dr. Dieter Czeschlik and Dr. Jutta Lindenborn, the Springer Life Sciences Editorial realized this book on microbial cell wall structures; these structures are important components for the interactions and stability of microbial cells in their environment.

Mainz, June 2009 Helmut König, Harald Claus, Ajit Varma

Contents

Contributors

Mehtap Abu-Qarn Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel

Terry J. Beveridge† Department of Microbiology, University of Guelph, Guelph, Ontario N1G2W1, Canada

Reinhold Brückner Department of Microbiology, University of Kaiserslautern, Paul Ehrlich Str., Geb. 23, 67663 Kaiserslautern, Germany, rbrueckn@rhrk.uni-kl.de

Marie-Pierre Chapot-Chartier INRA, UMR1319 Micalis, Domaine de Vilvert, F-78352 Jouy-en-Josas, France, Marie-Pierre.Chapot@jouy.inra.fr

Harald Claus Institute of Microbiology and Wine Research, Johannes Gutenberg-University, 55099 Mainz, Germany, hclaus@uni-mainz.de

David Corbett Faculty of Life Sciences, University of Manchester, Manchester, M139PT, UK, i.s.Roberts@manchester.ac.uk

Cristina De Castro Department of Organic Chemistry and Biochemistry, University of Naples Federico II, via Cintia, 4 I-80126 Napoli, Italy, cristina.decastro@unina.it

Everly Conway de Macario Center of Marine Biotechnology, University of Maryland Biotechnology Institute (UMBI), Columbus Center, 701 E. Pratt Street, Baltimore, MD 21202, USA, macarioe@umbi.umd.edu

Ulrich Dobrindt Institute for Molecular Infection Biology, University of Würzburg, Josef-Schneider-Str. 2, Bau D15, 97080 Würzburg, Germany, ulrich.dobrindt@mail.uni-wuerzburg.de

Eva-Maria Egelseer Department of NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 11, A-1190 Vienna, Austria, eva-maria.egelseer@boku.ac.at
Department of NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Strasse 33, 1180 Vienna, Austria, eva-maria.egelseer@boku.ac.at

Jerry Eichler Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel, jeichler@bgu.ac.il

Jörg Hacker Robert Koch-Institute, Nordufer 20, 13353 Berlin, Germany, HackerJ@rki.de

Alexander Hahn JWGU Frankfurt am Main, Cluster of Excellence Macromolecular Complexes, Department of Biosciences, Molecular Cell Biology of Plants, Centre of Membrane Proteomics, Max-von-Laue Str. 9, 60438 Frankfurt, Germany, hahn@bio.uni-frankfurt.de

Regine Hakenbeck Department of Microbiology, University of Kaiserslautern, Paul Ehrlich Str., Geb. 23, 67663 Kaiserslautern, Germany, hakenb@rhrk.uni-kl.de

Bernhard Henrich Department of Microbiology, University of Kaiserslautern, Paul Ehrlich Str., Geb. 23, 67663 Kaiserslautern, Germany, henrich@rhrk.uni-kl.de

Thomas Hudson Faculty of Life Sciences, University of Manchester, Manchester, M139PT, UK, i.s.Roberts@manchester.ac.uk

Nicola Ilk Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Strasse 33, 1180 Vienna, Austria, nicola.ilk@boku.ac.at

Thomas Kohler Cellular and Molecular Microbiology Section, Medical Microbiology Department, University of Tübingen, 72076 Tübingen, Germany, thomas.kohler@med.uni-tuebingen.de

Helmut König Institute of Microbiology and Wine Research, Johannes Gutenberg-University, 55099 Mainz, Germany, hkoenig@uni-mainz.de

Zvia Konrad Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel

Susan F. Koval Department of Microbiology and Immunology, University of Western Ontario, London, ON N6A 5C1, Canada, skoval@uwo.ca

Emir Kulauzovic Cellular and Molecular Microbiology Section, Medical Microbiology Department, University of Tübingen, 72076 Tübingen, Germany, emir.kulauzovic@med.uni-tuebingen.de

Rosa Lanzetta Department of Organic Chemistry and Biochemistry, University of Naples Federico II, via Cintia, 4 I-80126 Napoli, Italy, rosa.lanzetta@unina.it

Silke Litzinger Fachbereich Biologie, Universität Konstanz, 78457 Konstanz, Germany, Silke.Litzinger@uni-konstanz.de

Alberto J.L. Macario Center of Marine Biotechnology, University of Maryland Biotechnology Institute (UMBI), Columbus Center, 701 E. Pratt Street, Baltimore, MD 21202, USA, macarioster@gmail.com

Hilla Magidovich Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel

Christoph Mayer Fachbereich Biologie, Universität Konstanz, 78457 Konstanz, Germany, ch.mayer@uni-konstanz.de

Mohamed Merroun Institute of Radiochemistry, Research Center Dresden-Rossendorf, 01314 Dresden, Germany, m.merroun@fz-rossendorf.de

Paul Messner Department of NanoBiotechnology, Boku – University of Natural Resources and Applied Life Sciences, Muthgasse 11, A-1190 Vienna, Austria, paul.messner@boku.ac.at

Oliver Mirus JWGU Frankfurt am Main, Cluster of Excellence Macromolecular Complexes, Department of Biosciences, Molecular Cell Biology of Plants, Centre of Membrane Proteomics, Max-von-Laue Str. 9, 60438 Frankfurt, Germany, o.mirus@bio.uni-frankfurt.de

Antonio Molinaro Department of Organic Chemistry and Biochemistry, University of Naples Federico II, via Cintia, 4 I-80126 Napoli, Italy, molinaro@unina.it

Tobias Oelschlaeger Institute for Molecular Infection Biology, University of Würzburg, Josef-Schneider-Str. 2, Bau D15, 97080 Würzburg, Germany, t.oelschlaeger@mail.uni-wuerzburg.de

Michelangelo Parrilli Department of Organic Chemistry and Biochemistry, University of Naples Federico II, via Cintia, 4 I-80126 Napoli, Italy, michelangelo.parrilli@unina.it

Andreas Peschel Cellular and Molecular Microbiology Section, Medical Microbiology Department, University of Tübingen, 72076 Tübingen, Germany, andreas.peschel@uni-tuebingen.de

Noa Plavner Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel

Dietmar Pum Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Strasse 33, 1180 Vienna, Austria, dietmar.pum@boku.ac.at

Reinhard Rachel Institute of Microbiology and Centre for Electron Microscopy, Universität Regensburg, Universitätsstraße. 31, Regensburg D-93053, Germany, reinhard.rachel@biologie.uni-regensburg.de

Renate Radek Institute of Biology/Zoology, Protozoology, Free University of Berlin, Königin-Luise-Str. 1–3, Berlin 14195, Germany, rradek@zedat.fu-berlin.de

Maren Rautenberg Cellular and Molecular Microbiology Section, Medical Microbiology Department, University of Tübingen, 72076 Tübingen, Germany, maren.rautenberg@med.uni-tuebingen.de

Ian S. Roberts Faculty of Life Sciences, University of Manchester, Manchester, M13 9PT, UK, i.s.Roberts@manchester.ac.uk

Christina Schäffer Department of NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 11, A-1190 Vienna, Austria, christina.schaeffer@boku.ac.at
Department of NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Strasse 33, 1180 Vienna, Austria, christina.schaeffer@boku.ac.at

Enrico Schleiff Department of Biosciences, JWGU Frankfurt am Main, Cluster of Excellence Macromolecular Complexes, Centre of Membrane Proteomics, Molecular Cell Biology of Plants, Max-von-Laue Str. 9, 60438 Frankfurt, Germany, schleiff@bio.uni-frankfurt.de

Bernhard Schuster Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Strasse 33, 1180 Vienna, Austria, bernhard.schuster@boku.ac.at

Sonja Selenska-Pobell Institute of Radiochemistry, Research Center Dresden-Rossendorf, 01314 Dresden, Germany, s.selenska-pobell@fzd.de

Alba Silipo Department of Organic Chemistry and Biochemistry, University of Naples Federico II, via Cintia, 4 I-80126 Napoli, Italy, alba.silipo@unina.it

Uwe B. Sleytr Department of NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 11, A-1190 Vienna, Austria, uwe.sleytr@boku.ac.at
Department of NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor-Mendel-Strasse 33, 1180 Vienna, Austria, uwe.sleytr@boku.ac.at

Ajit Varma Amity Institute of Microbial Sciences (AIMS), Amity University, Noida 201303 UP, India, ajitvarma@aihmr.amity.edu

Reinhard Wirth Institute of Microbiology, University of Regensburg, 93053 Regensburg, Germany, Reinhard.Wirth@biologie.uni-regensburg.de

Guoqing Xia Cellular and Molecular Microbiology Section, Medical Microbiology Department, University of Tübingen, 72076 Tübingen, Germany, guoqing.xia@med.uni-tuebingen.de

Sophie Yurist-Doutsch Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel

Part I
Cell Wall Polymers and Structures of Bacteria

Chapter 1
The Murein Sacculus

Silke Litzinger and Christoph Mayer

1.1 Introduction

The murein is a characteristic and unique macromolecular component of the cell wall of almost all bacteria, missing in only a very few bacterial groups such as mycoplasma (mollicutes) and planctomycetes. It is composed of glycan threads interlinked by short peptides, and hence belongs to a class of polymers called peptidoglycans. The murein constitutes a huge single molecule that encases the entire cell and forms a closed bag-shaped structure, the murein sacculus. The sacculus stabilizes the fragile cytoplasmic membrane and the cytoplasm within and protects them from the adverse effects of the environment. It also determines the cell's characteristic shape and is essential to withstand the large internal osmotic pressure (turgor). Some billion years ago, when the domain Bacteria arose from prokaryotic progenitors, cells acquired this exoskeleton-like structural element, allowing the accumulation of high intracellular concentrations of nutrients that accompanies an increasing cell turgor. The assembly of the giant murein macromolecule external to the cell membrane and the preservation of its structural integrity during cell growth and division is a difficult task. In fact, it is so complex that up to now fundamental questions about the murein construction, its 3D structure and its role in morphogenesis are still insufficiently understood, although fundamental breakthroughs have been made in recent years in this area.

Further chapters within this publication will deal with topics related to murein. Besides its stabilizing function, the murein sacculus serves as a scaffold for anchoring cell wall components such as proteins (cell wall associated proteins) or acidic polymers (e.g., teichoic acids). The murein and its biosynthesis pathway serve as primary target for antibiotics, since the integrity of the sacculus is of critical importance to cell viability and its structure and biosynthesis is restricted

C. Mayer (✉)
Fachbereich Biologie, Universität Konstanz, Konstanz 78457, Germany
e-mail: ch.mayer@uni-konstanz.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_1,

to bacteria. Furthermore, the murein and its degradation products are important pathogen-associated molecular patterns and their involvement in pathogenicity is addressed later in this volume.

Necessarily, this chapter on murein can only give a general introduction and many aspects can be just briefly touched. More detailed information on most aspects covered in this chapter can be obtained from a comprehensive compilation of reviews that was published very recently in *FEMS Microbiology Reviews* (Barreteau et al. 2008; Bouhss et al. 2008; Coyette and van der Ende 2008; Mainardi et al. 2008; Vollmer 2008; Vollmer et al. 2008a, b) and gives further information related to murein/peptidoglycan (den Blaauwen et al. 2008; Dramsi et al. 2008; Sauvage et al. 2008; Zapun et al. 2008a, b). We also refer to excellent chapters on murein within book publications (Salton 1994; Park 1996; Foster and Popham 2002; Seltmann and Holst 2002; Scheffers 2007; Vollmer 2007).

1.2 A Short History of the Discovery of the Murein Sacculus

1.2.1 The Exoskeleton-like Surface Layer

Along with the discovery and visualization of bacteria by Antonie van Leeuwenhoek in 1675, it had been noticed that bacteria come in various shapes (cf. Porter 1976); coccoid, rod, curved and spiral-shaped cells can be observed under the microscope. The fact that most bacteria are not spheres instantly predicted that a rigid exoskeleton-like surface structure is responsible for the cell shape. However, it was not until the introduction of electron microscopy techniques that bacterial cell walls could be visualized (Mudd et al. 1941; Salton and Horne 1951; Salton 1952) and the multilayered architecture of the Gram-negative cell envelope (see Sect. 1.2.2) was discovered (Kellenberger and Ryter 1958; Weidel et al. 1960; Murray et al. 1965; De Petris 1967). In addition, electron microscopy played an important role in monitoring the homogeneity of cell fractionations and thus was a prerequisite for the isolation of pure cell wall preparations (cf. Salton 1964; Weidel and Pelzer 1964; Salton 1994). In the early 1960s Wolfhard Weidel and coworkers from Tübingen isolated thin cell envelopes of the same size and shape as the originating *Escherichia coli* cells. They recognized the bag-shaped structure of this compound and named it murein or murein sacculus (from "murus", Latin "wall" and "sacculus", Latin "sack") (Weidel et al. 1960; Weidel and Pelzer 1964).

1.2.2 Gram-Negative and -Positive Cell Walls

Whereas the murein sacculus of Gram-negative bacteria (e.g., *E. coli*), which locates between an inner and outer membrane, is very thin and sometimes not even visible in electron micrographs, Gram-positive bacteria are surrounded by

a much thicker murein layer. These observations, together with analysis of differences in the chemical composition of the cell walls (see Sect. 1.2.3), laid the basis for our current view of the fundamental differences in the cell wall structure of Gram-negative and Gram-positive bacteria. The classification goes back to the work of the Danish bacteriologist Hans Christian Gram. In 1884 he reported the development of a staining technique (cf. Popescu and Doyle 1996), which is now recognized as one of the most important taxonomic tools in microbiology and is still used as a first test performed for the differentiation of bacteria. The Gram staining is based on the ability of bacterial cell walls to retain the dyes crystal violet or methylene blue. The dye is first fixed by addition of a mordant (Lugol's iodine solution) and then treated with an organic solvent (ethanol or isopropanol). The microorganisms that retain the dye–iodine complex appear violet/purple and are classified as Gram-positive. Others, in which the stain is washed out, are referred to as Gram-negative. Some Gram-positive bacteria may lose the stain easily and therefore appear as a mixture of Gram-positive and Gram-negative bacteria (Gram-variable). Other microorganisms do not respond to the Gram staining, such as mycobacteria, rickettsia, and spirochetes, and require special stains to be visualized (e.g., acid-fast staining). Gram did not know that the differential behavior of bacteria in his staining approach is based on fundamental differences in the cell wall composition and structure (Beveridge and Davies 1983; Popescu and Doyle 1996). We know now that Gram-positive bacteria retain the dyes much more strongly than Gram-negative bacteria because their cell walls have a much thicker murein layer and lack an outer membrane. In addition, Gram-positive murein contains acidic polymers (see Chap. 6), to which the dye can bind.

1.2.3 Role of Lysozyme and Penicillin in the Discovery of Murein

The discovery of the murein sacculus and the elucidation of its chemical structure and biosynthetic pathway was closely linked to research on the function of two antibiotic agents, lysozyme and penicillin, both discovered by Fleming (1955) (cf. Geddes 2008 and reviews Jollès and Jollès 1984; Jollès 1996; Bennett and Chung 2001 and refs therein). Lysozyme has a fascinating effect on bacteria, as it completely "dissolves" bacterial cells, e.g., *Micrococcus luteus* (formerly named *M. lysodeicticus* for this reason), within seconds of incubation and releases a disaccharide product from the cell wall (Salton 1952). Addition of lysozyme to bacterial cells that are stabilized in iso-osmotic media leads to the formation of spheroplasts (Gram-negative) or protoplasts (Gram-positive). These are round cells that have lost their specific shape, are osmotically labile, and burst in more diluted media (Robinson et al. 1974; Yamato et al. 1975). Penicillin, the other antibiotic agent, is produced by the fungus *Penicillium notatum*. It has a similar morphological effect on bacteria and eventually also lyzes them (Hughes et al. 1949). Penicillin began its tremendous success as a chemotherapeutic antibiotic in the 1940s (cf. Russell 2002; Chain et al. 2005).

Since penicillin only affects actively growing cells, it can be applied to isolate auxotrophic mutants of bacteria. The penicillin-isolation technique for nongrowing mutants developed into an important strategy to identify metabolic pathways (Davis 1948, 1949; Lederberg and Zinder 1948). Penicillin was also a valuable tool for studying bacterial cell wall biosynthesis. Park and Johnson observed that uridine nucleotides accumulate in penicillin-treated *Staphylococcus aureus* cells (Park and Johnson 1949). Later on, the isolation and characterization of the nucleotides carrying amino sugars and D-amino acids unknown at that time, which become known as Park's nucleotides, led the way to the discovery of the biosynthetic pathway of the unique cell wall polymer, murein (see Sect. 1.2.4) (Park 1952; Park and Strominger 1957). Park and others also demonstrated that penicillin kills bacteria by inhibiting their ability to synthesize the cell wall (Park and Strominger 1957; Martin 1964; Tipper and Strominger 1965; Wise and Park 1965). Transpeptidases, enzymes required for the crosslinking of the murein sacculus (see Sect. 1.4.3), so-called penicillin-binding proteins, are specifically inhibited by penicillin, which covalently binds to the active site of the enzymes (Tipper and Strominger 1968). Unraveling the structure of small soluble fragments released from isolated cell walls with murein-hydrolyzing enzymes, e.g., lysozyme, completed the elucidation of the structure of the murein sacculus (Primosigh et al. 1961; Ghuysen 1968). Accordingly, the murein is a heteropolymer arranged of building blocks (repeating units) of disaccharide-peptides, also called muropeptides (Fig. 1.1), the very compound(s) that are released from murein by the action of lysozyme.

1.2.4 Cell Wall Growth and Growth Models

The presence of a single macromolecule encasing the bacterial cell creates the problem of safe and controlled enlargement and division without the risk of lysis of the cell. Shortly after the recognition of the bag-shaped murein, Weidel and Pelzer proposed the enzymatic balance model for the growth of the sacculus (Weidel and Pelzer 1964; also see Höltje 1998). Accordingly, lytic and synthetic enzymes function in a delicately balanced interplay, with the former autolysins introducing nicks into the murein structure and the latter murein synthases inserting new material at these sites. Therefore, bacteriolysis following the inhibition of a step of murein synthesis can be explained as being the consequence of an imbalance between the synthetic and autolytic activities (e.g. Schwartz and Weidel 1965; Sugai et al. 1997; Meisel et al. 2003). We know now that the cell wall is indeed a highly dynamic compartment. Despite being a rigid, stabilizing molecule, the murein undergoes permanent remodeling, degradation, and resynthesis during growth. In *E. coli* about half of the murein is degraded within one generation and the fragments are released during growth, a process called cell wall turnover. The order of magnitude by which turnover occurs had long been underestimated, since the degradation products are usually efficiently reutilized (cell wall recycling) (see Sect. 1.6) (Goodell 1985; Goodell and Schwarz 1985). The reason for the massive

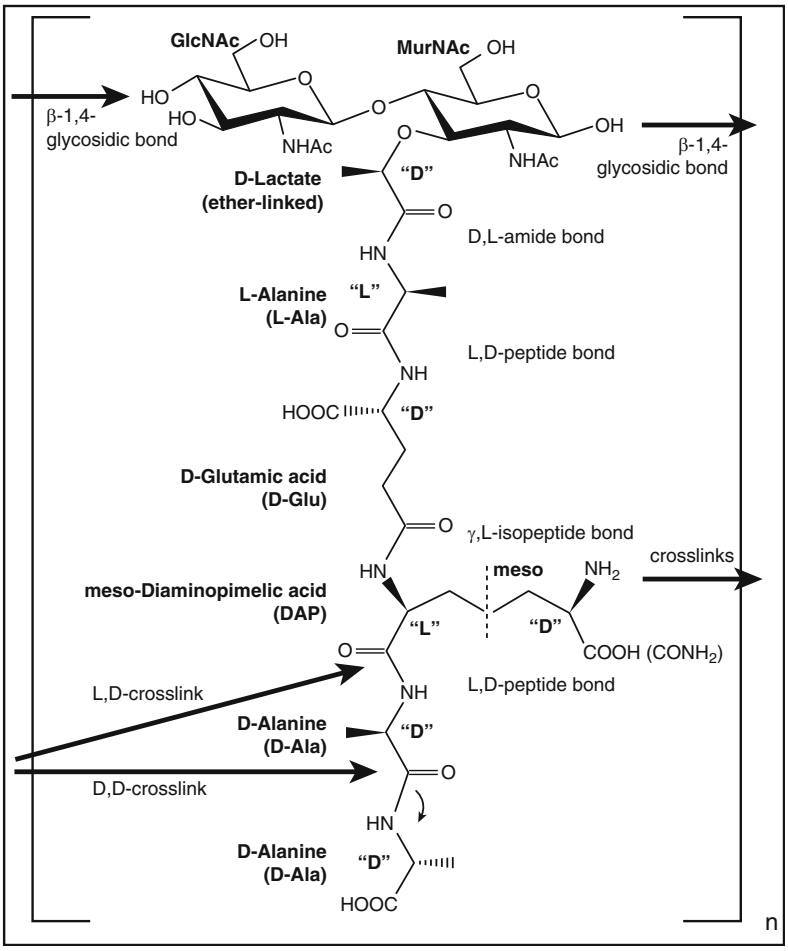

Fig. 1.1 Structure of the disaccharide-pentapeptide (GlcNAc-MurNAc-L-Ala-iso-D-Glu-DAP-D-Ala-D-Ala) building block of murein. The amino sugars are connected via β-1,4-glycosidic linkages, generating long glycan chains. The peptides are connected via a D,L-amide bond to the ether-linked D-lactate substituent of MurNAc. The D-Glu residue is linked via its γ-carboxyl group to the amino group of the L-center ("L") of DAP (isopeptide bond). The mirror plane of the meso-structure of DAP is indicated by a *dotted line*. The amino group of the D-center ("D") of DAP is required for crosslinking peptide chains either via D,D-peptide crosslinks, through attack of the carbonyl at the fourth amino acid (D-Ala) of a neighboring peptide chain (generated by penicillin-sensitive transpeptidases), or in some cases via L,D-peptide crosslinks, through attack of the carbonyl of the L-center of DAP (generated by penicillin-insensitive transpeptidases). The L-center of DAP is also the attachment side of Braun's lipoprotein (see text). Only about half of the peptides are present in crossslinks in *E. coli* and *B. subtilis*, a number that can vary significantly within other species. Also the peptide composition significantly varies within bacteria; the structure shown here holds for most Gram-negative and some Gram-positive bacteria (e.g., *Bacillus subtilis*, however about 99% of the D-carboxylate of DAP is amidated ($CONH_2$) in this organism, and up to 30% of the amino sugars are *N*-deacetylated, cf. Fig. 1.2)

degradation of the endogenous murein during cell growth is not clear; however, the process of murein synthesis is probably somehow intrinsically connected to cell lysis.

Cell wall growth might occur by insertion of new peptidoglycan material at specific points or randomly into the sacculus. Diffuse incorporation of murein along the lateral wall was confirmed for *E. coli* and *Bacillus* sp. (de Chastellier et al. 1975a, b; Cooper and Hsieh 1988; de Jonge et al. 1989; Nanninga 1991). Furthermore, Burman and Park (1984) showed by labeling experiments that newly synthesized strands of murein are initially inserted adjacent to old strands in *E. coli*. They concluded that in each generation about 90 separate membrane-associated protein complexes for murein synthesis would travel around the circumference of the cell, generating two new strands at a time (Burman et al. 1983a, b; Burman and Park 1984). Later, diffuse intercalation of murein into the side wall as well as enhanced synthesis at the division site was confirmed by D-cysteine labeling of murein (de Pedro et al. 1997, 2001, 2003). Koch argued in his surface stress theory that the cell wall expands by the force of turgor pressure (Koch et al. 1981, 1982; Koch 1985) and proposed that new covalent bonds have to be formed prior to hydrolysis to allow a safe enlargement of the stress-bearing murein layer, the so-called make-before-break hypothesis (Koch and Doyle 1985; Koch 1988, 1995). The observation that in Gram-positive bacteria labeled murein precursors are incorporated close to the membrane and are gradually displaced within the wall to the outside led Koch and Doyle (1985) to propose the inside-to-outside model for growth (Koch and Doyle 1985). Murein that becomes stretched upon moving to the outside would become stress-bearing and, hence, more susceptible to the activity of autolysins. An elegant growth model for Gram-negative bacteria, the three-for-one model, was provided by Joachim-Volker Höltje from Tübingen (Höltje 1996a, 1998; Höltje and Heidrich 2001; Vollmer and Höltje 2001). According to this model, three new glycan strands are attached in a relaxed conformation from below to the mature cell wall, which is under osmotic pressure, and are inserted simultaneously with removal of the so-called "old" docking strand. A multienzyme complex (holoenzyme) was proposed that closely ties murein synthesis with autolysis. The model explains the massive muropeptide release during growth, includes the make-before-break postulate, and furthermore suggests that the existing cell wall would serve as a template in order to maintain the cell's shape during growth, which is an intriguing feature of bacterial cells.

1.2.5 Cell Shape and Morphogenesis

Bacterial cells keep their characteristic shapes upon cell growth and division, a complex process that is not so far understood. Obviously, the shape of bacterial cells is fixed by the murein sacculus. However, is the shape of the sacculus an intrinsic feature of its chemical composition or does a special morphogenetic system exist which determines the shape of the sacculus? In 1971, Schwarz and

Leutgeb addressed this question. They showed that significant variations in the composition of murein were found in sacculi from cells grown in various media and harvested at different stages of growth, whereas the general shape of the cells remained unchanged. They concluded that this finding stands opposed to the assumption of a strict correlation between chemistry and shape of the sacculus. However, it is relatively easy to change the shape of the sacculus, e.g. by modifying the hypothetical morphogenetic system, such as by converting rod-shaped cells into spherical cells. Spheroplasts/protoplasts can reform a new spherical sacculus, which is indistinguishable, in chemical composition, from the rod-shaped sacculus (Schwarz and Leutgeb 1971). These findings were taken as evidence for the existence of a hypothetical morphogenetic system whose activity is reflected by the shape of the sacculus. In recent years the discovery of bacterial cytoskeleton elements has revived the field of bacterial morphogenesis (see Sect. 1.5) and has taken us a step towards understanding what determines cell shape, how shape is inherited, and how murein is involved in these processes.

1.2.6 Spatial Orientation and Structural Arrangement of the Murein

The topological organization of the murein is uncertain, since there is, so far, no direct way to approach this question. X-ray diffraction studies have confirmed the absence of semicrystalline structures in the murein of bacterial cell walls but do not indicate a completely unordered structure; diffraction rings indicate a periodicity of about 1 and 0.44 nm (Formanek and Rauscher 1979; Labischinski et al. 1979). Verwer et al. investigated the selective partial hydrolysis of peptide bridges of *E. coli* murein by an endopeptidase and discovered by electron microscopy that the preferential orientation of the glycan strands is more or less perpendicular to the long axis of the cell (Verwer et al. 1978). This supports a horizontal layer model of the murein sacculus of *E. coli* consisting of glycan strands (of peptidyldisaccharides of 1 nm periodicity) arranged parallel to the cell membrane that run along the short axis (Weidel and Pelzer 1964; Vollmer and Höltje 2001). Recently, the classical conception of the spatial orientation of glycan stands of the murein oriented in parallel to the cell membrane of bacteria has been challenged by computer-simulation studies (Dmitriev et al. 1999, 2000, 2003, 2004, 2005). The authors argued that a layer of murein would be unstable if constructed of short glycan chains connected by only few peptide bridges. Instead, they proposed a structure in which the glycan strands would protrude perpendicular from the cell membrane, the scaffold model, and in which peptide bridges would solely account for the stabilizing property of the sacculus. Vollmer and Höltje, however, argued against this model by careful consideration of experimental data, such as thickness, elasticity, amount, and covered surface area of the murein layer, as well as glycan chain length distribution and degree of crosslinking (Vollmer and Höltje 2004). They came to the conclusion that a scaffold model, at least for *E. coli,* is not

consistent with experimental data. However, we are still far away from a clear picture of the organization of the bacterial cell wall, and it is not even clear if the arrangement of the murein needs to be similar for all bacteria (cf. (Young 2006)). New analytical tools and approaches are highly desired to resolve the 3D structure (s) of murein. Recently, the complete resonance assignment of purified murein from *E. coli* was obtained by solid state NMR experiments and might in future provide details of the macromolecular structure and spatial distribution (Kern et al. 2008). Electron cryotomography (ECT) can in principle deliver structural information in atomic resolution and might provide information about the murein structure in the near future (Jensen and Briegel 2007). With another promising technique, atomic force microscopy (AFM), the inner surface of the *Bacillus* cell walls has been visualized very recently. Surprisingly, these studies revealed a regular macrostructure of thick, c. 50-nm-wide, peptidoglycan cables running basically across the short axis of the cell, indicating a murein structure that is very different from our current view of a layered murein (Hayhurst et al. 2008).

1.3 Analysis of the Chemical Structure and Composition of the Murein

1.3.1 Chemical Structure of the Murein

The murein is absolute essential for bacteria to resist the high intracellular turgor, which can reach pressures of 2–5 atmospheres in Gram-negative bacteria and up to 50 atm in Gram-positive bacteria (Archibald et al. 1993; Seltmann and Holst 2002). The remarkable tensile strength of the cell wall, combined with elasticity, is achieved by fairly inextensible glycan chains of variable length, held together by small elastic peptide crosslinks, allowing the sacculus to expand or shrink dependent on the cell's turgor (Ghuysen 1968; Höltje 1998). It has been estimated that the surface area of the sacculus can reversibly increase about threefold without rupture (Koch et al. 1987; Koch and Woeste 1992). The overall composition and structure of the murein is generally similar in bacteria. The rigid glycan chains are generated by alternating β-1,4-linked amino sugars *N*-acetylglucosamine (GlcNAc) and its 3-*O*-lactyl ether derivative, *N*-acetylmuramic acid (MurNAc) (Fig. 1.1). MurNAc is truly unique to bacteria and not even present in the second large group of peptidoglycans, the pseudomureins of Archaea (cf. Chap. 10). The flexible peptides of the murein are attached (via an D,L-amide bond) to the D-lactyl ether substituent of MurNAc (Fig. 1.1, *gray* substructure) and contain (in contrast to pseudomurein) noncodogenic D-amino acids (D-Glu, D-Ala), besides L-amino acids (L-Ala) and di-basic amino acids. These amino acids generate alternating D,L- and L,D-peptide/amide bonds. The D-L-D-L-D sequence in the peptides (beginning with the D-lactyl group of MurNAc and including the L-center of meso-diaminopimelic acid (DAP); Fig. 1.1) prevents the formation of α-helical structures, and thus gives the

peptides much more flexibility. It also makes the murein insensitive to cleavage by "normal" peptidases/proteinases, which are specific for L,L-peptide bonds.

The chemistry of the glycan strands shows only a few variations among different bacteria, such as *O*-acetylation (mainly at C6 of MurNAc) and *N*-deacetylation (of both GlcNAc and MurNAc to various degrees), whereas the peptide composition can differ quite significantly (Schleifer and Kandler 1972; Quintela et al. 1995a; Vollmer 2008; Vollmer et al. 2008a). The basic stem peptide structure of the murein is L-Ala-D-Glu-DAP-D-Ala-(D-Ala) (Fig. 1.1). The di-basic amino acid DAP is present in most Gram-negative species but is substituted with L-lysine (L-Lys) (in most Gram-positives) or with L-ornithine (L-Orn) (in Spirochetes, *Bifidobacterium globosum* and *Thermus thermophilus*) (Vollmer et al. 2008a). In *T. thermophilus* the δ-NH_2 of L-Orn is modified by glycine–glycine and in about 23% of total muropeptides the *N*-terminal glycine is substituted by a residue of phenylacetic acid (Quintela et al. 1995b). DAP is also used in some Gram-positive bacilli and in mycobacteria; however, in these organisms it is frequently amidated. In *B. subtilis* about 99% of the DAP is amidated at the D-carboxylate (Foster and Popham 2002). Rare di-basic amino acids at the third position of the peptide stem include D-Lys, L,L-DAP, meso-lanthonine, L-2,4-diaminobutyrate (L-Dab), L-homoserine (L-Hsr), and others (Vollmer et al. 2008a). The amino acids at the other positions at the peptide side chain are less frequently modified, but can be substituted by glycine, L-serine (at position 1) or threo-3-hydroxyglutamate (*Microbacterium lacticum*) (at position 2) (Vollmer et al. 2008a). A fifth amino acid in the peptide, usually a D-Ala, is used to perform the transpeptidase reaction (see Sect. 1.4.3), by which crosslinks are introduced in the peptide stems (Fig. 1.1). In some cases glycine, D-serine, or D-lactate can substitute for the amino sugar at position 5. These modifications are associated with the development of vancomycin resistance in enterococci (VRE), which is a result of weaker binding of the antibiotic vancomycin to the modified peptide (Boneca and Chiosis 2003).

1.3.2 Peptide Crosslinking

The peptide side chains can be interlinked by the amino group of a di-basic amino acid (DAP or L-Lys, etc.) of one peptide chain and a carboxyl group of another chain, thereby connecting the glycan strands within the murein (Fig. 1.1). Most peptide crosslinks in *E. coli* are of the D,D-type, formed between the amino group of the D-center of DAP (position 3) and the carboxyl group of D-Ala (position 4). The formation of the D,D-peptide crosslinks is catalyzed by specific D,D-transpeptidases, also called penicillin-binding proteins (see Sect. 1.4.3), which are inhibited in the presence of penicillin and other β-lactam antibiotics (Sauvage et al. 2008; Zapun et al. 2008a). However, in the murein of *E. coli* and other bacteria to some extent L,D-crosslinks also occur, which connect the L- and the D-center of DAP (Fig. 1.1). They are catalyzed by special penicillin-insensitive L,D-transpeptidases (Höltje 1998; Magnet et al. 2008). A large increase in L,D-crosslinks is generated by

cultivating bacteria in media containing β-lactam antibiotics, hence selecting for the formation of penicillin-insensitive crosslinks (Mainardi et al. 2002; Cremniter et al. 2006). A L,D-transpeptidase homologous to L,D-transpeptidase of *Enterococcus faecium* has been shown to be responsible for the attachment of the Braun's lipoprotein (MlpA) to *E. coli* murein (see Fig. 1.1) (Magnet et al. 2007b), which indicates that evolutionarily related domains have been tailored to use muropeptides or proteins as acyl acceptors in the L,D-transpeptidation reaction (Magnet et al. 2007a).

According to Schleifer and Kandler, up to some 100 peptide-linkage types can be differentiated (reviewed in their monomental work Schleifer and Kandler 1972). They introduced a system to classify peptidoglycan types (three-digit numbering, e.g., A1γ; see Table 1.1 and Fig. 1.2) and used it as a taxonomic tool for bacteria. However, it appeared that murein composition strongly depends on growth conditions and age or growth phase, which leads to significant variations in the murein composition within one species (Glauner et al. 1988; Glauner and Höltje 1990; Quintela et al. 1997). Furthermore, different linkage types might exist within one species. An alternative classification of murein was introduced by Jean-Marie Ghuysen (1968), which differentiates five di-basic amino acids (peptide types A to E: DAP, L-Lys, L-Lys-Gly, Hsr, L-Orn; for definition of the abbreviations see Table 1.1) and four types of crosslinks (types 1–4: direct crosslink; Gly, D-Asn or L-amino acids; peptides; or D-amino acids).

Besides dimeric muropeptides, there is a small proportion of trimeric and tetrameric structures in *E. coli* (see Fig. 1.2, *red arrows*) that contain three or four crosslinked peptides (Glauner and Höltje 1990). The majority of crosslinked muropeptides in *Bacillus* sp. are dimers and higher crosslinkage oligomers are only present in minute quantities (Severin et al. 2004). The number of crosslinkages varies significantly in bacterial species and is dependent on culture conditions, but usually Gram-negative bacteria are crosslinked to a lesser extent (up to 50% of

Table 1.1 Murein (peptidoglycan) classification, according to Schleifer and Kandler (1972)

Three digit identifier (e.g., A1α)	**A** Crosslinks between position 3 of the first and position 4 of the second of two amino acid side chains				**B** Crosslinks between position 2 of the first and position 4 of the second of two amino acid side chains	
	1 Direct crosslink	**2** Polymerized peptide unit	**3** Monocarboxylic L-amino acid or Glycine or both	**4** Di-Carboxylic-amino acid	**1** L-Di-amino acid	**2** D-Di-amino acid
α	L-Lys	–	L-Lys	L-Lys	L-Lys	L-Orn
β	L-Orn	–	L-Orn	L-Orn	L-Hsr	L-Hsr
γ	*m*-DAP	–	*m*-DAP	L,L-DAP	L-Glu	L-Dab
δ	–	–	–	–	L-Ala	–

Variations at position 3 of the stem peptide include: L-lysine (L-Lys), L-ornithine (L-Orn), meso-diaminopimelic acid (*m*-DAP), L-Hydroxy-serine (L-Hsr), L-glutamate (L-Glu), L-diaminobutyrate (L-Dab), and L-alanine (L-Ala). Examples of peptide crosslinkages of selected bacteria are depicted in Fig. 1.2

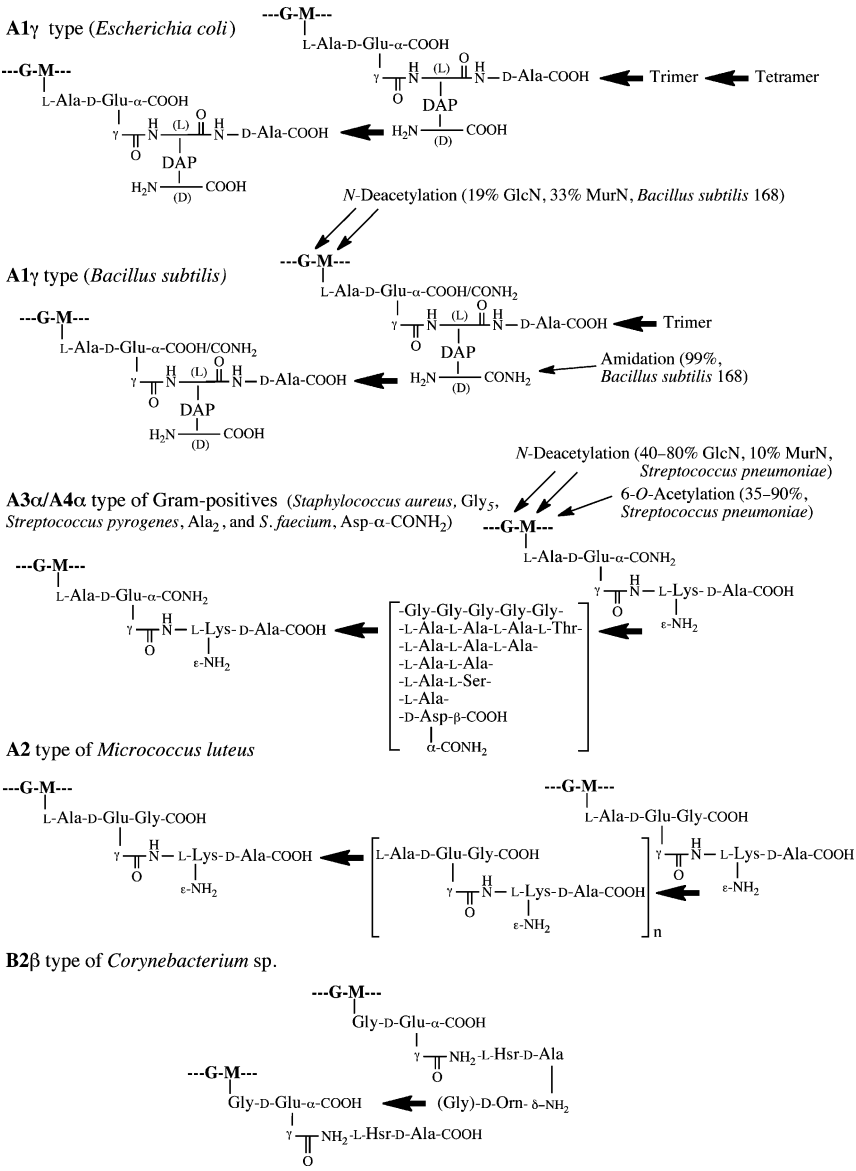

Fig. 1.2 Crosslinkage-types of the murein of selected bacterial species according to Schleifer and Kandler (1972). The glycan backbone is indicated, G stands for GlcNAc and M for MurNAc. The amino acids involved in crosslinkage are shown as partial structures; see legend to Fig. 1.1 and Table 1.1 for details and footnote to Table 1.1 for abbreviations used. Dimeric crosslinks are predominant in the murein of most bacteria (*black solid arrows*), but trimeric and even tetrameric crosslinks (*grey solid arrows*) have been identified

peptides are involved in crosslinks) compared to Gram-positive bacteria (up to 90% crosslinks). Sometimes confusing is the use of the term degree of crosslinkage, which is defined as 100 × (1/2 dimers + 2/3 trimers + 3/4 tetramers)/all muropeptides. By this definition, the degree of crosslinkage is equal to the molar percentage of peptides that act as donors in the crosslinking reaction. This is not the same parameter as the molar percentage of crosslinked peptides, which is a much larger value than the former. *E. coli* strains have a degree of crosslinkage of about 25%, and almost 50% of the peptides chains are part of crosslinked structures (Vollmer and Höltje 2004).

1.3.3 Isolation of Murein and Murein Content

Structural and compositional elucidation of murein requires the isolation and defined fragmentation of the macromolecule to yield muropeptides or linear, uncrosslinked peptidoglycan chains, which can be separated and identified (see Sect. 1.3.4). This, however, is not a trivial task. Preparation of pure murein is hampered by additional components of the cell envelope that are covalently linked to the murein; e.g., wall teichoic acids in Gram-positives (see Chap. 6) and lipoproteins in Gram-negatives (see Sect. 1.3.5) (Rosenthal and Dziarski 1994). Complete fragmentation of the murein is also difficult to obtain, due to great variations from the overall subunit structure (cf. Figs 1.1 and 1.2) and selective substrate specificities of murolytic enzymes. Structural modifications of the murein, such as *O*-acetylation, phosphorylation, amidation, etc., might interfere with fragmentation. In addition, some modifications (e.g., *N*-acetyl groups, phosphoryl groups) might be removed throughout the purification process (Vollmer 2008). For Gram-negative and Gram-positive bacteria, optimized murein isolation protocols are applicable (Höltje et al. 1975; Glauner et al. 1988; Harz et al. 1990; Rosenthal and Dziarski 1994; Atrih et al. 1999). Recently, an adaptation of these protocols was developed for the isolation of mycobacterial murein (Mahapatra et al. 2008).

In general, murein is extracted and purified by adding a bacterial cell suspension drop by drop to a boiling 8% SDS solution. This will instantly remove the lipid compounds of the cell envelope of Gram-negative bacteria, lyze the cells, and also denature the cell's own arsenal of murein hydrolases (autolysins), which might interfere with isolation of intact murein (Höltje et al. 1975; Glauner et al. 1988). After removal of the SDS by extensive washing and (ultra)centrifugation of the samples, the sediment is treated with nuclease, amylase and proteinase (e.g., pronase) to remove DNA, glycogen and proteins from the preparation. Isolation of Gram-positive murein was achieved by similar approaches, but usually requires an additional treatment with hydrofluoric acid to remove acid labile teichoic acid contaminants (de Jonge et al. 1992; Atrih et al. 1999; Dhalluin et al. 2005). Alternatively, disruption of cells with glass beads in a sonicator has been applied (Cummins and Harris 1956; Kowalski et al. 1970; Rosenthal and Dziarski 1994).

The amount of murein in the cell wall of bacteria can be deduced from quantification of specific cell wall components like DAP, either by direct measurement of the DAP content of isolated sacculi or by steady-state incorporation of radioactively labeled DAP over several generations (Braun et al. 1973; Wientjes et al. 1991). Thereby, an value of 3.5×10^6 DAP molecules per sacculus in the cell wall of *E. coli* was determined and the molecular mass of the macromolecule murein of *E. coli* was estimated to be 3.5×10^9 Da, which is in the mass range of the other large macromolecule of the bacterial cell, the chromosome. The data indicate an average surface area per disaccharide of about 2.5 nm^2, with a length of the disaccharide unit of 1.1 nm and an average distance between glycan strands calculated to be c. 2 nm. Combined with electron microscopic measurements of the surface area of the cells, this suggests a basically monolayered murein structure in *E. coli* (*cf.* Vollmer and Höltje 2004) that amounts to only 1–3% of the cell mass (or 3–10% of the cell's dry weight) (Park 1996). In Gram-positive bacteria the proportion of the dry weight of the murein varies considerably within species. In most Gram-positive bacteria, the murein layer is up to 10 times thicker than that of Gram-negatives (20–80 nm) and accounts for 20–60% of the dry weight of the organism (e.g., *Bacilli, Staphylococci*) (Rogers et al. 1974; Shockman and Barrett 1983; Archibald et al. 1993; Foster and Popham 2002).

1.3.4 Isolation of Muropeptides and Glycan Chains

Biochemical analysis of the murein composition generally requires digestion with murein hydrolases (e.g., chalaropsis or human serum muramidase, mutanolysin, cellosyl, L,D-amidase, D,D-endopeptidase, etc.) (Tipper et al. 1964; Verwer et al. 1978; Calandra and Cole 1980; Mollner and Braun 1984; Shockman et al. 1996; Vollmer et al. 2008b), to release soluble murein fragments (muropeptides), which can be separated by HPLC techniques (Glauner 1988; Glauner et al. 1988; Glauner and Höltje 1990; Harz et al. 1990; de Jonge et al. 1992; Atrih et al. 1999; Dhalluin et al. 2005). More than 50 different muropeptides were identified in murein preparations from *E. coli* (Glauner 1988; Glauner et al. 1988). Alternatively, a fluorophore-assisted carbohydrate electrophoresis method was developed to separate the major muropeptides (Li et al. 2004). A purely chemical method of releasing murein building blocks (acid hydrolysis) was developed by Schleifer and Kandler (Schleifer and Kandler 1967) and extensively used to extract the peptide portion of the murein of various bacteria and determine their amino acid composition (Schleifer and Kandler 1972). Under alkaline conditions the lactyl ether bond of muropeptides is cleaved to yield lactyl-peptides, which can be analyzed by thin-layer chromatography or modern LC-MS techniques (Arbeloa et al. 2004).

In order to obtain information about the length of glycan chains, endopeptidase and/or amidase treatment is performed. Glycan strands of up to 30 disaccharide units can be separated by a HPLC technique that has been developed to specifically address the issue of glycan strand length (Harz et al. 1990). The average length of glycan

strands of 21 disaccharide units was determined for *E. coli* murein, given that long glycan strands with more than 30 disaccharide units represent about 25–30% of the total material. Alternatively the average degree of polymerization can be determined by measuring the amount of 1,6-anhydroMurNAc. Glycan strands in *E. coli* and other Gram-negative bacteria (usually to a much lesser extent in Gram-positive bacteria) terminate at the reducing end with 1,6-anhydroMurNAc, an intramolecular glycoside that is generated by the C6 hydroxyl group of MurNAc reacting with the C1 hemiacetal upon murein cleavage by so-called lytic transglycosylases (see Sect. 1.6). Three to six percent of 1,6-anhydroMurNAc-containing molecules were determined in *E. coli,* from which value an average degree of oligomerization of 25–40 disaccharide units can be calculated (Glauner 1988). The glycan chains in the murein of *B. subtilis* were reported to range from 54 to 96 disaccharide units in length (Ward 1973). Very recently, however, Hayhurst et al. (2008) isolated glycan strands as long as 5 μm (equal to c. 5,000 disaccharide units) from *B. subtilis* and reported an average length of 1.3 μm (1,300 disaccharide units). In contrast, *S. aureus* glycan strands are much shorter at an average of 6–9 disaccharides (Tipper et al. 1967; Ward 1973; Boneca et al. 2000). Interestingly, the nonreducing end of the glycan chains of *S. aureus* were found to lack the GlcNAc moiety, which usually terminates the glycan strands in other bacteria (Boneca et al. 2000).

The glycan strands within the murein are not planar sheets of parallel or antiparallel oriented glycan chains, as in related cell wall polysaccharides of plants (cellulose) or arthropods (chitin). The glycan chains in the murein are helically twisted, caused by rejections of the bulky ether substituents at C3. A right-handed α-helical structure with four disaccharide-peptide subunits generating one turn would lead to a glycan thread from which peptides would protrude in all four directions in space. Such a structure was assumed in older literature and it fits well with the view of glycan strands in which every second peptide undergoes a cross-linking within plane (a maximum of 50% crosslinks can be generated), thereby building hexagonal meshes like a honeycomb (so-called tessera; Demchick and Koch 1996). However, a recent aqueous solution NMR structure of a short peptidoglycan fragment (tetrasaccharide di-pentapeptide) indicates that the right-handed glycan helix more likely has threefold symmetry (Meroueh et al. 2006). Assuming that the structure of the insoluble murein macromolecule can be deduced from the structure of a small soluble fragment, a threefold symmetry would not allow a planar arrangement of crosslinked glycans and it would be difficult to conform to the horizontal layer model of the murein (see Sect. 1.6).

1.3.5 Modifications in the Gram-Negative Murein Structure

In Gram-negative bacteria, the murein is embedded in two membranes, the cell membrane and the outer membrane. It is attached to the inner face of the outer membrane via covalent bonds to Braun's lipoprotein (MlpA/lpp) and noncovalent association with outer membrane protein A (OmpA) and lipoprotein PAL, both

carrying peptidoglycan-binding modules extending into the periplasm (Nikaido 2003). In *E. coli* some 10^5 MlpA molecules connect the murein to the outer membrane (Braun 1975). Like other lipoproteins, MlpA is bound to the inner leaflet of the outer membrane by modification of the SH side chain of the N-terminal cysteine with diacylglycerol, whereas the α-amino group of the cysteine, which is released by cleavage of the signal peptide, is *N*-acylated. The ε-amino group of the C-terminal Lys of MlpA forms an isopeptide bond with the L-center of DAP of the murein peptide stem (cf. Fig. 1.1 and Sect. 1.3.2). In this way, the 56-amino-acid lipoprotein MlpA connects the outer membrane and the murein sacculus (Braun and Bosch 1972; Höltje 1998). The role of PAL and OmpA interactions with the murein are not clear to date, although a function in structural integrity, OM assembly, and cell division has been proposed (Clavel et al. 1998; Nikaido 2003; Cascales and Lloubes 2004; Gerding et al. 2007).

Glycan strands of the murein of Gram-negative bacteria usually terminate with 1,6-anhydro MurNAc, as mentioned above (Sect. 1.3.4), which is an intramolecular glycoside modifying the reducing end of the glycan strands. In some Gram-negative bacteria *N*-acetyl substitutions of glucosamine in the glycan strands are removed. A lack of at least 70% of *N*-acetyl substitutions of glucosamine of the peptidoglycan from the Gram-negative bacterium *Rhodopseudomonas viridis* has been reported, which renders the murein of this organism resistant to lysozyme (Schmelzer et al. 1982).

Although cyanobacteria are related to Gram-negative bacteria, their cell wall contains features of the Gram-positives. First of all, the murein layer is considerably thicker than that of most Gram-negative bacteria; from about 10 nm thickness in *Synechococcus* (Golecki 1974) reaching 700 nm in large cyanobacteria like *Oscillatoria princeps* (Hoiczyk and Baumeister 1995). Moreover, the extent of crosslinking (53–63%) is more similar to that reported for Gram-positive bacteria and the cell wall contains secondary polysaccharides (Hoiczyk and Hansel 2000). However, the cell wall lacks teichoic acid and contains the typical Gram-negative DAP amino acid at position 3 of the peptide side chain.

1.3.6 Modifications in the Gram-Positive Murein Structure

The murein layer of Gram-positive bacteria is much thicker than that of Gram-negatives. The existence of multiple layers of murein in Gram-positive cell walls, as described in many textbooks, has not been demonstrated directly, and this concept may be an oversimplification. The cell wall of *Bacillus* cells usually appears as a relatively amorphous structure between 20 and 50 nm thick that is tightly opposed to the underlying protoplast in electron micrographs of conventionally fixed preparations (Shockman and Barrett 1983; Archibald et al. 1993). Cryo-electron-microscopy on frozen-hydrated bacteria has made it possible to observe more structural details of the cell wall in a close-to-native state and revealed the existence of a periplasmic space in *B. subtilis* as well as in *S. aureus*

(Matias et al. 2003; Matias and Beveridge 2005, 2006, 2007. Earlier fractionation studies had already provided evidence for the existence of a functional homolog of a periplasmic space in *B. subtilis* that contains about 9.8% of the total protein content (Merchante et al. 1995; Pooley et al. 1996).

In most Gram-positive bacteria the crosslinkage is not a direct connection of peptide side chains by a D,D-peptide/amide bond but involves an interpeptide bridge (Fig. 1.2). There exists a range of different interpeptide bridges ranging from one to seven amino acids, e.g., a pentaglycine bridge in *S. aureus* and an L-Ala-L-Ala dipeptide bridge in *Streptococcus pneumoniae* (cf. Table 1.1). Furthermore, Gram-positive bacteria frequently have a much higher degree of crosslinks; e.g., up to 90% of the peptide side chains of the murein of *Staphylococcus aureus* are involved in crosslinks (Gally and Archibald 1993).

Some Gram-positive pathogens, *Bacillus* sp. (*B. cerus, B. anthracis,* etc.) as well *S. aureus* and *Streptococcus* sp. have a high intrinsic resistance to lysozyme (Araki et al. 1972; Zipperle et al. 1984; Vollmer 2008). This has been attributed to either *O*-acetylation of MurNAc residues or de-*N*-acetylation of GlcNAc and MurNAc residues in the glycan backbone of the murein (Zipperle et al. 1984; Clarke and Dupont 1992; Severin et al. 2004; Bera et al. 2005, 2006; Psylinakis et al. 2005; Herbert et al. 2007). Although *O*-acetylation is more commonly observed in Gram-positive bacteria, it has also been recognized in some Gram-negative pathogens (*Neisseria* sp., *Helicobacter pylori* and *Proteus mirabilis*). *Bacillus subtilis* lacks *O*-acetylation of murein, but contains de-*N*-acetylated amino sugars. Interestingly, two *B. subtilis* laboratory strains (strains 168 and W23) significantly differ in the degree of de-*N*-acetylation (Vollmer 2008). In *B. subtilis* W23 only GlcNAc residues of the glycan backbone of murein are deacetylated (16%), whereas in *B. subtilis* 168 GlcNAc and MurNAc, are particially *N*-deacetylated (19 and 33%, respectively). Recently, peptidoglycan *O*-acetyltransferases and *N*-deacetylases have been cloned and characterized from *S. aureus, S. pneumoniae*, *B. subtilis* and *B. cereus* (Vollmer and Tomasz 2000; Fukushima et al. 2002; Bera et al. 2005, 2006; Psylinakis et al. 2005). *O*-acetyltransferases transfer the acetyl group to the C6 hydroxyl of MurNAc (Vollmer 2008), whereas the peptidoglycan *N*-deacetylases differ in their substrate specificity and deacetylate either GlcNAc or MurNAc residues or both.

PdaA of *B. subtilis* deacetylates GlcNAc, but not MurNAc residues (Fukushima et al. 2002; Blair and van Aalten 2004). It is required for the formation of muramic δ-lactam during sporulation of *B. subtilis* (Fukushima et al. 2002). The peptidoglycan of the outer, thick layer (cortex) of endospores has a characteristic structure. Every second MurNAc residue in the cortex is a muramic acid δ-lactam, which is generated by the action of PdaA and the amidase CwlD that removes the peptidyl side chain from de-*N*-acetylated MurNAc-peptides while forming an intramolecular δ-lactam ring between the free 2-amino group and the C3-lactyl substituent (Sekiguchi et al. 1995). In addition, approximately 23% of the stem peptides are present as single L-Ala and 26% have a tetrapeptide chain. Moreover, only about 3% of the cortex is crosslinked (Foster and Popham 2002). These unique features of the cortex murein are important for specific germination-specific lytic enzymes,

which cleave the cortex but leave the underlying primordial cell wall, which basically has the same structure as the vegetative cell wall, untouched (Popham et al. 1996; Makino and Moriyama 2002).

The cell wall of Gram-positives contains additional components not found within Gram-negatives. Most Gram-positive bacteria incorporate glycopolymers (CWGs) in their cell wall (Weidenmeier and Peschel 2008). Acidic polysaccharides, teichoic acids, or teichuronic acids are either membrane-anchored (lipoteichoic acids, LTA) or covalently attached to the murein sacculus (wall teichoic acids, WTA) through MurNAc-phosphodiester bonds and usually via a special linkage unit (cf. Chap. 6).

1.3.7 Mycobacterial Murein

Mycobacterium spp. produce a unique cell envelope structure which contains an outer membrane-like layer covering a complex mycolyl-arabinogalactan-peptidoglycan cell wall (Brennan and Nikaido 1995). Although the peptidoglycan within this complex is classified as the common type A1γ (Schleifer and Kandler 1972) (Fig. 1.2), it has some unique features, including the occurrence of *N*-glycolylmuramic acid (MurNGly) (Adam et al. 1969; Azuma et al. 1970; Wietzerbin-Falszpan et al. 1970). *N*-Glycolylation is a rare carbohydrate modification seen only for muramic acid in actinobacteria and for neuraminic acids (sialic acids) in eukaryotes. Only five other genera of bacteria – *Rhodococcus, Tsukamurella, Gordonia, Nocardia*, and *Micromonospora*, all belonging to the class Actinomycetales, which are closely related to mycobacteria – have *N*-glycolylmuramic acid in their peptidoglycan (Holt et al. 1994; Vollmer 2008). The glycoyl modification is introduced in the soluble, cytoplasmic precursors of murein biosynthesis (cf. Sect. 1.4). The *namH* gene encodes a UDP-MurNAc hydroxylase (monooxygenase), that oxidizes the acetamido group of the murein precursor in the presence of molecular oxygen and NADPH (Raymond et al. 2005). A *namH* mutant lacks *N*-glycolate modification and was rendered hypersensitive to lysozyme and β-lactam antibiotics. It has been hypothesized that the glycolyl (additional hydroxyl group) stabilizes the cell wall through hydrogen bonding (Brennan and Nikaido 1995). The peptidoglycan structure in the readily cultivable *M. smegmatis* and in *M. tuberculosis* contains both, *N*-glycolylated and *N*-acetylated, muramic acid residues. Other modifications include: amidation at the free carboxylic acid of DAP and D-Glu, direct crosslinkage between meso-diaminopimelic acid residues, and substitution of L-Ala by Gly in the peptide side chains (Petit et al. 1969; Azuma et al. 1970; Wietzerbin-Falszpan et al. 1973; Wietzerbin et al. 1974; Salton 1994; Petit et al. 1975; Phiet et al. 1976). In contrast, the peptidoglycan from in vivo derived non-cultivable *Mycobacterium leprae* has recently been shown to contain murein, which is exclusively *N*-acetylated, but carries glycine residues instead of D-Ala in the uncrosslinked peptide side chains (Draper et al. 1987; Mahapatra et al. 2008).

1.3.8 Cell Wall-less Bacteria and L-Forms

Under certain environmental conditions (including iso-osmotic mileu) some bacterial species, e.g. intracellular pathogens, can do without a murein layer. No β-lactamase activity was found in *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Planctomycetes,* which indicates the lack of murein (Claus et al. 2000). The Mollicutes (*Spiroplasma, Mycoplasma*, and *Acholeplasma*) are eubacteria derived from *Clostridia* by regressive evolution and genome reduction to produce the smallest and simplest self-replicating cells. They are characterized by a complete lack of a cell wall (Balish and Krause 2006; Trachtenberg 2006). A group of free-living bacteria that are abundant in aquatic habitats, which lack murein, are the Planctomycetes. They are morphologically different from other bacteria and very likely have secondarily lost their murein during evolution. The existence of murein in Chlamydiae has been debated for several years. The fact that Chlamydiae are susceptible to β-lactam antibiotics (and other antibiotics acting on the cell wall), but no muramic acid or murein could be detected is known in the literature as the chlamydial anomaly (Fox et al. 1990; Moulder 1993; Ghuysen and Goffin 1999). Penicillin binding proteins have been identified in Chlamydiae, all of which are monofunctional amidases but no gene involved in glycosyl transfer was recognized (Claus et al. 2000; McCoy and Maurelli 2005, 2006; Pavelka 2007). Recently, genome analyses revealed that a nearly complete set of murein biosynthetic genes exists, most of which were shown to be functional in vitro and expressed in Chlamydiae (Griffiths and Gupta 2002; McCoy et al. 2003; Skipp et al. 2005). Hence, some kind of murein layer very likely exists in Chlamydiae and it has been speculated that this layer might be solely composed of crosslinked peptides, which would explain the absence of glycosyl transferases in these organisms (Ghuysen and Goffin 1999).

Cell wall-less forms of bacteria, so-called L-forms, can be generated by treatment with penicillin and propagation under osmoprotected conditions. were first isolated in the 1930s by Emmy Klieneberger, and characterized as small, mycoplasma-like, spherical, and osmosensitive cells that grew only on plates of hypertonic complex medium. Unstable and stable forms can be distinguished by their property to return (revert) to their original morphology. Recently evidence has been provided that unstable, revertable L-forms might contain at least small amounts of murein required for cell division (Casadesus 2007).

1.4 Murein Biosynthesis

Murein biosynthesis is almost identical in Gram-positive and Gram-negative bacteria and occurs in three different cellular compartments: (1) the cytoplasm, where the soluble nucleotide precursors (UDP-GlcNAc and UDP-MurNAc-peptides, also known as “Park’s nucleotides”) are synthesized, (2) the cytoplasmic membrane, where lipid-linked intermediates (lipid I, lipid II) are generated at the inner surface of the cell membrane and translocated (lipid II) to the outer surface of the

cell membrane, and (3) the extracellular or periplasmic compartment, where the membrane-bound disaccharide-peptide units of lipid II are polymerized and cross-linked by murein synthases. Here we provide a brief overview of murein biosynthesis (see also Fig. 1.4). For more detailed information we refer to recent review articles (Archibald et al. 1993; van Heijenoort 1994; Höltje 1998; van Heijenoort 1998, 2001a, b, 2007; Barreteau et al. 2008; Bouhss et al. 2008; Sauvage et al. 2008; Vollmer and Bertsche 2008).

1.4.1 Peptidoglycan Precursor Synthesis

In the cytoplasm and on the inner side of the cytoplasmic membrane the assembly of the peptidoglycan precursors takes place in four stages of reactions:

1. Formation of UDP-GlcNAc (by GlmS; GlmM; GlmU)
2. Formation of UDP-MurNAc (by MurA (MurZ) and MurB)
3. Formation of UDP-MurNAc-peptides (by MurC to MurF)
4. Formation of lipid-linked murein precursors (by MraY (MurX), and MurG).

Formation of UDP-GlcNAc. UDP-GlcNAc is synthesized from fructose-6-phosphate by four enzymes (van Heijenoort 2001b; Barreteau et al. 2008). Glucosamine-6-phosphate synthase (GlmS), the first enzyme in the biosynthesis of UDP-GlcNAc, catalyzes the conversion of D-fructose-6-phosphate to D-glucosamine-6-phosphate, using L-glutamine as the source of ammonia but not exogenous ammonia (Badet et al. 1987). GlmS is a bifunctional enzyme that carries an N-terminal aminotransferase (glutaminase) and a C-terminal isomerase domain. Ammonia produced by glutamine hydrolysis is trapped by the first domain and channeled to the isomerase domain, where it acts as a nucleophile in a Schiff base reaction (Teplyakov et al. 1999, 2001, 2002; Mouilleron and Golinelli-Pimpaneau 2007; Mouilleron et al. 2008). We should mention here that the GlmS-catalyzed reaction is the central, rate-determining step and branching point of amino sugar metabolism in many organisms. Hence, it is tightly controlled and subject to interesting regulatory features that include riboswitches (*B. subtilis*) and small regulatory RNAs (*E. coli*) (Winkler et al. 2004; Kalamorz et al. 2007; Reichenbach et al. 2008; Urban and Vogel 2008). Phosphoglucosamine mutase (GlmM), the second enzyme, catalyzes the inter-conversion of glucosamine-6-phosphate and glucosamine-1-phosphate (Mengin-Lecreulx and van Heijenoort 1996; Jolly et al. 1999). In some Gram-positive pathogens GlmM is known as FemD, since it has been discovered as a Fem factor ("factor essential for methicillin resistance") (Berger-Bächi et al. 1992; Wu et al. 1996). GlmM is a typical hexose phosphate mutase that is activated by phosphorylation on a serine residue by glucosamine 1,6-diphosphate, the intermediate of the catalytic process. The third enzyme, GlmU, is a bifunctional enzyme that catalyzes acetyl and uridyl transfer. In a first step, GlmU acts as glucosamine-1-phosphate acetyltransferase, catalyzing the transfer of an acetyl group from acetyl-coenzyme A (AcCoA) to glucosamine-1-phosphate

(GlcN-1-P) yielding GlcNAc-1-phosphate. In a second step, GlmU acts as *N*-acetylglucosamine-1-phosphate uridyltransferase, catalyzing the transfer of UMP from UTP onto GlcNAc-1-phosphate to yield UDP-GlcNAc (Hove-Jensen 1992; Mengin-Lecreulx and van Heijenoort 1993, 1994; Brown et al. 1999).

Formation of UDP-MurNAc. UDP-MurNAc is formed in a two-step, addition-elimination mechanism by the enzymes MurA (formerly known as MurZ) and MurB (van Heijenoort 2001b; Barreteau et al. 2008). Transferase MurA catalyzes the transfer of enolpyruvate from phosphoenolpyruvate (PEP) to the 3-hydroxyl group of GlcNAc yielding UDP-GlcNAc-enolpyruvate. Thereafter the reductase MurB, a flavoprotein, converts the enolpyruvyl substituent to D-lactyl yielding UDP-MurNAc as final product. In the first half reaction of MurB, FAD is reduced to $FADH_2$ by NADPH, and in the second half reaction the vinyl enol is reduced by $FADH_2$ (Barreteau et al. 2008). MurA and MurB are essential in *E. coli* and other Gram-negative bacteria, while Gram-positive bacteria usually have two paralogs of MurA (MurA1 and A2). MurA is inhibited by the epoxide fosfomycin, a structural analog of PEP, which covalently traps the active site cysteine of MurA (Eschenburg et al. 2005).

Formation of UDP-MurNAc-peptides. The peptide chain is attached to UDP-MurNAc by an ATP-dependent stepwise addition of single amino acids catalyzed by three Mur ligases (MurC,D,E) (van Heijenoort 2001b; Barreteau et al. 2008; Vollmer and Bertsche 2008). The first amino acid (L-Ala) is added by ligase MurC and the second amino acid (D-Glu) is transferred by MurD (D-amino acid amino transferase). D-Glu is generated from L-Glu by glutamate racemase (MurI) in *E. coli*, whereas *B. subtilis* and other Gram-positive bacteria possess two glutamate racemases (RacE and RacE2/YrpC). The third amino acid, a di-amino acid (DAP in Gram-negative bacteria and most bacilli, but L-Lys in most Gram-positive bacteria, e.g., *S. aureus*), is added by ligase MurE. In some bacteria, like *B. subtilis,* the DAP residues are amidated on their free carboxyl group, prior to the action of the Mur-ligases, by an unknown mechanism (Mengin-Lecreulx and van Heijenoort 1994). In a last step, a D-Ala-D-Ala dipeptide is added by ligase MurF (Healy et al. 2000), generating positions 4 and 5 of the stem peptide, which are important for the crosslinkage reaction by transpeptidases (cf. Fig. 1.1). The dipeptide D-Ala-D-Ala, in turn, is generated by D,D-amino acid ligases (DdlA and B) (Zawadzke et al. 1991; Ellsworth et al. 1996).

In some Gram-negative bacteria like *E. coli* there is a fifth, but nonessential murein ligase, Mpl (Mengin-Lecreulx et al. 1996). Mpl is active in the murein recycling pathway (see below) and adds the intact tripeptide L-Ala-iso-D-Glu-DAP, which is released from cell wall turnover products, onto UDP-MurNAc (Park and Uehara 2008).

Formation of lipid-linked murein precursors. The formation of the lipid-linked intermediates begins with the transfer of the phospho-MurNAc-pentapeptide moiety of the cytoplasmic UDP-MurNAc-penta-peptide to the membrane acceptor undecaprenyl phosphate (C55 ~ P, also known as bactoprenol phosphate) to yield undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide (lipid I) (Anderson et al. 1965; van Heijenoort 2007; Barreteau et al. 2008; Bouhss et al. 2008). This first

membrane step is catalyzed by the transferase MraY (UDP-MurNAc-Ala-iso-D-Glu-DAP/L-Lys-D-Ala-D-Ala: undecaprenyl phosphate phospho-*N*-acetyl-muramoyl-pentapeptide transferase; formerly named MurX) on the inner side of the cytoplasmic membrane (Anderson et al. 1965; van Heijenoort 2001b; Barreteau et al. 2008; Bouhss et al. 2008). Thereafter, the *N*-acetylglucosamine transferase MurG catalyzes the addition of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to lipid I yielding undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide-GlcNAc (lipid II) (Anderson et al. 1965; van Heijenoort 2001b, 2007; Barreteau et al. 2008; Bouhss et al. 2008). MurG belongs to the glycosyltransferase superfamily B (GT-B), and the crystal structure of the *E. coli* enzyme in complex with the donor substrate UDP-GlcNAc has been solved (Hu et al. 2003).

In some Gram-positive bacteria, lipid II is further modified by glycine or L-amino acids into the side chain, which is performed by a unique family of nonribosomal, peptide bond-forming enzymes that use aminoacyl-tRNA as the substrate (Fem and Bpp transferases in *S. aureus* and *E. faecalis*, respectively). Fem factors ("factors essential for methicillin resistance") had been identified by transposon mutagenesis, and their inactivation reduces methicillin resistance (Berger-Bächi et al. 1992; Stranden et al. 1997). In *S. aureus* five glycine residues are added to the ε-amino-group of the L-Lys residue at position 3 by the action of three enzymes (Navarre and Schneewind 1999). FmhB specifically adds the first glycine residues, FemA the second and third ones, and finally FemB the fourth and fifth glycine residues of peptide cross-bridges (de Jonge et al. 1993; Henze et al. 1993; Ehlert et al. 1997; Navarre and Schneewind 1999; Rohrer et al. 1999; van Heijenoort 2001b; Foster and Popham 2002).

1.4.2 Translocation of Lipid II Across the Cell Membrane

Lipid II is translocated across the hydrophobic environment of the cytoplasmic membrane by a still unknown mechanism. It had been recognized that a protein is involved in the lipid II translocation, a hypothetical flippase, and that this event is coupled with murein synthesis (van Dam et al. 2007). RodA and FtsW were proposed as possible candidates for flippase, since these membrane proteins, in association with PBPs, were found to be essential for murein synthesis and/or result in filamentation (Ishino et al. 1986; Ikeda et al. 1989; Ehlert and Höltje 1996; Boyle et al. 1997). However, very recently, MurJ (MviN) was identified as a lipid II flippase of *E. coli* in a bioinformatic approach by searching for minimal neccessity of membrane proteins (Ruiz 2008). MurJ is essential and required for murein biosynthesis. Depletion of the corresponding gene causes accumulation of lipid II and the cells show morphological defects and eventually burst. The flippase was assigned to the multi-antimicrobial extrusion (MATE) superfamily to which drug-proton symporters belong, as well as RfbX (Wzx), WzxE, and SpoVB, all membrane proteins involved in the production of extracellular polysaccharides (Inoue et al. 2008; Ruiz 2008).

1.4.3 Peptidoglycan Polymerization by Murein Synthases

The last stage of peptidoglycan biosynthesis occurs at the outside of the cytoplasmic membrane and within the periplasmic space and/or cell wall compartment. In Gram-negative bacteria, murein synthases are monofunctional glycosyl transferases (GT), bifunctional glycosyl transferase/transpeptidases (GT/TP), or monofunctional transpeptidases (TP) (Park and Matsuhashi 1984; Di Berardino et al. 1996). MtgA, the monofunctional GT of *E. coli*, is capable of polymerizing glycan strands but does not crosslink the peptides (Di Berardino et al. 1996; Vollmer and Bertsche 2008). In *B. subtilis* monofunctional GT do not exist (Foster and Popham 2002). All murein synthases have a short N-terminal cytoplasmic part and are anchored to the cytoplasmic membrane by a transmembrane region (Vollmer and Bertsche 2008). In the literature GT often are erroneously called transglycosylases; however, they catalyze a glycosyl transfer rather than a transglycosylation reaction. GT transfer the growing, lipid-linked peptidoglycan strand, having the first MurNAc activated at the reducing end (C1) by a pyrophosphate (donor), onto the C-4 carbon of the *N*-acetylglucosamine residue of lipid II (acceptor) (Archibald et al. 1993). Hence, the peptidoglycan chain grows at the reducing end, but protrudes to the cell wall with its nonreducing end (cf. Fig. 1.3) (Lovering et al. 2007; Yuan et al. 2007). Polymerization of lipid II molecules by GT yields linear glycan strands of variable length, dependent on the enzyme's preference (Yuan et al. 2007).

The β-1,4-heteroglycan strands that have been polymerized by GT are crosslinked and incorporated into the preexisting cell wall by TP, which catalyze the crosslinking between peptide subunits (Archibald et al. 1993; Höltje 1998; Navarre and Schneewind 1999; van Heijenoort 2001b; Foster and Popham 2002; Scheffers and Pinho 2005). During this reaction, undecaprenyl-pyrophosphate is released and then dephosphorylated yielding the lipid carrier bactoprenol and flipped back to serve again as a lipid II carrier (El Ghachi et al. 2005; van Heijenoort 2007). Transpeptidation is a two-step mechanism for crosslinking the glycan strands of the murein through peptide bonds. In Gram-negative bacteria and bacilli, the peptides can be crosslinked directly between the amino group of DAP at position 3 of one peptide and the carboxy terminus of the D-Ala residue at position 4 of another peptide. In most Gram-positive bacteria the peptides are crosslinked through cross-bridge peptides such as pentaglycine in *S. aureus* and D-Ala-D-Ala in *S. pyogenes* (cf. Fig. 1.2 and Sect. 1.3.2). In the first step, the bond between the dipeptide D-Ala-D-Ala is cleaved with formation of an enzyme-substrate intermediate and concomitant release of the terminal D-Ala. The second step involves the transfer of the tetrapeptidyl group to the acceptor, which is the non-α amino group of the di-basic amino acid in a second stem peptide in bacteria with direct crosslinking (Gram-negative bacteria and bacilli) or the last amino acid of the cross-bridge peptides in most Gram-positve bacteria. Enzymes with D,D-TP activity are known as penicillin-binding proteins (PBPs), since they covalently bind penicillin and other β-lactam antibiotics, which structurally resembling the substrate of the D,D-TP, the D-Ala-D-Ala motif of the stem peptide.

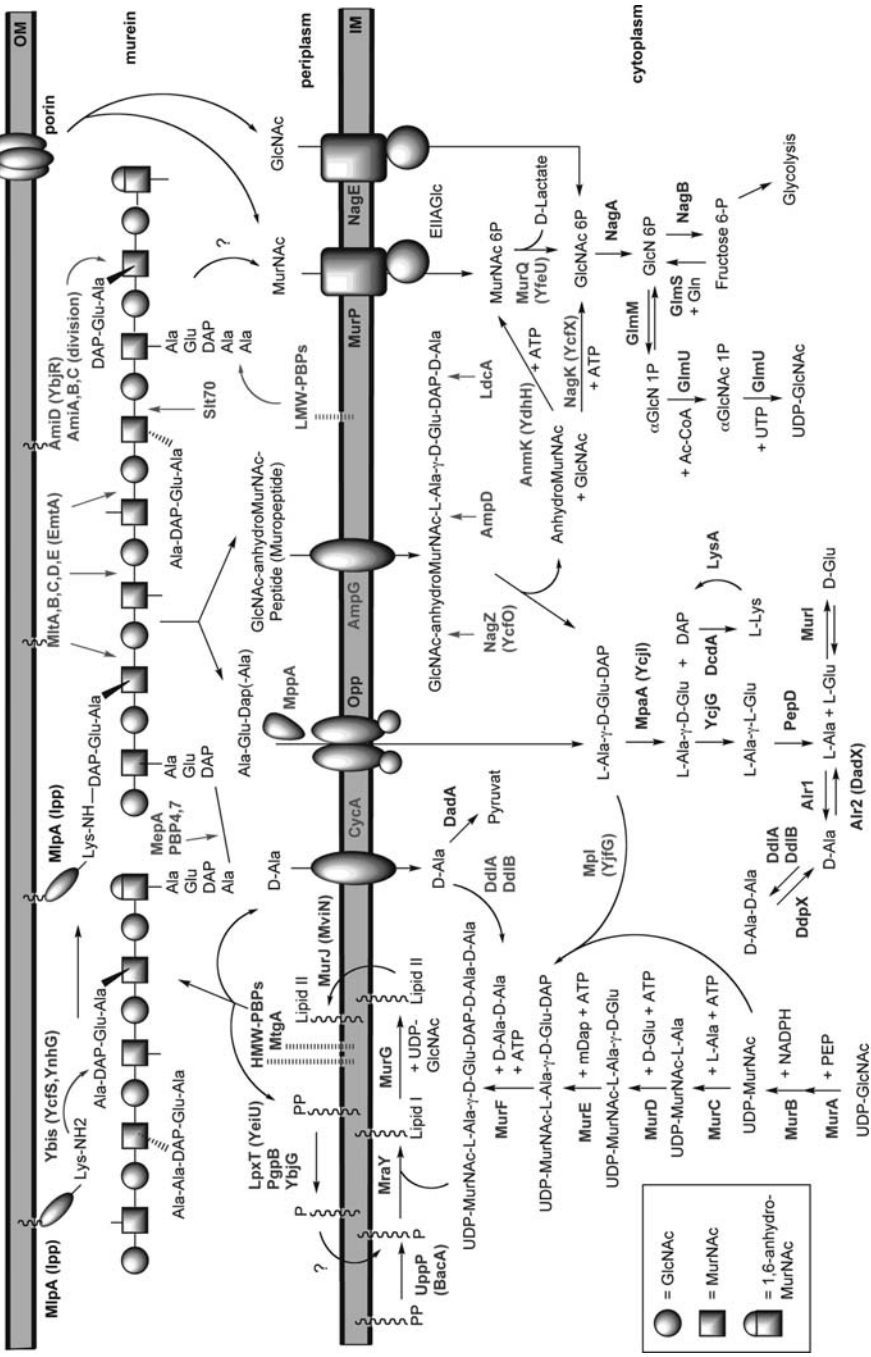

Fig. 1.3 Overview of murein biosynthesis, catabolism, and turnover/recycling in *E. coli* and interplay of the pathways. See text for more information on specific enzymes and also Table 1.2 for details on the specificity and function of high-molecular-weight penicillin-binding proteins (HMW-PBPs) and low-molecular-weight penicillin-binding proteins (LMW-PBP)

L,D-TP, another class of peptidoglycan crosslinking enzymes, were previously shown to bypass the D,D-TP activity of the classical penicillin-binding proteins (PBPs) leading to high-level cross-resistance to glycopeptide and β-lactam antibiotics (cf. Fig. 1.1) (Mainardi et al. 2002; Biarrotte-Sorin et al. 2006; Cremniter et al. 2006). L,D-TP homologues from *B. subtilis, Enterococcus faecalis* and *E. faecium* crosslink their cognate disaccharide-peptide subunits containing DAP, L-Lys-L-Ala-Ala, and L-Lys-D-iso-asparagine at the third position of the stem peptide, respectively (Dramsi et al. 2008). L,D-TP differ in their capacity to hydrolyze the tetrapeptide (L,D-carboxypeptidase activity) and pentapeptide (L,D-endopeptidase activity) stems, in addition to the common crosslinking activity. In contrast, sortases (see Chap. 9), which are enzymes that covalently bind proteins to the murein, such as StrA of *S. aureus* cleave the Thr-Gly peptide bond of the sorting signal and transfer the α-amino group of the pentaglycine to the carboxyl of the Thr side chain (L,L-TP reaction, protein sorting).

PBPs can be divided into two classes: high-molecular-weight PBPs (HMW-PBPs) and low-molecular-weight PBPs (LMW-PBPs) (Table 1.2) (Ghuysen 1991; Goffin and Ghuysen 1998). HMW-PBPs consist of two domains located in the periplasm. The C-terminal domain always has a penicillin-binding motif (penicillin-binding domain), which catalyzes the crosslinking of the peptides. HMW-PBPs can be further divided into class A and class B, depending on the activity of the N-terminal domain (Goffin and Ghuysen 1998), (Anderson et al. 1965). Class A PBPs are bifunctional enzymes and their N-terminal domain has GT activity. Hence, class A PBPs allow polymerization of glycan strands as well as formation of crosslinks between peptides. In contrast, class B PBPs are monofunctional TP with a noncatalytic periplasmic N-terminal domain whose function is unknown; presumably it participates in protein–protein interactions (Anderson et al. 1965; Terrak et al. 1999). In the murein sacculus of bacteria not all peptides are crosslinked and LMW-PBPs exist, which act as carboxypeptidases. D,D-Carboxypeptidases hydrolyze the peptide bonds between the terminal dipeptide D-Ala-D-Ala, thereby preventing crosslinking of that peptide.

The function of the PBPs has been investigated mainly in the model organisms *E. coli* and *B. subtilis* as well as pathogens (e.g., *S. aureus* and *S. pneumoniae*) through mutations in the different PBP coding genes (Table 1.2). PPB1A and PBP1B of *E. coli* are the class A murein synthases with high activity. Since they can complement each other, they are not essential for growth, but a deletion of both genes is lethal. PBP1C of *E. coli* is of minor abundance and cannot substitute PBP1A and PBP1B for cell growth. Time-course experiments of murein synthesized from radioactive lipid II precursors revealed that PBP1A, unlike PBP1B, requires the presence of polymerized glycan strands carrying monomeric peptides for crosslinking activity. PBP1A was capable of attaching nascent murein to nonlabeled sacculi by transpeptidation reactions, in which monomeric tri- and tetrapeptides in the sacculi were the acceptors (Bertsche et al. 2005; Born et al. 2006). In *B. subtilis* four class A PBPs exist. Mutation of PBP1A and PBP1B of *B. subtilis* shows slow growth, abnormal cell morphology, and formation of asymmetric sporulation septa (Scheffers and Errington 2004). Additional mutations of PBP2c and PBP4 result in further

Table 1.2 Penicillin-binding proteins (PBPs) of selected bacteria: classification and function

Species	High-molecular weight PBPs				Low-molecular weight PBPs		
	Class A (transferase/ transpeptidase)	Class B (transpeptidases)					
		Elongation	Division	Sporulation or unknown function	Carboxypeptidases	Endopeptidases	β-Lactamaseor unknown function
B. subtilis	PBP1 (*ponA*) PBP2c (*pbpF*) PBP4 (*pbpD*) PBP2d (*pbpG*)	PBP2a (*pbpA*) PbpH (*pbpH*)	PBP2b (*pbpB*)	PBP3 (*pbpC*) PBP4b (*pbpI*) SpoVD (*spoVD*)	PBP5 (*dacA*) PBP5P* (*dacB*) PBP4a (*dac*C) DacF (dacF)	PBP4* (*pbpE*) PbpX (*pbpX*)	YbbE (*ybbE*)
E. coli	PBP1a (*ponA*)	PBP2 (*pbpA*)	PBP3/FtsI (*pbpB*)		PBP5 (*dacA*)	PBP4 (*dacB*)	AmpC (*ampC*)
	PBP1b (*ponB*)				PBP6 (*dacC*)	PBP7 (*pbpG*)	AmpH (*ampH*)
	PBP1c (*pbpC*)				PBP6b (*dacD*)	MepA (*mepA*)	PBP4b (*yfeW*)
S. aureus	PBP2 (*pbpB*/*pbp2*)	PBP3 (*pbpC*)	PBP1 (*pbpA*)	PBP2a (*mecA*)	PBP4 (*pbp*D)[a]		
S.pneumoniae	PBP1a (*pbp1a*) PBP1b (*pbp1b*) PBP2a (*pbp2a*)	PBP2b (*pbp2b*)	PBP2x (*pbp2x*)		PBP3 (*dacA*)		

[a]transpeptidase activity

reduction of the growth, while these mutations alone have no effect on growth (Scheffers and Errington 2004). PBP2c and 2d, as well as 4b, have no obvious role in vegetative murein synthesis but do play a role in spore cell wall synthesis (McPherson et al. 2001; Wei et al. 2004). For an overview of the complete sets of PBPs of selected bacteria and their classification and function, see Table 1.2.

Class B PBPs of *E. coli,* PBP2, and *B. subtilis,* PBP2a and PbpH, are involved in maintenance of the cell shape during elongation, while PBP3 of *E. coli* and PBP2b of *B. subtilis* are required for cell division (Spratt 1975; Spratt and Pardee 1975; Yanouri et al. 1993; Wei et al. 2003). Interestingly, the monofunctional class B transpeptidases of *E. coli* were also shown to differ in substrate specificity; PBP3 has a preference for tripeptide and PBP2 for pentapeptide side chains (Botta and Park 1981; Pisabarro et al. 1985; Begg et al. 1990).

Recently, the structure of a bifunctional murein synthase (PBP2, the only bifunctional PBP of *Staphylococcus aureus*) including its transpeptidase (TP) and GT domains was solved in complex with the substrate analog moenomycin (Lovering et al. 2007). The GT adopts a fold distinct from those of other transferase classes and provides insights into the progessive catalytic mechanism and key interactions required for enzyme inhibition (Fig. 1.4) (Lovering et al. 2007; Yuan et al. 2007, 2008).

1.5 Localization of Murein Synthesis and Morphogenesis

The characteristic shape of bacteria has traditionally been attributed to the cell wall. Undoubtedly, the exoskeleton-like murein is responsible for maintaining the characteristic form of the cell. However, it has become evident in recent years that other factors in turn direct the murein biosynthesis machinery during cell expansion and division, thereby controlling the shape of the sacculus. Insertion of new murein into the existing sacculus has to be precisely regulated in space and time during the bacterial cell cycle to generate two new daughter cells of defined cell shape. In rod-shaped bacteria, longitudinal growth, by which a regular cylindrical surface is generated (elongation), needs to alternate with constrictive growth, by which a transversal wall at the cell center is formed that further develops into new cell poles (cell division).

There are many reports in the literature of mutant strains impaired in cell morphology. Mostly, these changes affect conversion of a rod into a coccus (there is no report of a morphological change the other way around), formation of short rods or minicells, and the occurrence of unseparated filamentous cells or chains of cells (Goodell and Schwarz 1975; Nanninga 1998; den Blaauwen et al. 2008; Zapun et al. 2008b). These findings indicated very early that cell elongation and division are separate events, which are controlled by a range of delicately balanced factors (cf. (Höltje 1998; Nanninga 1998)). Mutations affecting cell shape frequently locate in genes involved in cell wall synthesis; however, in addition, morphogenes were identified which have no obvious connection to murein

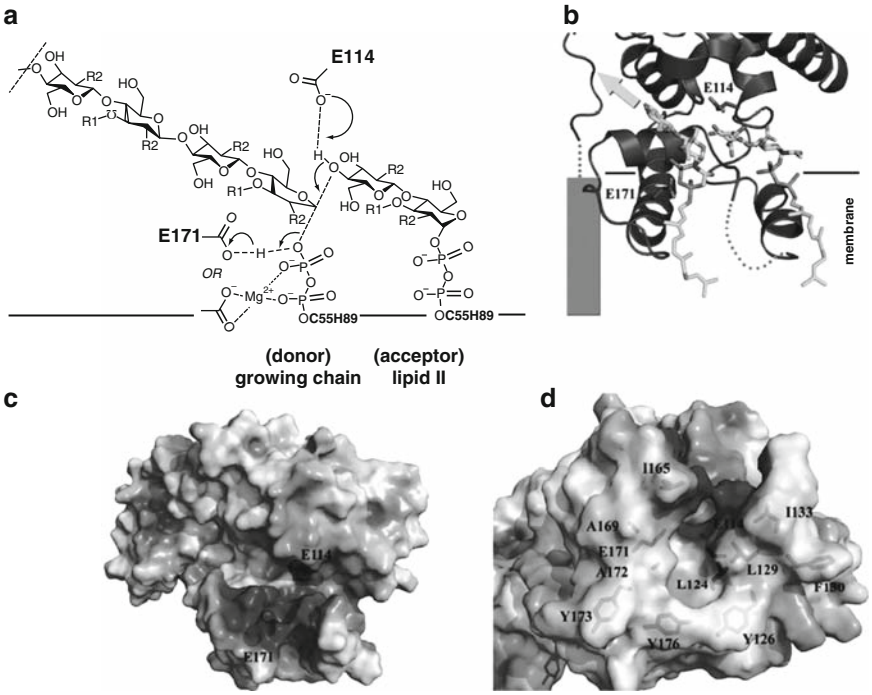

Fig. 1.4 Structure of the bifunctional GT/TP of *Staphylococcus aureus* (PBP2) and proposed mechanism for lipid II polymerization by the GT domain. To simplify these diagrams, the peptide substituents on lipid II have been omitted. (**a**) Schematic for lipid II polymerization. For clarity, R1 and R2 groups are used in place of the *O*-lactyl and NHAc groups, respectively. In this model, lipid II is the acceptor (*right side*), and the growing glycan chain is the donor (*left side*); both are bound via undecaprenyl ($C_{55}H_{89}$) pyrophosphate. Residue E114 acts to deprotonate the acceptor 4-OH group, which concomitantly attacks C1 of the donor, in an SN2-like reaction that inverts the α-linked precursors into a β-1,4-linked product. Residue E171 may assist this process by direct protonation of the phosphate-sugar bond or by stabilizing the pyrophosphate group through interaction with a divalent cation. (**b**) Spatial representation of the lipid II polymerization model. The membrane interface (*horizontal black line*), transmembrane region (*vertical blue rectangle*), and missing polypeptide in the structure (*dotted blue line*) are shown for effect. The protein structure is unmodified from the moenomycin-bound complex, with the growing glycan-chain donor (*left side*) modeled over the moenomycin structure and the lipid II acceptor (*right side*) fitted manually between the glycan-chain donor and the E114 catalytic residue (shown with E171 in stick form). After polymerization, the product would be translocated in the direction denoted by the *yellow arrow*. (**c**) Detail of active-site pockets and cleft. Residues E114 and E171 are shown in space-filling form. The electrostatic potentials (*red*, negative; *blue*, positive) indicate a conserved region of positive charge across the middle of the pocket. This region binds the phosphoric acid diester group of the moenomycin structure and is located in a position to bind both pyrophosphates in the substrate model. (**d**) Details of the hydrophobic platform of the GT fold presumably interacting with the membrane. The view is approximately 90° from (**c**), with residues shown in stick form. *Green*, hydrophobic platform; *gray*, E114 and E171. The picture was taken from Lovering et al. (2007). Reprinted with permission from AAAS

synthesis (Normark 1969; Matsuzawa et al. 1973; Lutkenhaus et al. 1980; Aldea et al. 1988). Round phenotypes can be generated by inhibition of a range of enzymes or mutations/deletions of the corresponding genes (rod mutants), e.g., *rodA* and *mreD* (*rodB*), *mreC* (and *pbp2),* or by overexpression (e.g., *bolA*) (Matsuzawa et al. 1973; Spratt and Pardee 1975; Wachi et al. 1987, 1989; Aldea et al. 1988; Varley and Stewart 1992; Henriques et al. 1998; de Pedro et al. 2001). These proteins are involved in the organization of murein synthesis during elongation. Moreover, "filamentation thermosensitive mutants" (*fts* mutants) were isolated that exhibited normal cell morphology at low temperature, but grew as elongated cells upon shift to 42°C (Pagès et al. 1975; Ishino et al. 1989). These proteins are localized at midcell and are involved in cell division (den Blaauwen et al. 2008).

1.5.1 Bacteria Cytoskeletal Elements Direct Murein

In the late 1990s prokaryotic actin and tubulin homologs (Mbl, MreB, and FtsZ, respectively) were discovered among bacterial morphogenes (Erickson 1995; Löwe and Amos 1998; van den Ent et al. 2001a). High-resolution fluorescence microscopy using GFP-fusion proteins allowed the identification of their cell cycle-dependent distribution within living cells (see recent reviews: van den Ent et al. 2001b; Errington 2003; Young 2003; Cabeen and Jacobs-Wagner 2005; Scheffers and Pinho 2005; Cabeen and Jacobs-Wagner 2007; Carballido-Lopez and Formstone 2007; Osborn and Rothfield 2007; den Blaauwen et al. 2008; Zapun et al. 2008b).

The involvement of actin-like cytoskeletal elements (MreB and its paralogs Mbl and MreBH) in cell shape determination and localization of the peptidoglycan synthesis machinery were first recognized in *B. subtilis* and later in other bacteria (Jones et al. 2001; Daniel and Errington 2003; Shih et al. 2003; Figge et al. 2004). They form dynamic helical structures just beneath the cytoplasmic membrane extending from pole to pole in rod-shaped cells as analyzed by fluorescence microscopy using GFP-fusions (Jones et al. 2001; Daniel and Errington 2003; Errington et al. 2003). Intriguingly, orthologs of MreB have not been found in most genomes of coccoid bacteria (Zapun et al. 2008b). Thus, the absence of a cylindrical sidewall in cocci may be caused by the absence of an actin-like cytoskeleton that directs murein synthesis towards longitudinal growth. MreB (Gram-negative rods) and Mbl (Gram-positive rods) are essential for murein synthesis along the lateral wall, and hence for the rod shape of the cell. They colocalize with further morphogenetic proteins and cell wall synthesizing enzymes (Cabeen and Jacobs-Wagner 2005, 2007; Scheffers and Pinho 2005).

Widely distributed in bacteria, both in rod shaped and coccoid cells, is the tubulin homolog FtsZ (Goehring and Beckwith 2005). FtsZ polymerizes in a GTP-dependent fashion and builds a highly dynamic ring-like structure in the cell center. The formation of the Z-ring is the first step towards cell division and is required for the localization of other components, which assemble into the divisome at the cell center (den Blaauwen et al. 2008).

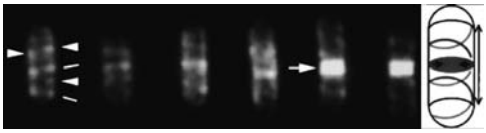

Fig. 1.5 Staining of *B. subtilis* cells with a fluorescent vancomycin derivative that binds nascent murein (lipid II) reveals different growth modes. (**a**) *Lines* and *arrowheads* indicate tilted bands and peripheral dots, respectively, that are characteristic of a helical mode staining. The *arrow* points to a densely stained region representing a division site. The staining pattern suggest two modes of cell wall growth, lateral insertion along a helical pattern of the side wall and localized growth at mid-cell, which is schematized in (**b**). *Gray lines* and *ovals* show the sites of nascent wall synthesis during cell elongation and division. *Light* and *dark gray* provide perspective, with *dark gray* at the front of the cell and *light* at the back. *Arrows* show the directions of cell elongation or division driven by the wall synthesis. Reprinted from Daniel and Errington (2003) with permission of Elsevier

The murein insertion into the cell wall of growing *Bacillus* cells was visualized by an elegant method using fluorescent derivatives of the peptidoglycan-binding antibiotics such as vancomycin and ramoplanin, which label the externalized but unincorporated murein precursor lipid II and nascent murein (Daniel and Errington 2003; Tiyanont et al. 2006). Helical staining patterns could be visualized along the cylindrical wall of *B. subtilis* cells during elongation as well as at mid-cell during cell division, under conditions minimizing the inhibitory effects of the antibiotics (Fig. 1.5). No staining, however, was observed at the cell poles, which is consistent with the observation that cell wall synthesis and turnover at the cell poles is absent or very low (Mobley et al. 1984). Whether the helical pattern is directly influenced by the Mbl/MreB cytoskeleton is at present controversial and the mechanisms controlling when and where new peptidoglycan is deposited are far from being understood. However, it is presumed that the murein synthesis complex is directed by the bacterial cytoskeleton including Mbl/MreB, which forms helical cables within cells, and FtsZ, which forms a contractile ring structure at mid-cell. The discovery of bacterial cytoskeleton elements and the visualization of their distribution and colocalization with murein synthases has revolutionized our view of morphogenesis of bacteria and has opened an exciting new field of microbial cell biology.

1.5.2 Interaction with Murein Synthases and Assembly of Elongase and Divisome Complexes

It is an appealing view that the external assembly of murein is directed by the interactions of cytoskeleton elements functioning as scaffolds for the murein synthesis machinery. The elongation of rod-shaped bacteria is dependent on a multienzyme complex named elongase that inserts murein at discrete sites, while using the actin cytoskeleton as a tracking device (den Blaauwen et al. 2008). Upon initiation of cell division a switch from dispersed to localized murein synthesis occurs at mid-cell. The accumulation of the tubulin homolog FtsZ at mid-cell

initiates the assembly of a multienzyme complex of division, the divisome, and forms a contractile ring structure that drives inward synthesis of murein and invagination of the membrane, generating a septum that matures into new hemispherical poles (den Blaauwen et al. 2008). In *E. coli* PBP1b and PBP3 are associated with the divisome, whereas PBP1a and PBP2 are connected with the elongase (Spratt 1975; Spratt and Pardee 1975). However, a clear assignment to either of the complexes is not absolute and the proteins can substitute for each other. Colocalization of the complete set of PBPs expressed during vegetative growth of *B. subtilis* and the cytoskeletal elements was analyzed by fluorescence microscopy using green fluorescent protein (GFP) fusions (Scheffers et al. 2004; Scheffers and Pinho 2005; Scheffers 2007). PBP1 and PBP2b were found to localize specifically to the septum indicating distinctive roles in division, whereas PBP2a, PbpH, PBP3, and PBP4a localize specifically to the lateral wall (for the complete set of PBPs of selected bacteria and their function see Table 1.2). The results further implicate PBP5 and possibly PBP4 in lateral wall growth. Localization of PBPs to the septum was found to be strictly dependent on FtsZ, but the GFP-PBP fluorescence patterns were not detectably altered in the absence of MreB or Mbl (Scheffers et al. 2004).

Near the end of the cell cycle a septum is formed at the cell division plane. Two new poles are generated that differ from the old poles of the previous round of cell division. Old poles have been found to be more static than new poles with respect to cell wall assembly and turnover (de Pedro et al. 1997). In rod-shaped bacteria such as *E. coli* and *B. subtilis* there is not much turnover of the murein of the poles during growth, hence the polar caps are basically inert. This is in sharp contrast to *Corynebacterium sp.* and streptomycetes, which predominantly grow at the cell pole or hyphal tip, respectively. New cell wall material is incorporated at the tip as demonstrated by fluorescently labeled nascent murein (Schwedock et al. 1997; Daniel and Errington 2003). It is assumed that murein is inserted in a flexible form, gets stretched by the turgor pressure that is the driving force for growth, and becomes more rigid further back from the tip (Flardh 2003).

In contrast to rod-shaped bacteria, cell wall synthesis in spherical cocci only takes place at the division site. Ovoid cells, e.g. streptococci, synthesize the cell wall at the septum and the so-called “equatorial ring” (Scheffers and Pinho 2005). Interestingly, FtsZ is absent from members of the phyla Chlamydiae and Planctomycetes (Pilhofer et al. 2008). Their last common ancestor likely possessed an FtsZ-based cell division mechanism and the organisms may have shifted independently to a non-FtsZ-based cell division mechanism (Pilhofer et al. 2008).

1.6 Murein Remodeling, Turnover, and Recycling

Despite being a rigid, stabilizing structure, the murein sacculus is a highly dynamic compartment that is continuously remodeled, degraded, and resynthesized. Thereby it responds to changes in the environment by modulating the protective function of the cell wall. Surprisingly, cell growth and division is accompanied by a massive

release of cell wall fragments (muropeptides). Lysis of the endogenous cell wall involves a range of autolytic enzymes that target virtually every covalent linkage that connects the building blocks within the murein structure (i.e., *N*-acetylglucosaminidases, lysozyme-like *N*-acetylmuramidases, lytic transglycosylases, L-Ala-muramoyl amidases, D,D- and L,D-endo- and carboxypeptidases). The clear assignment of physiological function to most autolysins is a difficult task, since these enzymes are highly redundant and hence difficult to assess with genetic methods. Compared to the biosynthesis of the murein sacculus, its breakdown (turnover) and the reutilization of the turnover products (recycling) are far less understood. Here we will summarize the current knowledge of cell wall turnover and recycling of *E. coli*. An overview of these pathways and the connection to cell wall biosynthesis is given in Fig. 1.3. The autolytic system of Gram-positive bacteria will be described in Chap. 16 of this volume. For further details we also refer to an excellent recent review on cell wall recycling (Park and Uehara 2008).

1.7 Turnover

In 1985, Goodell reported the unexpected finding that *E. coli* breaks down half of its murein in each generation and reutilizes the turnover products (Goodell 1985). The reason why there is such a massive degradation of the endogenous cell wall during growth of *E. coli* is still mysterious. Apparently, a delicately balanced system of synthetic and lytic activities is required to maintain a functional sacculus (cf. Sect. 1.2.4). The turnover of the murein sacculus might be an intrinsic consequence of the mechanism of cell wall growth, involving a multienzyme complex that links murein synthases and autolysins. Indeed, some interactions of lytic enzymes (Slt70, MltA and MltB) and murein synthases (PBP1A, PBP1B, PBP2, PBP3) have been demonstrated (Vollmer and Bertsche 2008).

The autolytic system of *E. coli* includes one soluble (Slt70) and five membrane-anchored lytic transglycosylases MltA, B, C, D, E (EmtA), which are lipoproteins and are located at the inner leaflet of the outer membrane. EmtA is an endo-specific lytic transglycosylase, whereas Slt70, MltA, and MltB are exo-enzymes that act from the reducing end, which carries a 1,6-anhydroMurNAc moiety (Vollmer and Bertsche 2008). Lytic transglycosylases cleave the glycan strand of murein between MurNAc and GlcNAc, like lysozyme, but do not catalyze a hydrolysis reaction. Instead they catalyze an intramolecular transglycosylation reaction, in which the 6-OH group of the MurNAc residue attacks its own reducing end, generating a glycan strand that terminates with 1,6-anhydroMurNAc (Fig. 1.6). Moreover, *E. coli* contains five *N*-acetymuramoyl-L-Ala amidases, i.e., three periplasmic enzymes (AmiA, B, C), one lipoprotein (AmiD), which is anchored to the inner face of the outer membrane, and one cytoplasmic enzyme AmpD, which has a substrate preference for 1,6-anhydroMurNAc-peptides. The three peptidoglycan-endopeptidases of *E. coli* all contribute to formation of the septum and separation of the newly formed daughter cells (PBP4, PBP7, and MepA). The penicillin-insensitive

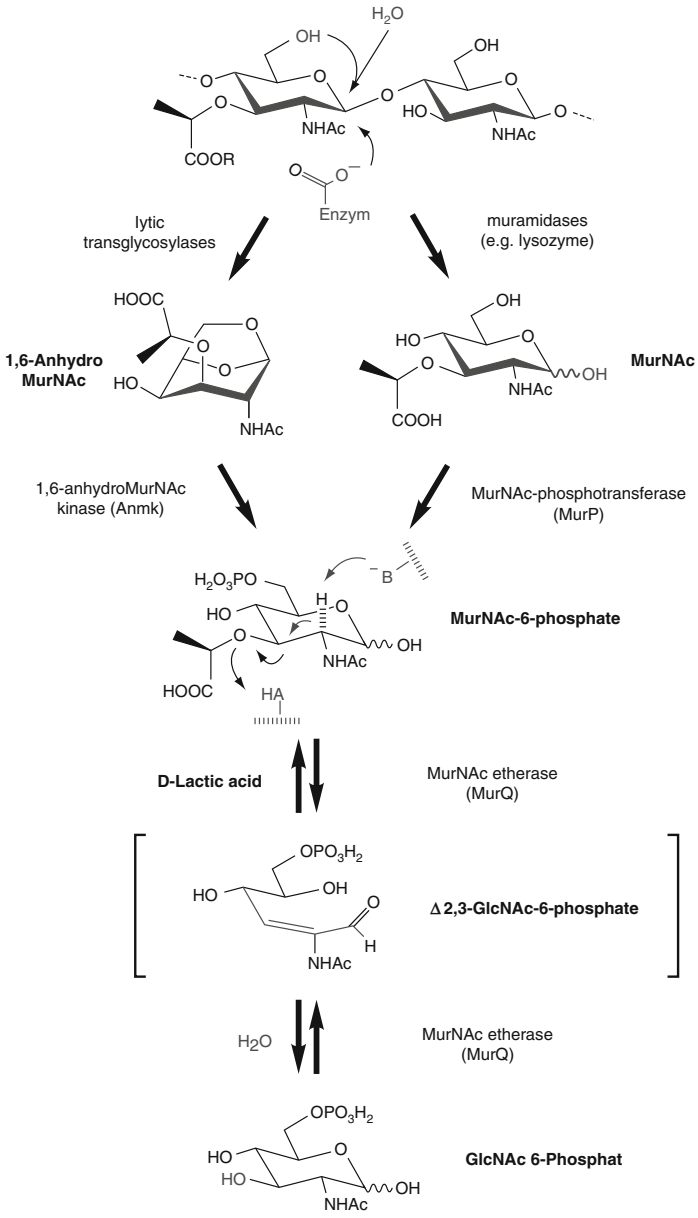

Fig. 1.6 Turnover/recycling pathways of the cell wall sugars 1,6-anhydroMurNAc and MurNAc, which are generated by cleavage of the murein by lytic transglycosylases and lysozyme, respectively. The sugars are phosphorylated at 6-OH by an anhydroMurNAc-specific kinase (AnmK) and a MurNAc-specific phosphotransferase system (MurP). The lactyl ether substituent of the product, MurNAc-6-phosphate, is hydrolyzed by MurNAc etherase (MurQ), which catalyzes an lyase-type mechanism, yielding GlcNAc-6-phosphate and D-lactic acid and involving the formation of an α,β-unsaturated sugar intermediate (Jaeger et al. 2005; Hadi et al. 2008; Jaeger and Mayer 2008a)

MepA hydrolyzes both D,D,-and L,D-crosslinks (Vollmer and Bertsche 2008). D,D-Carboxypeptidases (PBP5, 6, 6B) capable of removing C-terminal D-amino acids are PBPs of so far unknown function (cf. Table 1.2). However, it can be assumed that they antagonize D,D-transpeptidases, thereby controlling crosslinking.

Some of the last proteins to be recruited to the Z-ring in *E. coli* are the peptidoglycan-hydrolyzing enzymes AmiC and EnvC (Bernhardt and de Boer 2003, 2004). These enzymes digest the PGN layers connecting two recently developed daughter cells. EnvC is a metallo-endopeptidase related to lysostaphin, an enzyme that degrades staphylococcal murein by cleaving the interpeptide bridges at Gly–Gly bonds (Bernhardt and de Boer 2004). Intriguingly, *E. coli* does not carry glycine peptide bridges; however, muropeptides carrying glycine have been detected in *E. coli* although their role and distribution within the murein is unknown (Höltje 1998).

1.7.1 Recycling

The massive turnover of about 50% of the endogenous cell wall was long overlooked, because the turnover products, at least in *E. coli*, are not lost in the medium, but are efficiently reutilized (cf. Fig. 1.3). The tripeptide (L-Ala-iso-D-Glu-DAP) can be taken up by the general oligopeptide ABC-transporter Opp recruiting a specific periplasmic binding protein MppA (Park 1993; Park et al. 1998). The intact tripeptide is shuttled to the murein biosynthesis pathway but also might be degraded to single amino acids, dependent on the metabolic requirement. The major recycling pathway, however, involves the uptake of 1,6-anhydroMurNAc-containing peptidyldisaccharides (muropeptides) by the secondary transporter AmpG (Jacobs et al. 1994; Cheng and Park 2002). They are released from the murein by membrane bound (Mlt) or soluble (Slt70) periplasmic lytic transglycosylases (Fig. 1.6) (Höltje 1996b; Scheurwater et al. 2007). The muropeptides are further hydrolyzed in the cytoplasm by NagZ, a β-*N*-acetylglucosaminidase (Cheng et al. 2000; Vötsch and Templin 2000), AmpD, an anhydro-MurNAc-L-Ala amidase (Höltje et al. 1994; Jacobs et al. 1994, 1995), and LdcA, a L,D-carboxypeptidase (Templin et al. 1999), yielding amino sugars and peptides/amino acids. In the recent years, the recycling pathway of the amino sugars has been elucidated. This pathway involves the phosphorylation of GlcNAc and 1,6-anhydroMurNAc by specific kinases (NagK, AnmK) (Uehara and Park 2004; Uehara et al. 2005) and the conversion of MurNAc-6-phosphate to GlcNAc-6-phosphate by an etherase that cleaves off the D-lactic acid residue of MurNAc-6-phosphate (Jaeger et al. 2005; Uehara et al. 2006; Jaeger and Mayer 2008a, b). The etherase of *E. coli* MurQ was shown to catalyze a lyase-type mechanism that acts by syn-elimination of D-lactic acid yielding an α,β-unsaturated aldehyde with E-stereochemistry and a syn-addition of water to form the final product, GlcNAc-6-phosphate (cf. Fig. 1.6) (Jaeger et al. 2005; Hadi et al. 2008). GlcNAc-6-phosphate is deacetylated by NagA and the product glucosamine-6-phosphate either enters glycolysis (upon deamination by NagB yielding

fructose-6-phosphate) or the biosynthesis pathway upon isomerization via GlmM and activation by GlmU, which generates UDP-GlcNAc, the precursor for murein synthesis (Fig. 1.3) (Mayer and Boos 2005).

Recycling is not essential under laboratory conditions, and since murein represents only about 2% of the cell mass of Gram-negative bacteria, the benefit in terms of energy recovery is rather limited. Therefore, keeping the enzymes of the recycling pathway must provide some other advantages for the cell. It has long been speculated that the murein turnover and recycling pathway and the muropeptides generated by these processes might have crucial functions in the regulation of physiological processes, e.g., the initiation of cytokinesis, stationary phase or dormant state entry. Recently, evidence has been provided that muropeptides can be sensed by the cell involving two-component regulatory systems and serine/threonine kinase that carry putative murein binding, so-called PASTA domains (penicillin-binding protein and serine/threonine kinase associated domains) (Yeats et al. 2002; Jones and Dyson 2006; Palmer and Stoodley 2007; Fiuza et al. 2008). The demonstration of a regulatory function of muropeptides was provided earlier by the elucidation of the induction of a chromosomal β-lactamase (AmpC) in enterobacteria (Jacobs et al. 1997; Kraft et al. 1999; Uehara and Park 2002). Uptake and accumulation of a "wrong" turnover product (anhydroMurNAc-pentapeptide instead of anhydroMurNAc-tripeptide), upon inactivation of PBPs by β-lactam antibiotics, resulted in the AmpR-dependent activation of AmpC (Park and Uehara 2008). Hence, bacteria are able to sense the state of their cell wall through recycling products (Park 1995).

Turnover has been reported in some Gram-positive bacteria. *Bacillus* sp. and *Lactobacillus* sp. degrade their endogenous wall to an extent similar to that of *E. coli* (Mauck and Glaser 1970; Chaloupka and Kreckova 1971; Mauck et al. 1971; Boothby et al. 1973; Daneo-Moore et al. 1975; Doyle et al. 1988). However, it is so far unclear whether the turnover products are reutilized. Many enzymes dedicated to the process of scavenging the murein turnover products have been conserved in bacteria (cf. Table 1.2 and supplementary material of Park and Uehara 2008). However, some differences occur, e.g., the utilization of MurNAc through an etherase is restricted to only about half of the bacterial species (Jaeger and Mayer 2008a). However, we have recognized the presence of orthologs of the MurNAc etherase of *E. coli,* particularly in Gram-positives (Jaeger and Mayer 2008a). Hence, a recycling or rescueing pathway for muropeptides very likely exists in these organisms.

1.8 Conclusions

A renewed interest in the structure and dynamics of the bacterial cell wall has emerged from recent discoveries in cutting-edge fields of molecular microbiology. The cell wall and especially its main stabilizing component, the murein or peptidoglycan, is involved in a range of important features of prokaryotic cells, for instance: (1) cell cycle control, cell division, and morphology – involving the newly discovered components of the divisome and the bacterial cytoskeleton, (2)

cellular heterogeneity, biofilm formation, and multicellular (social) behavior – involving newly discovered signaling molecules and cascades that control cell differentiation and motility, and (3) starvation response, dormant state entry, and resuscitation – involving novel autolytic pathways and complex regulatory circuits, which are close to being understood. Despite an array of new discoveries within these fields, fundamental problems are still unanswered. The goal will be the understanding, as a whole, of how the cell wall is modified and remodeled dependent on the physiological state of the cell and the environmental conditions.

Moreover, murein and, in particular, small muropeptides derived from it are potent biological effectors and major pathogen-associated molecular "patterns". They are recognized by the host innate immune system and are associated with a range of stimulatory activities in man such as adjuvanticity, activation of macrophages, cytotoxicity, induction of arthritis, or modulation of slow-wave sleep (recently reviewed in Boneca 2005; Cloud-Hansen et al. 2006; Chaput and Boneca 2007). It has been known for a long time that muramyl-dipeptide (MDP; MurNAc-L-Ala-iso-D-Gln) is an important immuno-stimulatory compound present in Freund's adjuvant. More recently, human cytosolic receptors (NOD, nucleotide-binding oligomerization domain proteins) were identified that function as sensors for muropeptides. NOD1 recognizes DAP-containing cell wall fragments (e.g., MurNAc-L-Ala-iso-D-Glu-DAP) derived from Gram-negative bacteria with iso-D-Glu-DAP being the minimal recognition structure. NOD2 is a sensor for MurNAc-dipeptide (MDP) derived from Gram-positive bacteria (Girardin et al. 2003a, b, c). Depending on the bacterial species considered, murein fragments are either released in the growth medium or are efficiently reutilized for cell wall synthesis (i.e., recycling). In general, microbial pathogens have developed sophisticated strategies to evade or modulate the host's defense system. Hence, they should also optimize or modify cell wall turnover/recycling upon interaction with (and growth within) human cells, to minimize the release of proinflammatory muropeptides.

Last but not least, the murein remains a primary target for antibiotics, and the recent progress in elucidation of structures of murein synthases may led to the discovery of novel classes of antibiotics that target so far unexplored reactions in murein metabolism. Despite being known for 50 years now, the bacterial murein sacculus remains an interesting research topic and will keep us excited for quite some time.

References

Adam A, Petit JF, Wietzerbin-Falszpan J, Sinay P, Thomas DW, Lederer E (1969) L'acide *N*-glycolyl-muramique, constituant des parois de *Mycobacterium smegmatis*: identification par spectrometrie de masse. FEBS Lett 4:87–92

Aldea M, Hernandez-Chico C, de la Campa AG, Kushner SR, Vicente M (1988) Identification, cloning, and expression of *bolA*, an *ftsZ*-dependent morphogene of *Escherichia coli*. J Bacteriol 170:5169–5176

Anderson JS, Matsuhashi M, Haskin MA, Strominger JL (1965) Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide: presumed membrane transport intermediates in cell wall synthesis. Proc Natl Acad Sci USA 53:881–889

Araki Y, Nakatani T, Nakayama K, Ito E (1972) Occurrence of *N*-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls from *Bacillus cereus*. J Biol Chem 247:6312–6322

Arbeloa A, Hugonnet JE, Sentilhes AC, Josseaume N, Dubost L, Monsempes C, Blanot D, Brouard JP, Arthur M (2004) Synthesis of mosaic peptidoglycan cross-bridges by hybrid peptidoglycan assembly pathways in Gram-positive bacteria. J Biol Chem 279:41546–41556

Archibald AR, Hancock IC, Harwood CR (1993) Cell wall structure, synthesis, and turnover. In: Sonenshein AL, Hoch JA, Losick R (eds) *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology, and molecular genetics. ASM Press, Washington, DC, pp 381–410

Atrih A, Bacher G, Allmaier G, Williamson MP, Foster SJ (1999) Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. J Bacteriol 181:3956–3966

Azuma I, Thomas DW, Adam A, Ghuysen JM, Bonaly R, Petit JF, Lederer E (1970) Occurrence of *N*-glycolylmuramic acid in bacterial cell walls. A preliminary survey. Biochim Biophys Acta 208:444–451

Badet B, Vermoote P, Haumont PY, Lederer F, LeGoffic F (1987) Glucosamine synthetase from *Escherichia coli*: purification, properties, and glutamine-utilizing site location. Biochemistry 26:1940–1948

Balish MF, Krause DC (2006) *Mycoplasmas*: a distinct cytoskeleton for wall-less bacteria. J Mol Microbiol Biotechnol 11:244–255

Barreteau H, Kovac A, Boniface A, Sova M, Gobec S, Blanot D (2008) Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol Rev 32:168–207

Begg KJ, Takasuga A, Edwards DH, Dewar SJ, Spratt BG, Adachi H, Ohta T, Matsuzawa H, Donachie WD (1990) The balance between different peptidoglycan precursors determines whether *Escherichia coli* cells will elongate or divide. J Bacteriol 172:6697–6703

Bennett JW, Chung KT (2001) Alexander Fleming and the discovery of penicillin. Adv Appl Microbiol 49:163–184

Bera A, Herbert S, Jakob A, Vollmer W, Götz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan *O*-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol Microbiol 55:778–787

Bera A, Biswas R, Herbert S, Götz F (2006) The presence of peptidoglycan *O*-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity. Infect Immun 74:4598–4604

Berger-Bächi B, Strassle A, Gustafson JE, Kayser FH (1992) Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 36:1367–1373

Bernhardt TG, de Boer PA (2003) The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. Mol Microbiol 48:1171–1182

Bernhardt TG, de Boer PA (2004) Screening for synthetic lethal mutants in *Escherichia coli* and identification of EnvC (YibP) as a periplasmic septal ring factor with murein hydrolase activity. Mol Microbiol 52:1255–1269

Bertsche U, Breukink E, Kast T, Vollmer W (2005) In vitro murein peptidoglycan synthesis by dimers of the bifunctional transglycosylase-transpeptidase PBP1B from *Escherichia coli*. J Biol Chem 280:38096–38101

Beveridge TJ, Davies JA (1983) Cellular responses of *Bacillus subtilis* and *Escherichia coli* to the Gram stain. J Bacteriol 156:846–858

Biarrotte-Sorin S, Hugonnet JE, Delfosse V, Mainardi JL, Gutmann L, Arthur M, Mayer C (2006) Crystal structure of a novel β-lactam-insensitive peptidoglycan transpeptidase. J Mol Biol 359:533–538

Blair DE, van Aalten DM (2004) Structures of *Bacillus subtilis* PdaA, a family 4 carbohydrate esterase, and a complex with *N*-acetyl-glucosamine. FEBS Lett 570:13–19
Boneca IG (2005) The role of peptidoglycan in pathogenesis. Curr Opin Microbiol 8:46–53
Boneca IG, Chiosis G (2003) Vancomycin resistance: occurrence, mechanisms and strategies to combat it. Expert Opin Ther Targets 7:311–328
Boneca IG, Huang ZH, Gage DA, Tomasz A (2000) Characterization of *Staphylococcus aureus* cell wall glycan strands, evidence for a new β-*N*-acetylglucosaminidase activity. J Biol Chem 275:9910–9918
Boothby D, Daneo-Moore L, Higgins ML, Coyette J, Shockman GD (1973) Turnover of bacterial cell wall peptidoglycans. J Biol Chem 248:2161–2169
Born P, Breukink E, Vollmer W (2006) In vitro synthesis of cross-linked murein and its attachment to sacculi by PBP1A from *Escherichia coli*. J Biol Chem 281:26985–26993
Botta GA, Park JT (1981) Evidence for involvement of penicillin-binding protein 3 in murein synthesis during septation but not during cell elongation. J Bacteriol 145:333–340
Bouhss A, Trunkfield AE, Bugg TD, Mengin-Lecreulx D (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol Rev 32:208–233
Boyle DS, Khattar MM, Addinall SG, Lutkenhaus J, Donachie WD (1997) *ftsW* is an essential cell-division gene in *Escherichia coli*. Mol Microbiol 24:1263–1273
Braun V (1975) Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim Biophys Acta 415:335–377
Braun V, Bosch V (1972) Sequence of the murein-lipoprotein and the attachment site of the lipid. Eur J Biochem 28:51–69
Braun V, Gnirke H, Henning U, Rehn K (1973) Model for the structure of the shape-maintaining layer of the *Escherichia coli* cell envelope. J Bacteriol 114:1264–1270
Brennan PJ, Nikaido H (1995) The envelope of *mycobacteria*. Annu Rev Biochem 64:29–63
Brown K, Pompeo F, Dixon S, Mengin-Lecreulx D, Cambillau C, Bourne Y (1999) Crystal structure of the bifunctional *N*-acetylglucosamine 1-phosphate uridyltransferase from *Escherichia coli*: a paradigm for the related pyrophosphorylase superfamily. EMBO J 18:4096–4107
Burman LG, Park JT (1984) Molecular model for elongation of the murein sacculus of *Escherichia coli*. Proc Natl Acad Sci USA 81:1844–1848
Burman LG, Raichler J, Park JT (1983a) Evidence for diffuse growth of the cylindrical portion of the *Escherichia coli* murein sacculus. J Bacteriol 155:983–988
Burman LG, Reichler J, Park JT (1983b) Evidence for multisite growth of *Escherichia coli* murein involving concomitant endopeptidase and transpeptidase activities. J Bacteriol 156:386–392
Cabeen MT, Jacobs-Wagner C (2005) Bacterial cell shape. Nat Rev Microbiol 3:601–610
Cabeen MT, Jacobs-Wagner C (2007) Skin and bones: the bacterial cytoskeleton, cell wall, and cell morphogenesis. J Cell Biol 179:381–387
Calandra GB, Cole RM (1980) Lysis and protoplast formation of group B streptococci by mutanolysin. Infect Immun 28:1033–1037
Carballido-Lopez R, Formstone A (2007) Shape determination in *Bacillus subtilis*. Curr Opin Microbiol 10:611–616
Casadesus J (2007) Bacterial L-forms require peptidoglycan synthesis for cell division. Bioessays 29:1189–1191
Cascales E, Lloubes R (2004) Deletion analyses of the peptidoglycan-associated lipoprotein Pal reveals three independent binding sequences including a TolA box. Mol Microbiol 51:873–885
Chain E, Florey HW, Gardner AD, Heatley NG, Jennings MA, Orr-Ewing J, Sanders AG (2005) The Classic: penicillin as a chemotherapeutic agent. Clin Orthop Relat Res 439:23–26
Chaloupka J, Kreckova P (1971) Turnover of mucopeptide during the life cycle of *Bacillus megaterium*. Folia Microbiol (Praha) 16:372–382
Chaput C, Boneca IG (2007) Peptidoglycan detection by mammals and flies. Microbes Infect 9:637–647
Cheng Q, Park JT (2002) Substrate specificity of the AmpG permease required for recycling of cell wall anhydro-muropeptides. J Bacteriol 184:6434–6436

Cheng Q, Li H, Merdek K, Park JT (2000) Molecular characterization of the β-*N*-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. J Bacteriol 182:4836–4840

Clarke AJ, Dupont C (1992) *O*-acetylated peptidoglycan: its occurrence, pathobiological significance, and biosynthesis. Can J Microbiol 38:85–91

Claus H, Martin HH, Jantos CA, König H (2000) A search for β-lactamase in *chlamydiae, mycoplasmas, planctomycetes*, and cyanelles: bacteria and bacterial descendants at different phylogenetic positions and stages of cell wall development. Microbiol Res 155:1–6

Clavel T, Germon P, Vianney A, Portalier R, Lazzaroni JC (1998) TolB protein of *Escherichia coli* K-12 interacts with the outer membrane peptidoglycan-associated proteins Pal, Lpp and OmpA. Mol Microbiol 29:359–367

Cloud-Hansen KA, Peterson SB, Stabb EV, Goldman WE, McFall-Ngai MJ, Handelsman J (2006) Breaching the great wall: peptidoglycan and microbial interactions. Nat Rev Microbiol 4:710–716

Cooper S, Hsieh ML (1988) The rate and topography of cell wall synthesis during the division cycle of *Escherichia coli* using *N*-acetylglucosamine as a peptidoglycan label. J Gen Microbiol 134:1717–1721

Coyette J, van der Ende A (2008) Peptidoglycan: the bacterial Achilles heel. FEMS Microbiol Rev 32:147–148

Cremniter J, Mainardi JL, Josseaume N, Quincampoix JC, Dubost L, Hugonnet JE, Marie A, Gutmann L, Rice LB, Arthur M (2006) Novel mechanism of resistance to glycopeptide antibiotics in *Enterococcus faecium*. J Biol Chem 281:32254–32262

Cummins CS, Harris H (1956) The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. J Gen Microbiol 14:583–600

Daneo-Moore L, Coyette J, Sayare M, Boothby D, Shockman GD (1975) Turnover of the cell wall peptidoglycan of *Lactobacillus acidophilus*. The presence of a fraction immune to turnover. J Biol Chem 250:1348–1353

Daniel RA, Errington J (2003) Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. Cell 113:767–776

Davis BD (1948) Isolation of biochemically deficient mutants of bacteria by penicillin. J Am Chem Soc 70:4267

Davis BD (1949) The isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc Natl Acad Sci USA 35:1–10

de Chastellier C, Frehel C, Ryter A (1975a) Cell wall growth of *Bacillus megaterium*: cytoplasmic radioactivity after pulse-labeling with tritiated diaminopimelic acid. J Bacteriol 123:1197–1207

de Chastellier C, Hellio R, Ryter A (1975b) Study of cell wall growth in *Bacillus megaterium* by high-resolution autoradiography. J Bacteriol 123:1184–1196

de Jonge BL, Wientjes FB, Jurida I, Driehuis F, Wouters JT, Nanninga N (1989) Peptidoglycan synthesis during the cell cycle of *Escherichia coli*: composition and mode of insertion. J Bacteriol 171:5783–5794

de Jonge BL, Chang YS, Gage D, Tomasz A (1992) Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. J Biol Chem 267:11248–11254

de Jonge BL, Sidow T, Chang YS, Labischinski H, Berger-Bächi B, Gage DA, Tomasz A (1993) Altered muropeptide composition in *Staphylococcus aureus* strains with an inactivated *femA* locus. J Bacteriol 175:2779–2782

de Pedro MA, Quintela JC, Höltje JV, Schwarz H (1997) Murein segregation in *Escherichia coli*. J Bacteriol 179:2823–2834

de Pedro MA, Donachie WD, Höltje JV, Schwarz H (2001) Constitutive septal murein synthesis in *Escherichia coli* with impaired activity of the morphogenetic proteins RodA and penicillin-binding protein 2. J Bacteriol 183:4115–4126

de Pedro MA, Schwarz H, Koch AL (2003) Patchiness of murein insertion into the sidewall of *Escherichia coli*. Microbiology 149:1753–1761

de Petris S (1967) Ultrastructure of the cell wall of *Escherichia coli* and chemical nature of its constituent layers. J Ultrastruct Res 19:45–83
Demchick P, Koch AL (1996) The permeability of the wall fabric of *Escherichia coli* and *Bacillus subtilis*. J Bacteriol 178:768–773
den Blaauwen T, de Pedro MA, Nguyen-Disteche M, Ayala JA (2008) Morphogenesis of rod-shaped sacculi. FEMS Microbiol Rev 32:321–344
Dhalluin A, Bourgeois I, Pestel-Caron M, Camiade E, Raux G, Courtin P, Chapot-Chartier MP, Pons JL (2005) Acd, a peptidoglycan hydrolase of *Clostridium difficile* with *N*-acetylglucosaminidase activity. Microbiology 151:2343–2351
Di Berardino M, Dijkstra A, Stuber D, Keck W, Gubler M (1996) The monofunctional glycosyltransferase of *Escherichia coli* is a member of a new class of peptidoglycan-synthesising enzymes. FEBS Lett 392:184–188
Dmitriev BA, Ehlers S, Rietschel ET (1999) Layered murein revisited: a fundamentally new concept of bacterial cell wall structure, biogenesis and function. Med Microbiol Immunol 187:173–181
Dmitriev BA, Ehlers S, Rietschel ET, Brennan PJ (2000) Molecular mechanics of the mycobacterial cell wall: from horizontal layers to vertical scaffolds. Int J Med Microbiol 290:251–258
Dmitriev BA, Toukach FV, Schaper K-J, Holst O, Rietschel ET, Ehlers S (2003) Tertiary structure of bacterial murein: the scaffold model. J Bacteriol 185:3458–3468
Dmitriev BA, Toukach FV, Holst O, Rietschel ET, Ehlers S (2004) Tertiary structure of *Staphylococcus aureus* cell wall murein. J Bacteriol 186:7141–7148
Dmitriev B, Toukach F, Ehlers S (2005) Towards a comprehensive view of the bacterial cell wall. Trends Microbiol 13:569–574
Doyle RJ, Chaloupka J, Vinter V (1988) Turnover of cell walls in microorganisms. Microbiol Rev 52:554–567
Dramsi S, Magnet S, Davison S, Arthur M (2008) Covalent attachment of proteins to peptidoglycan. FEMS Microbiol Rev 32:307–320
Draper P, Kandler O, Darbre A (1987) Peptidoglycan and arabinogalactan of *Mycobacterium leprae*. J Gen Microbiol 133(Pt 5):1187–1194
Ehlert K, Höltje JV (1996) Role of precursor translocation in coordination of murein and phospholipid synthesis in *Escherichia coli*. J Bacteriol 178:6766–6771
Ehlert K, Schröder W, Labischinski H (1997) Specificities of FemA and FemB for different glycine residues: FemB cannot substitute for FemA in staphylococcal peptidoglycan pentaglycine side chain formation. J Bacteriol 179:7573–7576
El Ghachi M, Derbise A, Bouhss A, Mengin-Lecreulx D (2005) Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. J Biol Chem 280:18689–18695
Ellsworth BA, Tom NJ, Bartlett PA (1996) Synthesis and evaluation of inhibitors of bacterial D-alanine:D-alanine ligases. Chem Biol 3:37–44
Erickson HP (1995) FtsZ, a prokaryotic homolog of tubulin? Cell 80:367–370
Errington J (2003) The bacterial actin cytoskeleton. ASM News 69:608–614
Errington J, Daniel RA, Scheffers DJ (2003) Cytokinesis in bacteria. Microbiol Mol Biol Rev 67:52–65
Eschenburg S, Priestman M, Schonbrunn E (2005) Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) is essential for product release. J Biol Chem 280:3757–3763
Figge RM, Divakaruni AV, Gober JW (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. Mol Microbiol 51:1321–1332
Fiuza M, Canova MJ, Zanella-Cleon I, Becchi M, Cozzone AJ, Mateos LM, Kremer L, Gil JA, Molle V (2008) From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division. J Biol Chem 283:18099–18112

Flardh K (2003) Growth polarity and cell division in Streptomyces. Curr Opin Microbiol 6:564–571
Fleming A (1955) The story of penicillin. Bull Georgetown Univ Med Cent 8:128–132
Formanek H, Rauscher R (1979) Electron diffraction studies of the peptidoglycan of bacterial cell walls. Ultramicroscopy 3:337–342
Foster SJ, Popham DL (2002) Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers, and capsules. In: Sonenshein AL, Hoch JA, Losick R (eds) *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, DC, pp 21–41
Fox A, Rogers JC, Gilbart J, Morgan S, Davis CH, Knight S, Wyrick PB (1990) Muramic acid is not detectable in *Chlamydia psittaci* or *Chlamydia trachomatis* by gas chromatography-mass spectrometry. Infect Immun 58:835–837
Fukushima T, Yamamoto H, Atrih A, Foster SJ, Sekiguchi J (2002) A polysaccharide deacetylase gene (*pdaA*) is required for germination and for production of muramic δ-lactam residues in the spore cortex of *Bacillus subtilis*. J Bacteriol 184:6007–6015
Gally D, Archibald AR (1993) Cell wall assembly in *Staphylococcus aureus*: proposed absence of secondary crosslinking reactions. J Gen Microbiol 139:1907–1913
Geddes A (2008) 80th Anniversary of the discovery of penicillin: an appreciation of Sir Alexander Fleming. Int J Antimicrob Agents 32:373
Gerding MA, Ogata Y, Pecora ND, Niki H, de Boer PA (2007) The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in *E. coli*. Mol Microbiol 63:1008–1025
Ghuysen JM (1968) Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol Rev 32:425–464
Ghuysen JM (1991) Serine β-lactamases and penicillin-binding proteins. Annu Rev Microbiol 45:37–67
Ghuysen JM, Goffin C (1999) Lack of cell wall peptidoglycan versus penicillin sensitivity: new insights into the chlamydial anomaly. Antimicrob Agents Chemother 43:2339–2344
Girardin SE et al. (2003a) Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. Science 300:1584–1587
Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ (2003b) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 278:8869–8872
Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D (2003c) Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J Biol Chem 278:41702–41708
Glauner B (1988) Separation and quantification of muropeptides with high-performance liquid chromatography. Anal Biochem 172:451–464
Glauner B, Höltje JV (1990) Growth pattern of the murein sacculus of *Escherichia coli*. J Biol Chem 265:18988–18996
Glauner B, Höltje JV, Schwarz U (1988) The composition of the murein of *Escherichia coli*. J Biol Chem 263:10088–10095
Goehring NW, Beckwith J (2005) Diverse paths to midcell: assembly of the bacterial cell division machinery. Curr Biol 15:R514–526
Goffin C, Ghuysen JM (1998) Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol Mol Biol Rev 62:1079–1093
Golecki JR (1974) [Fine structure of the cell wall in various Cyanophyceae]. Zentralbl Bakteriol [German] 228:189–192
Goodell EW (1985) Recycling of murein by *Escherichia coli*. J Bacteriol 163:305–310
Goodell EW, Schwarz U (1975) Sphere-rod morphogenesis of *Escherichia coli*. J Gen Microbiol 86:201–209
Goodell EW, Schwarz U (1985) Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*. J Bacteriol 162:391–397

Griffiths E, Gupta RS (2002) Protein signatures distinctive of *chlamydial* species: horizontal transfers of cell wall biosynthesis genes *glmU* from archaea to *chlamydiae* and *murA* between *chlamydiae* and *Streptomyces*. Microbiology 148:2541–2549

Hadi T, Dahl U, Mayer C, Tanner ME (2008) Mechanistic studies on *N*-acetylmuramic acid 6-phosphate hydrolase (MurQ): an etherase involved in peptidoglycan recycling. Biochemistry 47:11547–11558

Harz H, Burgdorf K, Höltje JV (1990) Isolation and separation of the glycan strands from murein of *Escherichia coli* by reversed-phase high-performance liquid chromatography. Anal Biochem 190:120–128

Hayhurst EJ, Kailas L, Hobbs JK, Foster SJ (2008) Cell wall peptidoglycan architecture in *Bacillus subtilis*. Proc Natl Acad Sci USA 105:14603–14608

Healy VL, Lessard IA, Roper DI, Knox JR, Walsh CT (2000) Vancomycin resistance in *enterococci*: reprogramming of the D-Ala-D-Ala ligases in bacterial peptidoglycan biosynthesis. Chem Biol 7:R109–119

Henriques AO, Glaser P, Piggot PJ, Moran CP Jr (1998) Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. Mol Microbiol 28:235–247

Henze U, Sidow T, Wecke J, Labischinski H, Berger-Bächi B (1993) Influence of *femB* on methicillin resistance and peptidoglycan metabolism in *Staphylococcus aureus*. J Bacteriol 175:1612–1620

Herbert S, Bera A, Nerz C, Kraus D, Peschel A, Goerke C, Meehl M, Cheung A, Götz F (2007) Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog 3:e102

Hoiczyk E, Baumeister W (1995) Envelope structure of four gliding filamentous *cyanobacteria*. J Bacteriol 177:2387–2395

Hoiczyk E, Hansel A (2000) *Cyanobacterial* cell walls: news from an unusual prokaryotic envelope. J Bacteriol 182:1191–1199

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Philadelphia

Höltje J-V (1996a) A hypothetical holoenzyme involved in the replication of the murein sacculus of *Escherichia coli*. Microbiology 142(Pt 8):1911–1918

Höltje J-V (1996b) Lytic transglycosylases. In: Jollès P (ed) Lysozymes: model enzymes in biochemistry and biology. Birkhäuser Verlag, Basel, Switzerland, pp 425–429

Höltje J-V (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol Mol Biol Rev 62:181–203

Höltje J-V, Heidrich C (2001) Enzymology of elongation and constriction of the murein sacculus of *Escherichia coli*. Biochimie 83:103–108

Höltje J-V, Mirelman D, Sharon N, Schwarz U (1975) Novel type of murein transglycosylase in *Escherichia coli*. J Bacteriol 124:1067–1076

Höltje J-V, Kopp U, Ursinus A, Wiedemann B (1994) The negative regulator of β-lactamase induction AmpD is a *N*-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol Lett 122:159–164

Hove-Jensen B (1992) Identification of *tms*-26 as an allele of the *gcaD* gene, which encodes *N*-acetylglucosamine 1-phosphate uridyltransferase in *Bacillus subtilis*. J Bacteriol 174: 6852–6856

Hu Y, Chen L, Ha S, Gross B, Falcone B, Walker D, Mokhtarzadeh M, Walker S (2003) Crystal structure of the MurG:UDP-GlcNAc complex reveals common structural principles of a superfamily of glycosyltransferases. Proc Natl Acad Sci USA 100:845–849

Hughes WH, Kramer IR, Fleming A (1949) The effect of penicillin on morphology. J Gen Microbiol 3:23

Ikeda M, Sato T, Wachi M, Jung HK, Ishino F, Kobayashi Y, Matsuhashi M (1989) Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein, which function in cell division, cell elongation, and spore formation, respectively. J Bacteriol 171:6375–6378

Inoue A, Murata Y, Takahashi H, Tsuji N, Fujisaki S, Kato JI (2008) Involvement of an essential gene, *mviN*, in murein synthesis in *Escherichia coli*. J Bacteriol 190:7298–7301

Ishino F, Park W, Tomioka S, Tamaki S, Takase I, Kunugita K, Matsuzawa H, Asoh S, Ohta T, Spratt BG et al. (1986) Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin-binding protein 2 and rodA protein. J Biol Chem 261:7024–7031

Ishino F, Jung HK, Ikeda M, Doi M, Wachi M, Matsuhashi M (1989) New mutations *fts*-36, *fts*-33, and *ftsW* clustered in the *mra* region of the *Escherichia coli* chromosome induce thermosensitive cell growth and division. J Bacteriol 171:5523–5530

Jacobs C, Huang LJ, Bartowsky E, Normark S, Park JT (1994) Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J 13: 4684–4694

Jacobs C, Joris B, Jamin M, Klarsov K, Van Beeumen J, Mengin-Lecreulx D, van Heijenoort J, Park JT, Normark S, Frere JM (1995) AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol Microbiol 15:553–559

Jacobs C, Frere JM, Normark S (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible β-lactam resistance in gram-negative bacteria. Cell 88:823–832

Jaeger T, Mayer C (2008a) *N*-acetylmuramic acid 6-phosphate lyases (MurNAc etherases): role in cell wall metabolism, distribution, structure, and mechanism. Cell Mol Life Sci 65:928–939

Jaeger T, Mayer C (2008b) The transcriptional factors MurR and catabolite activator protein regulate *N*-acetylmuramic acid catabolism in *Escherichia coli*. J Bacteriol 190:6598–6608

Jaeger T, Arsic M, Mayer C (2005) Scission of the lactyl ether bond of *N*-acetylmuramic acid by *Escherichia coli* "etherase". J Biol Chem 280:30100–30106

Jensen GJ, Briegel A (2007) How electron cryotomography is opening a new window onto prokaryotic ultrastructure. Curr Opin Struct Biol 17:260–267

Jollès P (1996) From the discovery of lysozyme to the characterization of several lysozyme families. In: Jollès P (ed) Lysozymes: model enzymes in biochemistry and biology. Birkhäuser Verlag, Basel, Switzerland, pp 3–5

Jollès P, Jollès J (1984) What's new in lysozyme research? Always a model system, today as yesterday. Mol Cell Biochem 63:165–189

Jolly L, Ferrari P, Blanot D, Van Heijenoort J, Fassy F, Mengin-Lecreulx D (1999) Reaction mechanism of phosphoglucosamine mutase from *Escherichia coli*. Eur J Biochem 262:202–210

Jones G, Dyson P (2006) Evolution of transmembrane protein kinases implicated in coordinating remodeling of Gram-positive peptidoglycan: inside versus outside. J Bacteriol 188:7470–7476

Jones LJ, Carballido-Lopez R, Errington J (2001) Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. Cell 104:913–922

Kalamorz F, Reichenbach B, Marz W, Rak B, Görke B (2007) Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*. Mol Microbiol 65:1518–1533

Kellenberger E, Ryter A (1958) Cell wall and cytoplasmic membrane of *Escherichia coli*. J Biophys Biochem Cytol 4:323–326

Kern T, Hediger S, Muller P, Giustini C, Joris B, Bougault C, Vollmer W, Simorre JP (2008) Toward the characterization of peptidoglycan structure and protein-peptidoglycan interactions by solid-state NMR spectroscopy. J Am Chem Soc 130:5618–5619

Koch AL (1985) How bacteria grow and divide in spite of internal hydrostatic pressure. Can J Microbiol 31:1071–1084

Koch AL (1988) Biophysics of bacterial walls viewed as stress-bearing fabric. Microbiol Rev 52:337–353

Koch AL (1995) Bacterial growth and form: evolution and biophysics. Chapman and Hall, New York

Koch AL, Doyle RJ (1985) Inside-to-outside growth and turnover of the wall of Gram-positive rods. J Theor Biol 117:137–157

Koch AL, Woeste S (1992) Elasticity of the sacculus of *Escherichia coli*. J Bacteriol 174: 4811–4819

Koch AL, Higgins ML, Doyle RJ (1981) Surface tension-like forces determine bacterial shapes: *Streptococcus faecium*. J Gen Microbiol 123:151–161

Koch AL, Higgins ML, Doyle RJ (1982) The role of surface stress in the morphology of microbes. J Gen Microbiol 128:927–945

Koch AL, Lane SL, Miller JA, Nickens DG (1987) Contraction of filaments of *Escherichia coli* after disruption of cell membrane by detergent. J Bacteriol 169:1979–1984

Kowalski JJ, Tipper DJ, Berman DT (1970) Preparation of cell wall antigens of *Staphylococcus aureus*. Infect Immun 2:54–59

Kraft AR, Prabhu J, Ursinus A, Höltje JV (1999) Interference with murein turnover has no effect on growth but reduces β-lactamase induction in *Escherichia coli*. J Bacteriol 181:7192–7198

Labischinski H, Barnickel G, Bradaczek H, Giesbrecht P (1979) On the secondary and tertiary structure of murein. Low and medium-angle X-ray evidence against chitin-based conformations of bacterial peptidoglycan. Eur J Biochem 95:147–155

Lederberg J, Zinder N (1948) Concentration of biochemical mutants of bacteria with penicillin. J Am Chem Soc 70:4267

Li SY, Höltje JV, Young KD (2004) Comparison of high-performance liquid chromatography and fluorophore-assisted carbohydrate electrophoresis methods for analyzing peptidoglycan composition of *Escherichia coli*. Anal Biochem 326:1–12

Lovering AL, de Castro LH, Lim D, Strynadka NC (2007) Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis. Science 315:1402–1405

Löwe J, Amos LA (1998) Crystal structure of the bacterial cell-division protein FtsZ. Nature 391:203–206

Lutkenhaus JF, Wolf-Watz H, Donachie WD (1980) Organization of genes in the *ftsA-envA* region of the *Escherichia coli* genetic map and identification of a new *fts* locus (*ftsZ*). J Bacteriol 142:615–620

Magnet S, Arbeloa A, Mainardi JL, Hugonnet JE, Fourgeaud M, Dubost L, Marie A, Delfosse V, Mayer C, Rice LB, Arthur M (2007a) Specificity of L, D-transpeptidases from Gram-positive bacteria producing different peptidoglycan chemotypes. J Biol Chem 282:13151–13159

Magnet S, Bellais S, Dubost L, Fourgeaud M, Mainardi JL, Petit-Frere S, Marie A, Mengin-Lecreulx D, Arthur M, Gutmann L (2007b) Identification of the L, D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. J Bacteriol 189:3927–3931

Magnet S, Dubost L, Marie A, Arthur M, Gutmann L (2008) Identification of the L, D-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. J Bacteriol 190:4782–4785

Mahapatra S, Crick DC, McNeil MR, Brennan PJ (2008) Unique structural features of the peptidoglycan of *Mycobacterium leprae*. J Bacteriol 190:655–661

Mainardi JL, Morel V, Fourgeaud M, Cremniter J, Blanot D, Legrand R, Frehel C, Arthur M, Van Heijenoort J, Gutmann L (2002) Balance between two transpeptidation mechanisms determines the expression of β-lactam resistance in *Enterococcus faecium*. J Biol Chem 277:35801–35807

Mainardi JL, Villet R, Bugg TD, Mayer C, Arthur M (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS Microbiol Rev 32:386–408

Makino S, Moriyama R (2002) Hydrolysis of cortex peptidoglycan during bacterial spore germination. Med Sci Monit 8:RA119–127

Martin HH (1964) Composition of the mucopolymer in cell walls of the unstable and stable L-form of *Proteus mirabilis*. J Gen Microbiol 36:441–450

Matias VR, Beveridge TJ (2005) Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. Mol Microbiol 56:240–251

Matias VR, Beveridge TJ (2006) Native cell wall organization shown by cryo-electron microscopy confirms the existence of a periplasmic space in *Staphylococcus aureus*. J Bacteriol 188: 1011–1021

Matias VR, Beveridge TJ (2007) Cryo-electron microscopy of cell division in *Staphylococcus aureus* reveals a mid-zone between nascent cross walls. Mol Microbiol 64:195–206

Matias VR, Al-Amoudi A, Dubochet J, Beveridge TJ (2003) Cryo-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and *Pseudomonas aeruginosa*. J Bacteriol 185:6112–6118

Matsuzawa H, Hayakawa K, Sato T, Imahori K (1973) Characterization and genetic analysis of a mutant of *Escherichia coli* K-12 with rounded morphology. J Bacteriol 115:436–442

Mauck J, Glaser L (1970) Turnover of the cell wall of *Bacillus subtilis* W-23 during logarithmic growth. Biochem Biophys Res Commun 39:699–706

Mauck J, Chan L, Glaser L (1971) Turnover of the cell wall of Gram-positive bacteria. J Biol Chem 246:1820–1827

Mayer C, Boos W (2005) 29 March 2005, posting date. Chapter 3.4.1, Hexose/pentose and hexitol/pentitol metabolism. In: Böck A, Curtiss III R, Kaper JB, Neidhardt FC, Nyström T, Rudd KE, and Squires CL (eds) EcoSal – *Escherichia coli* and *Salmonella*: cellular and molecular biology. [Online.] http://www.ecosal.org. ASM Press, Washington, DC

McCoy AJ, Maurelli AT (2005) Characterization of *Chlamydia* MurC-Ddl, a fusion protein exhibiting D-alanyl-D-alanine ligase activity involved in peptidoglycan synthesis and D-cycloserine sensitivity. Mol Microbiol 57:41–52

McCoy AJ, Maurelli AT (2006) Building the invisible wall: updating the *chlamydial* peptidoglycan anomaly. Trends Microbiol 14:70–77

McCoy AJ, Sandlin RC, Maurelli AT (2003) In vitro and in vivo functional activity of *Chlamydia* MurA, a UDP-*N*-acetylglucosamine enolpyruvyl transferase involved in peptidoglycan synthesis and fosfomycin resistance. J Bacteriol 185:1218–1228

McPherson DC, Driks A, Popham DL (2001) Two class a high-molecular-weight penicillin-binding proteins of *Bacillus subtilis* play redundant roles in sporulation. J Bacteriol 183:6046–6053

Meisel U, Höltje JV, Vollmer W (2003) Overproduction of inactive variants of the murein synthase PBP1B causes lysis in *Escherichia coli*. J Bacteriol 185:5342–5348

Mengin-Lecreulx D, van Heijenoort J (1993) Identification of the *glmU* gene encoding *N*-acetylglucosamine-1-phosphate uridyltransferase in *Escherichia coli*. J Bacteriol 175: 6150–6157

Mengin-Lecreulx D, van Heijenoort J (1994) Copurification of glucosamine-1-phosphate acetyltransferase and *N*-acetylglucosamine-1-phosphate uridyltransferase activities of *Escherichia coli*: characterization of the *glmU* gene product as a bifunctional enzyme catalyzing two subsequent steps in the pathway for UDP-*N*-acetylglucosamine synthesis. J Bacteriol 176:5788–5795

Mengin-Lecreulx D, van Heijenoort J (1996) Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. J Biol Chem 271:32–39

Mengin-Lecreulx D, van Heijenoort J, Park JT (1996) Identification of the *mpl* gene encoding UDP-*N*-acetylmuramate: L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase in *Escherichia coli* and its role in recycling of cell wall peptidoglycan. J Bacteriol 178:5347–5352

Merchante R, Pooley HM, Karamata D (1995) A periplasm in *Bacillus subtilis*. J Bacteriol 177:6176–6183

Meroueh SO, Bencze KZ, Hesek D, Lee M, Fisher JF, Stemmler TL, Mobashery S (2006) Three-dimensional structure of the bacterial cell wall peptidoglycan. Proc Natl Acad Sci USA 103:4404–4409

Mobley HL, Koch AL, Doyle RJ, Streips UN (1984) Insertion and fate of the cell wall in *Bacillus subtilis*. J Bacteriol 158:169–179
Mollner S, Braun V (1984) Murein hydrolase (*N*-acetyl-muramyl-L-alanine amidase) in human serum. Arch Microbiol 140:171–177
Mouilleron S, Golinelli-Pimpaneau B (2007) Conformational changes in ammonia-channeling glutamine amidotransferases. Curr Opin Struct Biol 17:653–664
Mouilleron S, Badet-Denisot MA, Golinelli-Pimpaneau B (2008) Ordering of *C*-terminal loop and glutaminase domains of glucosamine-6-phosphate synthase promotes sugar ring opening and formation of the ammonia channel. J Mol Biol 377:1174–1185
Moulder JW (1993) Why is *Chlamydia* sensitive to penicillin in the absence of peptidoglycan? Infect Agents Dis 2:87–99
Mudd S, Polevitzky K, Anderson TF, Chambers LA (1941) Bacterial morphology as shown by the electron microscope: II. The bacterial cell-wall in the genus *Bacillus*. J Bacteriol 42:251–264
Murray RG, Steed P, Elson HE (1965) The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other Gram-negative bacteria. Can J Microbiol 11:547–560
Nanninga N (1991) Cell division and peptidoglycan assembly in *Escherichia coli*. Mol Microbiol 5:791–795
Nanninga N (1998) Morphogenesis of *Escherichia coli*. Microbiol Mol Biol Rev 62:110–129
Navarre WW, Schneewind O (1999) Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 63:174–229
Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656
Normark S (1969) Mutation in *Escherichia coli* K-12 mediating spherelike envelopes and changes tolerance to ultraviolet irradiation and some antibiotics. J Bacteriol 98:1274–1277
Osborn MJ, Rothfield L (2007) Cell shape determination in *Escherichia coli*. Curr Opin Microbiol 10:606–610
Pagès J-M, Piovant M, Lazdunski A, Lazdunski C (1975) On the control of septation in *Escherichia coli*. Biochimie 57:303–313
Palmer RJ Jr, Stoodley P (2007) Biofilms 2007: broadened horizons and new emphases. J Bacteriol 189:7948–7960
Park JT (1952) Uridine-5′-pyrophosphate derivatives. III. Amino acid-containing derivatives. J Biol Chem 194:897–904
Park JT (1993) Turnover and recycling of the murein sacculus in oligopeptide permease-negative strains of *Escherichia coli*: indirect evidence for an alternative permease system and for a monolayered sacculus. J Bacteriol 175:7–11
Park JT (1995) Why does *Escherichia coli* recycle its cell wall peptides? Mol Microbiol 17:421–426
Park JT (1996) The Murein Sacculus. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*, 2nd edn. ASM Press, Washington, DC, pp 48–57
Park JT, Johnson MJ (1949) Accumulation of labile phosphate in *Staphylococcus aureus* grown in the presence of penicillin. J Biol Chem 179:585–592
Park W, Matsuhashi M (1984) *Staphylococcus aureus* and *Micrococcus luteus* peptidoglycan transglycosylases that are not penicillin-binding proteins. J Bacteriol 157:538–544
Park JT, Strominger JL (1957) Mode of action of penicillin. Science 125:99–101
Park JT, Uehara T (2008) How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). Microbiol Mol Biol Rev 72:211–227
Park JT, Raychaudhuri D, Li H, Normark S, Mengin-Lecreulx D (1998) MppA, a periplasmic binding protein essential for import of the bacterial cell wall peptide L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate. J Bacteriol 180:1215–1223
Pavelka MS Jr (2007) Another brick in the wall. Trends Microbiol 15:147–149
Petit JF, Adam A, Wietzerbin-Falszpan J, Lederer E, Ghuysen JM (1969) Chemical structure of the cell wall of *Mycobacterium smegmatis*. I. Isolation and partial characterization of the peptidoglycan. Biochem Biophys Res Commun 35:478–485

Petit JF, Wietzerbin J, Das BC, Lederer E (1975) Chemical structure of the cell wall of *Mycobacterium tuberculosis* var. bovis, strain BCG. Z Immunitätsforsch Exp Klin Immunol 149:118–125

Phiet PH, Wietzerbin J, Zissman E, Petit JF, Lederer E (1976) Analysis of the cell wall of five strains of *Mycobacterium tuberculosis* BCG and of an attenuated human strain, W 115. Infect Immun 13:677–681

Pilhofer M, Rappl K, Eckl C, Bauer AP, Ludwig W, Schleifer KH, Petroni G (2008) Characterization and evolution of cell division and cell wall synthesis genes in the bacterial phyla *Verrucomicrobia, Lentisphaerae, Chlamydiae*, and *Planctomycetes* and phylogenetic comparison with rRNA genes. J Bacteriol 190:3192–3202

Pisabarro AG, de Pedro MA, Vazquez D (1985) Structural modifications in the peptidoglycan of *Escherichia coli* associated with changes in the state of growth of the culture. J Bacteriol 161:238–242

Pooley HM, Merchante R, Karamata D (1996) Overall protein content and induced enzyme components of the periplasm of *Bacillus subtilis*. Microb Drug Resist 2:9–15

Popescu A, Doyle RJ (1996) The Gram stain after more than a century. Biotech Histochem 71:145–151

Popham DL, Helin J, Costello CE, Setlow P (1996) Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc Natl Acad Sci USA 93:15405–15410

Porter JR (1976) Antony van Leeuwenhoek: tercentenary of his discovery of bacteria. Bacteriol Rev 40:260–269

Primosigh J, Pelzer H, Maass D, Weidel W (1961) Chemical characterization of mucopeptides released from the *E. coli* B cell wall by enzymic action. Biochim Biophys Acta 46:68–80

Psylinakis E, Boneca IG, Mavromatis K, Deli A, Hayhurst E, Foster SJ, Varum KM, Bouriotis V (2005) Peptidoglycan *N*-acetylglucosamine deacetylases from *Bacillus cereus*, highly conserved proteins in *Bacillus anthracis*. J Biol Chem 280:30856–30863

Quintela JC, Caparros M, de Pedro MA (1995a) Variability of peptidoglycan structural parameters in Gram-negative bacteria. FEMS Microbiol Lett 125:95–100

Quintela JC, Pittenauer E, Allmaier G, Aran V, de Pedro MA (1995b) Structure of peptidoglycan from *Thermus thermophilus* HB8. J Bacteriol 177:4947–4962

Quintela JC, de Pedro MA, Zollner P, Allmaier G, Garcia-del Portillo F (1997) Peptidoglycan structure of *Salmonella typhimurium* growing within cultured mammalian cells. Mol Microbiol 23:693–704

Raymond JB, Mahapatra S, Crick DC, Pavelka MS Jr (2005) Identification of the *namH* gene, encoding the hydroxylase responsible for the *N*-glycolylation of the mycobacterial peptidoglycan. J Biol Chem 280:326–333

Reichenbach B, Maes A, Kalamorz F, Hajnsdorf E, Görke B (2008) The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. Nucleic Acids Res 36:2570–2580

Robinson JP, Robinson RD, Hash JH (1974) Electron microscopy of *Staphylococcus aureus* cells and cell walls after treatment with lysozyme *Chalaropsis*. J Bacteriol 117:900–903

Rogers HJ, Pooley HM, Thurman PF, Taylor C (1974) Wall and membrane growth in bacilli and their mutants. Ann Microbiol (Paris) 125B:135–147

Rohrer S, Ehlert K, Tschierske M, Labischinski H, Berger-Bächi B (1999) The essential *Staphylococcus aureus* gene *fmhB* is involved in the first step of peptidoglycan pentaglycine interpeptide formation. Proc Natl Acad Sci USA 96:9351–9356

Rosenthal RS, Dziarski R (1994) Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol 235:253–285

Ruiz N (2008) Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. Proc Natl Acad Sci USA 105:15553–15557

Russell AD (2002) Mechanisms of antimicrobial action of antiseptics and disinfectants: an increasingly important area of investigation. J Antimicrob Chemother 49:597–599

Salton MR (1952) Cell wall of *Micrococcus lysodeikticus* as the substrate of lysozyme. Nature 170:746–747
Salton MRJ (1964) The bacterial cell wall. Elsevier, Amsterdam
Salton MR (1994) The bacterial cell envelope – a historical perspective. In: Ghuysen J-M, Hakenbeck R (eds) Bacterial cell wall. Elsevier, Amsterdam, pp 1–22
Salton MR, Horne RW (1951) Studies of the bacterial cell wall. I. Electron microscopical observations on heated bacteria. Biochim Biophys Acta 7:19–42
Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32:234–258
Scheffers DJ (2007) The cell wall of *Bacillus subtilis*. In: Graumann P (ed) *Bacillus* – cellular and molecular biology. Caister Academic Press, Norfolk, UK, pp 331–373
Scheffers DJ, Errington J (2004) PBP1 is a component of the *Bacillus subtilis* cell division machinery. J Bacteriol 186:5153–5156
Scheffers DJ, Pinho MG (2005) Bacterial cell wall synthesis: new insights from localization studies. Microbiol Mol Biol Rev 69:585–607
Scheffers DJ, Jones LJ, Errington J (2004) Several distinct localization patterns for penicillin-binding proteins in *Bacillus subtilis*. Mol Microbiol 51:749–764
Scheurwater E, Reid CW, Clarke AJ (2007) Lytic transglycosylases: bacterial space-making autolysins. Int J Biochem Cell Biol 40:586–591
Schleifer KH, Kandler O (1967) [On the chemical composition of the cell wall of streptococci. I. The amino acid sequence of the murein of *Str. thermophilus* and *Str. faecalis*]. Arch Mikrobiol [German] 57:335–364
Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477
Schmelzer E, Weckesser J, Warth R, Mayer H (1982) Peptidoglycan of *Rhodopseudomonas viridis*: partial lack of *N*-acetyl substitution of glucosamine. J Bacteriol 149:151–155
Schwartz U, Weidel W (1965) [On the mechanism of action of penicillin. II. Demonstration of a penicillin-induced hydrolytic degradation of murein and murein precursors in *E. Coli* B.]. Z Naturforsch B [German] 20:153–157
Schwarz U, Leutgeb W (1971) Morphogenetic aspects of murein structure and biosynthesis. J Bacteriol 106:588–595
Schwedock J, McCormick JR, Angert ER, Nodwell JR, Losick R (1997) Assembly of the cell division protein FtsZ into ladder-like structures in the aerial hyphae of *Streptomyces coelicolor*. Mol Microbiol 25:847–858
Sekiguchi J, Akeo K, Yamamoto H, Khasanov FK, Alonso JC, Kuroda A (1995) Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis*. J Bacteriol 177:5582–5589
Seltmann G, Holst O (2002) Periplasmic space and rigid layer. In: Seltmann G, Holst O (eds) The bacterial cell wall. Springer, Berlin, pp 103–132
Severin A, Tabei K, Tomasz A (2004) The structure of the cell wall peptidoglycan of *Bacillus cereus* RSVF1, a strain closely related to *Bacillus anthracis*. Microb Drug Resist 10:77–82
Shih YL, Le T, Rothfield L (2003) Division site selection in *Escherichia coli* involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. Proc Natl Acad Sci USA 100:7865–7870
Shockman GD, Barrett JF (1983) Structure, function, and assembly of cell walls of Gram-positive bacteria. Annu Rev Microbiol 37:501–527
Shockman GD, Daneo-Moore L, Kariyama R, Massidda O (1996) Bacterial walls, peptidoglycan hydrolases, autolysins, and autolysis. Microb Drug Resist 2:95–98
Skipp P, Robinson J, O′Connor CD, Clarke IN (2005) Shotgun proteomic analysis of *Chlamydia trachomatis*. Proteomics 5:1558–1573
Spratt BG (1975) Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc Natl Acad Sci USA 72:2999–3003

Spratt BG, Pardee AB (1975) Penicillin-binding proteins and cell shape in *E. coli*. Nature 254:516–517

Stranden AM, Ehlert K, Labischinski H, Berger-Bächi B (1997) Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a *femAB* null mutant of methicillin-resistant *Staphylococcus aureus*. J Bacteriol 179:9–16

Sugai M, Yamada S, Nakashima S, Komatsuzawa H, Matsumoto A, Oshida T, Suginaka H (1997) Localized perforation of the cell wall by a major autolysin: *atl* gene products and the onset of penicillin-induced lysis of *Staphylococcus aureus*. J Bacteriol 179:2958–2962

Templin MF, Ursinus A, Höltje JV (1999) A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*. EMBO J 18:4108–4117

Teplyakov A, Obmolova G, Badet-Denisot MA, Badet B (1999) The mechanism of sugar phosphate isomerization by glucosamine 6-phosphate synthase. Protein Sci 8:596–602

Teplyakov A, Obmolova G, Badet B, Badet-Denisot MA (2001) Channeling of ammonia in glucosamine-6-phosphate synthase. J Mol Biol 313:1093–1102

Teplyakov A, Leriche C, Obmolova G, Badet B, Badet-Denisot MA (2002) From Lobry de Bruyn to enzyme-catalyzed ammonia channelling: molecular studies of D-glucosamine-6P synthase. Nat Prod Rep 19:60–69

Terrak M, Ghosh TK, van Heijenoort J, van Beeumen J, Lampilas M, Aszodi J, Ayala JA, Ghuysen J-M, Nguyen-Distèche M (1999) The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of *Escherichia coli*. Mol Microbiol 34:350–364

Tipper DJ, Strominger JL (1965) Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci USA 54:1133–1141

Tipper DJ, Strominger JL (1968) Biosynthesis of the peptidoglycan of bacterial cell walls. XII. Inhibition of cross-linking by penicillins and cephalosporins: studies in *Staphylococcus aureus* in vivo. J Biol Chem 243:3169–3179

Tipper DJ, Strominger JL, Ghuysen JM (1964) Staphylolytic enzyme from *Chalaropsis*: mechanism of action. Science 146:781–782

Tipper DJ, Strominger JL, Ensign JC (1967) Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. VII. Mode of action of the bacteriolytic peptidase from Myxobacter and the isolation of intact cell wall polysaccharides. Biochemistry 6:906–920

Tiyanont K, Doan T, Lazarus MB, Fang X, Rudner DZ, Walker S (2006) Imaging peptidoglycan biosynthesis in *Bacillus subtilis* with fluorescent antibiotics. Proc Natl Acad Sci USA 103:11033–11038

Trachtenberg S (2006) The cytoskeleton of *spiroplasma*: a complex linear motor. J Mol Microbiol Biotechnol 11:265–283

Uehara T, Park JT (2002) Role of the murein precursor UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala in repression of β-lactamase induction in cell division mutants. J Bacteriol 184:4233–4239

Uehara T, Park JT (2004) The *N*-acetyl-D-glucosamine kinase of *Escherichia coli* and its role in murein recycling. J Bacteriol 186:7273–7279

Uehara T, Suefuji K, Valbuena N, Meehan B, Donegan M, Park JT (2005) Recycling of the anhydro-*N*-acetylmuramic acid derived from cell wall murein involves a two-step conversion to *N*-acetylglucosamine-phosphate. J Bacteriol 187:3643–3649

Uehara T, Suefuji K, Jaeger T, Mayer C, Park JT (2006) MurQ etherase is required by *Escherichia coli* in order to metabolize anhydro-*N*-acetylmuramic acid obtained either from the environment or from its own cell wall. J Bacteriol 188:1660–1662

Urban JH, Vogel J (2008) Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation. PLoS Biol 6:e64

van Dam V, Sijbrandi R, Kol M, Swiezewska E, de Kruijff B, Breukink E (2007) Transmembrane transport of peptidoglycan precursors across model and bacterial membranes. Mol Microbiol 64:1105–1114

van den Ent F, Amos L, Lowe J (2001a) Bacterial ancestry of actin and tubulin. Curr Opin Microbiol 4:634–638
van den Ent F, Amos LA, Löwe J (2001b) Prokaryotic origin of the actin cytoskeleton. Nature 413:39–44
van Heijenoort J (1994) Biosynthesis of the bacterial peptidoglycan unit. In: Ghuysen J-M, Hakenbeck R (eds) Bacterial cell wall. Elsevier, Amsterdam, pp 39–54
van Heijenoort J (1998) Assembly of the monomer unit of bacterial peptidoglycan. Cell Mol Life Sci 54:300–304
van Heijenoort J (2001a) Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycobiology 11:25R–36R
van Heijenoort J (2001b) Recent advances in the formation of the bacterial peptidoglycan monomer unit. Nat Prod Rep 18:503–519
van Heijenoort J (2007) Lipid intermediates in the biosynthesis of bacterial peptidoglycan. Microbiol Mol Biol Rev 71:620–635
Varley AW, Stewart GC (1992) The *divIVB* region of the *Bacillus subtilis* chromosome encodes homologs of *Escherichia coli* septum placement (*minCD*) and cell shape (*mreBCD*) determinants. J Bacteriol 174:6729–6742
Verwer RW, Nanninga N, Keck W, Schwarz U (1978) Arrangement of glycan chains in the sacculus of *Escherichia coli*. J Bacteriol 136:723–729
Vollmer W (2007) Structure and biosynthesis of the murein (Peptidoglycan) sacculus. In: Ehrmann M (ed) The periplasm. ASM Press, Washington, DC, pp 198–213
Vollmer W (2008) Structural variation in the glycan strands of bacterial peptidoglycan. FEMS Microbiol Rev 32:287–306
Vollmer W, Bertsche U (2008) Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. Biochim Biophys Acta 1778:1714–1734
Vollmer W, Höltje JV (2001) Morphogenesis of *Escherichia coli*. Curr Opin Microbiol 4: 625–633
Vollmer W, Höltje JV (2004) The architecture of the murein (peptidoglycan) in Gram-negative bacteria: vertical scaffold or horizontal layer(s)? J Bacteriol 186:5978–5987
Vollmer W, Tomasz A (2000) The *pgdA* gene encodes for a peptidoglycan *N*-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. J Biol Chem 275:20496–20501
Vollmer W, Blanot D, de Pedro MA (2008a) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32:149–167
Vollmer W, Joris B, Charlier P, Foster S (2008b) Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev 32:259–286
Vötsch W, Templin MF (2000) Characterization of a β-*N*-acetylglucosaminidase of *Escherichia coli* and elucidation of its role in muropeptide recycling and β-lactamase induction. J Biol Chem 275:39032–39038
Wachi M, Doi M, Tamaki S, Park W, Nakajima-Iijima S, Matsuhashi M (1987) Mutant isolation and molecular cloning of *mre* genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in *Escherichia coli*. J Bacteriol 169:4935–4940
Wachi M, Doi M, Okada Y, Matsuhashi M (1989) New *mre* genes *mreC* and *mreD*, responsible for formation of the rod shape of *Escherichia coli* cells. J Bacteriol 171:6511–6516
Ward JB (1973) The chain length of the glycans in bacterial cell walls. Biochem J 133:395–398
Wei Y, Havasy T, McPherson DC, Popham DL (2003) Rod shape determination by the *Bacillus subtilis* class B penicillin-binding proteins encoded by *pbpA* and *pbpH*. J Bacteriol 185: 4717–4726
Wei Y, McPherson DC, Popham DL (2004) A mother cell-specific class B penicillin-binding protein, PBP4b, in *Bacillus subtilis*. J Bacteriol 186:258–261
Weidel W, Pelzer H (1964) Bagshaped macromolecules – a new outlook on bacterial cell walls. Adv Enzymol 26:193–232
Weidel W, Frank H, Martin HH (1960) The rigid layer of the cell wall of *Escherichia coli* strain B. J Gen Microbiol 22:158–166

Weidenmeier C, Peschel A (2008) Teichoic acids and releated cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Microb Rev 6:276–287

Wientjes FB, Woldringh CL, Nanninga N (1991) Amount of peptidoglycan in cell walls of Gram-negative bacteria. J Bacteriol 173:7684–7691

Wietzerbin J, Das BC, Petit JF, Lederer E, Leyh-Bouille M, Ghuysen JM (1974) Occurrence of D-alanyl-(D)-meso-diaminopimelic acid and meso-diaminopimelyl-meso-diaminopimelic acid interpeptide linkages in the peptidoglycan of Mycobacteria. Biochemistry 13:3471–3476

Wietzerbin-Falszpan J, Das BC, Azuma I, Adam A, Petit JF, Lederer E (1970) Isolation and mass spectrometric identification of the peptide subunits of *mycobacterial* cell walls. Biochem Biophys Res Commun 40:57–63

Wietzerbin-Falszpan J, Das BC, Gros C, Petit JF, Lederer E (1973) The amino acids of the cell wall of *Mycobacterium tuberculosis* var. *bovis*, strain BCG. Presence of a poly(L-glutamic acid). Eur J Biochem 32:525–532

Winkler WC, Nahvi A, Roth A, Collins JA, Breaker RR (2004) Control of gene expression by a natural metabolite-responsive ribozyme. Nature 428:281–286

Wise EM Jr, Park JT (1965) Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc Natl Acad Sci USA 54:75–81

Wu S, de Lencastre H, Sali A, Tomasz A (1996) A phosphoglucomutase-like gene essential for the optimal expression of methicillin resistance in *Staphylococcus aureus:* molecular cloning and DNA sequencing. Microb Drug Resist 2:277–286

Yamato I, Anraku Y, Hirosawa K (1975) Cytoplasmic membrane vesicles of *Escherichia coli*. A simple method for preparing the cytoplasmic and outer membranes. J Biochem (Tokyo) 77:705–718

Yanouri A, Daniel RA, Errington J, Buchanan CE (1993) Cloning and sequencing of the cell division gene *pbpB*, which encodes penicillin-binding protein 2B in *Bacillus subtilis*. J Bacteriol 175:7604–7616

Yeats C, Finn RD, Bateman A (2002) The PASTA domain: a β-lactam-binding domain. Trends Biochem Sci 27:438

Young KD (2003) Bacterial shape. Mol Microbiol 49:571–580

Young KD (2006) Too many strictures on structure. Trends Microbiol 14:155–156

Yuan Y, Barrett D, Zhang Y, Kahne D, Sliz P, Walker S (2007) Crystal structure of a peptidoglycan glycosyltransferase suggests a model for processive glycan chain synthesis. Proc Natl Acad Sci USA 104:5348–5353

Yuan Y, Fuse S, Ostash B, Sliz P, Kahne D, Walker S (2008) Structural analysis of the contacts anchoring moenomycin to peptidoglycan glycosyltransferases and implications for antibiotic design. ACS Chem Biol 3:429–436

Zapun A, Contreras-Martel C, Vernet T (2008a) Penicillin-binding proteins and β-lactam resistance. FEMS Microbiol Rev 32:361–385

Zapun A, Vernet T, Pinho MG (2008b) The different shapes of cocci. FEMS Microbiol Rev 32:345–360

Zawadzke LE, Bugg TD, Walsh CT (1991) Existence of two D-alanine:D-alanine ligases in *Escherichia coli*: cloning and sequencing of the *ddlA* gene and purification and characterization of the DdlA and DdlB enzymes. Biochemistry 30:1673–1682

Zipperle GF Jr, Ezzell JW Jr, Doyle RJ (1984) Glucosamine substitution and muramidase susceptibility in *Bacillus anthracis*. Can J Microbiol 30:553–559

Chapter 2
Occurrence, Structure, Chemistry, Genetics, Morphogenesis, and Functions of S-Layers

Paul Messner, Christina Schäffer, Eva-Maria Egelseer, and Uwe B. Sleytr

2.1 Introduction

S-layers, as probably the most abundant bacterial cellular proteins, are being studied with regard to structure, synthesis, assembly, and function and provide excellent models for studying a proteinaceous cell component and its evolutionary relationships within the prokaryotic world. Recently, S-layers have also shown a considerable application potential in biotechnology, biomimetics, biomedicine, and molecular nanotechnology. In the past few years, several reports have been published on these application aspects, demonstrating the change of perception that has been taken place in this field of research (see Chaps. 16 and 17).

In this chapter, we give a general overview of occurrence, location, and structure of S-layers and then focus particularly on those S-layer-carrying organisms that have attracted special attention within the past decade because of their scientific, medical, or public implications (e.g. *Bacillus anthracis* S-layer proteins EA1 and Sap).

2.2 Occurrence, Location, and Structure

In the course of evolution, prokaryotic organisms have developed a considerable diversity in their supramolecular architectures. Although not a universal feature, many prokaryotic organisms possess a monomolecular array of proteinaceous subunits as the outermost component of the cell envelope (Sleytr 1978; Sleytr et al. 1988; Sleytr and Messner 2009). S-layers represent an almost universal

P. Messner (✉)
Department of NanoBiotechnology, Boku – University of Natural Resources and Applied Life Sciences, Muthgasse 11, A-1190 Vienna, Austria
e-mail: paul.messner@boku.ac.at

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_2,

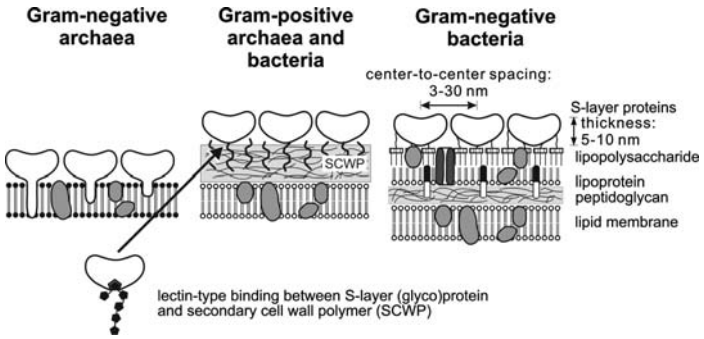

Fig. 2.1 Schematic illustration of major classes of prokaryotic cell envelopes containing crystalline cell surface layers (S-layer (glyco)proteins). (**a**) Cell-envelope structure of Gram-negative archaea with S-layers as the only cell-wall component external to the cytoplasmic membrane. (**b**) The cell envelope as observed in Gram-positive archaea and bacteria. In bacteria, the rigid wall component is primarily composed of peptidoglycan. SCWPs are only present in bacteria. In archaea, other wall polymers (e.g. pseudomurein or methanochondroitin) are found. (**c**) Cell-envelope profile of Gram-negative bacteria, composed of a thin peptidoglycan layer and an outer membrane. If present, the S-layer is closely associated with the lipopolysaccharide of the outer membrane (modified from Sleytr and Beveridge 1999)

feature of archaeal cell envelopes (see Chaps. 7–9) and have been detected in hundreds of different species of nearly every phylogenetic group of bacteria (Sleytr et al. 1996b, 2002; Åvall-Jääskeläinen and Palva 2005; Claus et al. 2005). Despite the fact that considerable variations exist in the structure and chemistry of prokaryotic envelopes (Fig. 2.1), S-layers have apparently coevolved with these diverse structures. In most archaea, S-layers are attached or inserted to the plasma membrane. In Gram-positive bacteria and Gram-positive archaea, the regular array assembles on the surface of the rigid wall matrix which is mainly composed of peptidoglycan or pseudomurein, respectively (e.g., see Chap. 1). In Gram-negative bacteria, the S-layer is attached to the lipopolysaccharide (LPS) component of the outer membrane. For some organisms, two superimposed S-layer lattices composed of different S-layer proteins have been described. S-layer-like monomolecular arrays of proteins have also been observed in bacterial sheaths (Beveridge and Graham 1991), spore coats (Holt and Leadbetter 1969), and on the surface of the cell wall of eukaryotic algae (Roberts et al. 1985).

The location and ultrastructure of S-layers of a great variety of organisms have been investigated by electron microscopy (EM) (Sleytr and Messner 1983) and atomic force microscopy (AFM) (Müller et al. 1996). The most suitable EM procedure for identifying S-layers on intact cells is freeze-etching (Fig. 2.2) (Sleytr and Glauert 1975; Sleytr 1978; Sleytr and Messner 1989). High resolution studies on the mass distribution of the lattices were performed on negatively stained preparations or unstained, ice-embedded samples. Two- and three-dimensional image analysis involving computer-image reconstruction revealed structural information down to approximately 1 nm (for reviews see Baumeister et al. 1989; Hovmöller 1993;

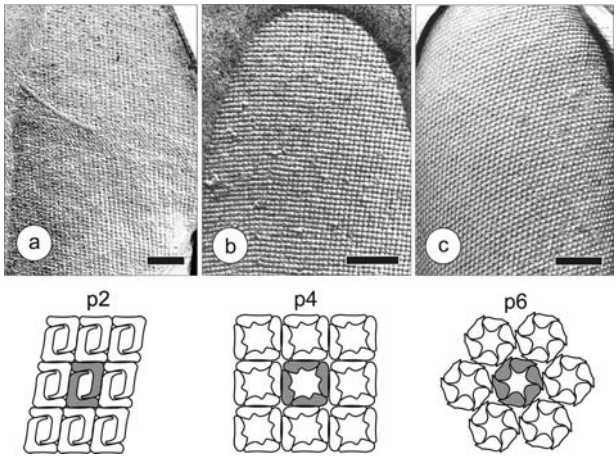

Fig. 2.2 Electron micrograph of freeze-etched preparations of intact cells of (**a**) *Geobacillus stearothermophilus* NRS 2004/3a, (**b**) *Desulfotomaculum nigrificans* NCIB 8706, and (**c**) *Thermoanaerobacter thermohydrosulfuricus* L111-69, showing oblique (p2), square (p4), and hexagonal (p6) lattice symmetry, respectively. The schematic illustration of the respective space groups shows the unit cells that are the building blocks of the S-layer lattice. Bar, 100 nm (modified from Sleytr and Messner 1983)

Beveridge 1994; Sleytr et al. 1996a). More recently, high-resolution studies on the mass distribution of S-layers were also obtained using AFM under aqueous conditions (Müller et al. 1999; Sleytr et al. 1999) and tertiary structure prediction, based on amino acid sequences (Horejs et al. 2008). A common feature of bacterial S-layers is their smooth outer and more corrugated inner surface. Archaeal S-layers frequently reveal pillar-like domains on the inner surface (see Chap. 9).

S-layer subunits can be aligned in lattices with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry (Fig. 2.2). Hexagonal symmetry is predominant among archaea (for compilation see Sleytr et al. 1996b, 2002). The morphological units generally have center-to-center spacings of approximately 3–30 nm. In S-layers, one or even more distinct classes of pores could be observed. Pore sizes were determined to be in the range of approximately 2–8 nm and pores can occupy 30–70% of the surface area.

2.3 Isolation, Chemistry, and Domains

Because of the diversity in the supramolecular structure of prokaryotic cell envelopes, different disruption and isolation procedures for S-layers have been developed. Usually, they are isolated from purified cell-wall fragments by the addition of hydrogen-bond breaking agents (e.g. guanidine hydrochloride or urea) (for review see Messner and Sleytr 1988; Schuster et al. 2005) or detergents or by

cation substitution (e.g. Na^+ or Li^+ replacing Ca^{2+}) (Koval and Murray 1984). Extraction and disintegration experiments revealed that the inter-subunit bonds in the S-layer are stronger than those binding the subunits to the supporting envelope (Sleytr 1975). Special isolation procedures are required for S-layers in those archaea where they are associated with the plasma membrane (see Chap. 9).

Chemical and genetic analyses on many S-layers revealed that they are generally composed of a single protein or glycoprotein species with molecular masses ranging from 40 to 170 kD (for reviews see Sleytr et al. 1993, 1999, 2001a, 2002; Sumper and Wieland 1995; Messner and Schäffer 2003; Åvall-Jääskeläinen and Palva 2005; Claus et al. 2005).

S-layers from bacteria are often weakly acidic proteins, typically containing 40–60% hydrophobic amino acids, and possess few or no sulphur-containing amino acids. The pIs of these proteins range from 4 to 6 although the S-layer proteins from lactobacilli and some archaea (e.g. *Methanothermus fervidus*) have pIs ranging from 8 to 10.

Comparative studies on S-layer genes of organisms from different taxonomic affiliations have shown that homologies between non-related organisms are low, although their amino acid compositions show no significant difference. High homologies are commonly explained by evolutionary relationships but other factors such as growth conditions and environmental stress may also be responsible for structural homologies of S-layer genes. For example, if present in Bacillaceae, high sequence identities are found at the N-terminus. In some strains, S-layer homology (SLH) motives (Lupas et al. 1994) are involved in the attachment of S-layer proteins to polysaccharides (secondary cell wall polymers) that are linked to the underlying peptidoglycan layer (Sára 2001; Mader et al. 2004; Huber et al. 2005). Among the S-layer proteins of *Geobacillus stearothermophilus* strains investigated so far, strain PV72/p2 possesses three N-terminal SLH domains that have been shown to be involved in cell wall attachment of the S-layer protein (compare with Sect. 2.6.4, Fig. 2.3a). In S-layer proteins without SLH domains, positively charged amino acids contained at either the conserved N-terminal region (Fig. 2.3b) or at the C-terminal region (Fig. 2.3c) interact with a peptidoglycan-associated secondary cell wall polymer via direct electrostatic interactions or hydrogen bonds, thereby mediating attachment to the cell wall (Sára 2001; Schäffer and Messner 2005; Egelseer et al. 2008; see also Sect. 2.6.4). As recently evaluated by surface plasmon resonance studies for the S-layer protein SbsC of *G. stearothermophilus* ATCC12980^T, this interaction is highly specific (Ferner-Ortner et al. 2007; compare with Sect. 2.6.4).

Traditionally, S-layers have been studied by TEM techniques and, as a result, much is known about their ultrastructure. Little is known, however, about S-layer protein structure–function relationships. In recent years, with the advent of recombinant DNA technologies, valuable new clues to the structural organization of S-layer proteins have been obtained. Methods such as deletion analysis, cloning of domains, linker mutagenesis, and cysteine scanning mutagenesis have been used (Mesnage et al. 1999; Howorka et al. 2000; Jarosch et al. 2001; Smit et al. 2001).

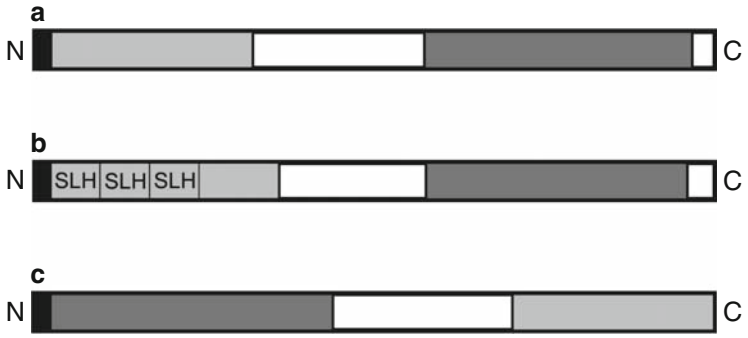

Fig. 2.3 Schematic drawing of the principal organization of S-layer protein domains. (**a**) S-layer protein without SLH domains and N-terminal cell wall anchoring (e.g., *G. stearothermophilus* wild-type strains ATCC 12980 and NRS 2004/a); (**b**) S-layer protein with SLH domains at the N-terminus and N-terminal cell wall anchoring (e.g., *G. stearothermophilus* PV72/p2 and *Lysinibacillus sphaericus* CCM 2177); (**c**) S-layer proteins without SLH domains and C-terminal cell wall anchoring (e.g., *Aneurinibacillus thermoaerophilus* L420-91 and *Lactobacillus acidophilus* ATCC 4356). On the proteins, the signal peptide (*closed square*), the S-layer cell wall binding domain (*open rectangle*) and the crystallization domain (*closed rectangle*) are indicated. Domains as indicated are not to scale in relation to the S-layer protein. N, N-terminus; C, C-terminus

A few post-translational modifications are known to occur in S-layer proteins, including protein phosphorylation and protein glycosylation. S-layer glycoproteins are among the best studied examples of glycosylated prokaryotic proteins (Eichler and Adams 2005; Logan 2006; Messner et al. 2008). They are widely distributed in the major lineages of Archaea, as well as among Bacteria; in the latter lineage, they have been demonstrated mainly within Gram-positive taxa (e.g., *Aneurinibacillus*, *Geobacillus*, *Clostridium*, *Desulfotomaculum*, *Paenibacillus*, *Thermoanaerobacter*, *Thermoanaerobacterium, Lactobacillus*) (Messner 1996; Messner and Schäffer 2000). Complete structural analyses of S-layer glycoprotein glycans from these Gram-positive organisms have been elaborated exclusively by our group. Only recently, glycosylated S-layer proteins have also been convincingly described in the Gram-negative species *Tannerella forsythia* (Lee et al. 2006b). The glycan chains and linkages of bacterial and archaeal glycoproteins are significantly different from those of eukaryotes (Sleytr et al. 2002; Messner and Schäffer 2003; Schäffer and Messner 2004). Most archaeal S-layer glycoprotein glycans consist of only short heterosaccharides, usually not built of repeating units. The predominant linkage types are *N*-glycosidic bonds. The opposite situation is found with bacteria where up to now only *O*-glycosidic linkages have been found. From comparative chemical and structural studies it appears that, at least in most bacteria, S-layers have little or no taxonomic value and may merely be considered as strain-specific characteristics. Moreover, individual strains revealed the capability to synthesize and assemble more than one type of S-layer (glyco)protein array.

2.4 Genetics, Biosynthesis, and Assembly

If present, S-layer proteins are among the most abundant cellular proteins, with a total protein biosynthesis effort of up to 20% being devoted to S-layer protein biosynthesis. The high amount of S-layer protein subunits is required for a complete coverage with a closed S-layer lattice during all stages of the bacterial growth cycle. This is ensured at the molecular level by a combination of strong S-layer gene promoters and high mRNA stability. More recently, the increased knowledge on diverse glycan structures of bacterial S-layer glycoproteins has led to detailed studies of the biosynthesis of these prokaryotic glycoproteins (see Sect. 2.6.4; Eichler and Adams 2005; Messner et al. 2008).

In the context of gene regulation of S-layer biosynthesis, it was interesting to observe that some bacteria can express different S-layer proteins. For example, in the pathogen *Campylobacter fetus,* synthesis of different S-layers enables the organism to circumvent the host's immune response (for a summary see Blaser 1998). S-layer variation might have evolved as important strategy of the respective bacteria to respond to changing environmental conditions. S-layer variation leads to the synthesis of alternate S-layer proteins, either by the expression of different S-layer genes or by recombination of partial coding sequences, and has been described in both pathogens and non-pathogens (Sára et al. 1996; Dworkin and Blaser 1997; Scholz et al. 2001; Jakava-Viljanen et al. 2002).

S-layers represent a fascinating system for studying the dynamic process of self-assembly of a supramolecular biological structure (Sleytr et al. 2001b, 2005). Detailed studies on selected S-layers from bacteria have shown that isolated subunits assemble spontaneously into regular arrays after removal of the disrupting agent used for their isolation (see Chap. 16). Studies on the in vivo morphogenesis of S-layers demonstrated that at high growth rates approximately 500 subunits per second must be synthesized, translocated to the cell surface, and incorporated into the pre-existing S-layer lattice. Differences in the net surface charge and specific (e.g. lectin-type) interactions between the inner and outer surface of the S-layer proteins to the supporting layer have been shown to be essential for proper orientation (Sleytr et al. 2002). Ultrastructural data indicate that during growth S-layer subunits (protomers) must have the ability to recrystallize on the supporting envelope layer, assuming a low free energy arrangement (Sleytr and Plohberger 1980; Sleytr and Messner 1989) with the intrinsic tendency to assume a continuously regular lattice during cell growth (Sleytr 1975, 1981; Sleytr and Glauert 1975).

2.5 Function

Considering that S-layer-carrying organisms are ubiquitous in the biosphere, the supramolecular concept of a closed, isoporous, protein meshwork has the potential to fulfil a broad spectrum of functions. When bacteria are no longer exposed to

Table 2.1 Functions of S-layers (modified after Sleytr et al. 2002)

General functions	Specific functions
Cell shape determination and maintenance	Determination of cell shape and cell division in archaea that possess S-layers as exclusive wall component
Isoporous membrane structure	Molecular sieve in the ultrafiltration range
	Delineating a compartment (periplasm) in Gram-positive bacteria
	Prevention of non-specific adsorption of macromolecules
	Hindrance of molecules to reach the cell wall proper (e.g. lytic enzymes)
Protective coat	Prevention of predation of Gram-negative bacteria by bacterial viruses (*Bdellovibrio bacteriovorous*)
Surface recognition and cell adhesion to substrates	Physicochemically and morphologically well-defined matrices
	Masking of net-negative charges of peptidoglycan-containing layer in Bacillaceae
Adhesion zone for exoenzymes	High-molecular-weight amylase of *Geobacillus stearothermophilus* wild-type strains
	Pullulanase and glycosyl hydrolases of *Thermoanaerobacter thermosulfurigenes*
Pathogenicity and cell adhesion	Virulence factor of pathogenic organisms
	Important role in invasion and survival within the host
	Specific binding of host molecules
	Protective coat against complement killing
	Ability to associate with macrophages and to resist the effect of proteases
	Production of immunologically non-crossreactive S-layers (S-layer variation)
Template for fine grain mineralization	Induction of precipitation of gypsum and calcite in *Synechococcus* strains and shedding of mineralized S-layers

natural environmental selection pressures, S-layers can be lost, indicating that the considerable biosynthesis effort is only required in natural habitats. In functional terms, S-layers are generally part of complex envelope structures (Fig. 2.1) and consequently should not be considered as isolated layers. Many of the functions assigned to S-layers are still hypothetical and not based on firm experimental data (for compilation see Table 2.1) (for reviews see Sleytr 1997; Sleytr and Beveridge 1999; Sára and Sleytr 2000; Sleytr et al. 2002; Engelhardt 2007).

2.6 Specific S-Layer-Carrying Organisms

Since the last comprehensive review on S-layer proteins in 1996 (Sleytr et al. 1996b), a large number of reports have appeared in the literature, documenting the universal occurrence of S-layers in the prokaryotic world. These informations are summarized in Table 2.2 including a complete coverage of GenBank accession numbers of S-layer structural genes and presently known data on surface layer glycosylation (*slg*) gene clusters.

Table 2.2 Bacterial S-layers (new entries are in alphabetical order, since 1995; for older references see Sleytr et al. (1996b) and Messner and Sleytr (1992)). In addition, a complete list of GenBank accession numbers of bacterial S-layer genes (see also Sleytr et al. 2002) and glycosylation-related genes is given

Organism[a]	S-layer lattice type[b]/ molecular mass (kDa)/ dimensions (nm)	S-layer gene/GenBank accession no.	Glycosylation/ GenBank accession no.	Reference
Aeromonas hydrophila, strain TF7	S/52/12	*ahsA*/L37348	–	Thomas and Trust (1995)
A. hydrophila, strain J-1	–/52/–	–/–	–	Yan et al. (1996); Bi et al. (2007)
A. hydrophila, serotype O:14 and O:81 strains	S/54, 53/–	–/–	–	Esteve et al. (2004)
Aeromonas salmonicida, strain A450	S/49/12.5	*vapA*/M64655	–	Chu et al. (1991)
A. salmonicida, several strains	–/~50/–	–/–	–	Madetoja et al. (2003)
Aeromonas sp., serotype O:11, several strains	–/–/–	–/–	–	Merino et al. (1996)
Alicyclobacillus tolerans strain K1^T	H/–/15-16	–/–	–	Duda et al. (2006)
Ammonifex degensii DSM 10501^T	S/–/11.0	–/–	–	Huber et al. (1996)
Anaerobranca gottschalkii	P/–/–	–/–	–	Prowe and Antranikian (2001)
Aneurinibacillus aneurinilyticus (*Bacillus aneurinolyticus*) JCM 9024^T	–/–/–	–/–	–	Shida et al. (1994); Heyndrickx et al. (1997)
A. thermoaerophilus (*Bacillus thermoaerophilus*), strain L420-91^T	S/116/10.0	*satA*/AY395578	*O*-glycan, *slg* cluster/ AY442352	Kosma et al. (1995a); Meier-Stauffer et al. (1996); Novotny et al. (2004a); Davis et al. (2007); Messner et al. (2008)
A. thermoaerophilus DSM 10155/G$^+$	S/153/10.0	*satB*/AY395579	*O*-glycan, *slg* cluster/ AF324836	Kosma et al. (1995b); Meier-Stauffer et al. (1996); Novotny et al. (2004a)
A. thermoaerophilus DSM 10155/G$^-$	S/–/10.0	*satC*/AY422725	–	Novotny et al. (2004a)
Anoxybacillus pushchinensis DSM 12423^T	–/–/–	–/–	–	Pikuta et al. (2000a)
Bacillus anthracis, Sterne derivative, substrain 9131	EA1: O/94/6.9 & 8.8 Sap: O/94/18.4 & 8.1	*eag*/X99724 *sap*/Z36946	–	Etienne-Toumelin et al. (1995); Mesnage et al. (1997); Couture-Tosi et al. (2002)
B. anthracis, strains Sterne 34F2, STI-1	Sap: –/94/–	–/–	–	Mikshis et al. (2006)
B. anthracis, several strains	Sap: –/–/– EA1:–/–/–	BA0885/AE016879 BA0887/AE016879	– –	Chitlaru et al. (2007)
B. anthracis, several strains	–/–/–	–/–	–	Zasada et al. (2005)
Bacillus borstelensis JCM 9022^T	–/–/–	–/–	–	Shida et al. (1995)

Bacillus cereus, several strains	O/97/–	–/–	–	Kotiranta et al. (1998)
B. cereus, several strains	–/~90/–	–/–	–	Mignot et al. (2001)
Bacillus formosus JCM 9169^T	–/–/–	–/–	–	Shida et al. (1995)
Bacillus licheniformis, strain NM 105	S/94/–	*olpA*/U38842	–	Zhu et al. (1996)
Bacillus pseudofirmus (*Bacillus firmus*), strain OF4	O/90-95/9.2 & 12.8	*slpA*/AF242295	–	Gilmour et al. (2000)
Bacillus reuszeri JCM 9170^T	–/–/–	–/–	–	Shida et al. (1995)
Bacillus smithii, several strains	O/132-138/ ~11.2 & 10.3	–/–	+	Messner et al. (1997)
Bacillus thermosphaericus, strains P-11, P-67	–/–/–	–/–	–	Andersson et al. (1995)
Bacillus thuringiensis ssp. *finitimus*, strain CTC	–/100/–	*ctc*/AJ012290	–	Sun et al. (2001); Zhang et al. (2007)
	–/100/–	*ctc2*/AY460125	–	
B. thuringiensis ssp. *galleriae* NRRL 4045	O/91/8.5 & 7.2	*slpA*/AJ249446	–	Mesnage et al. (2001)
B. thuringiensis ssp. *israelensis*, strain 4Q2	–/–/–	*slpA*/X62090	–	Mesnage et al. (2001)
B. thuringiensis ssp. *israelensis*, two strains	–/–/–	–/–	–	Wiwat et al. (1995)
B. thuringiensis ssp. *konkukian*	–/102/–	–/–	–	Lee et al. (2006a)
B. thuringiensis, several strains	–/–/–	–/–	–	Wang et al. (2004); Xu et al. (2004)
	–/65-130/–	*cry020*/–	–,+	
Bacillus sp., several strains	O,S,H/66-255/5-23	–/–	–,+	Sidhu and Olsen (1997)
blue filamentous bacterial community	H/–/27	–/–	–	Takacs et al. (2001)
Brevibacillus brevis (*Bacillus brevis*), strain 47	OWP: H/130/14.5	–/M14238	–	Tsuboi et al. (1986); Tsuboi et al. (1988)
	MWP: H/150/14.5	–/P06546	–	
B. brevis, strain HPD31	H/135/18.1	*HWP*/D90050	–	Ebisu et al. (1990)
Caloramator viterbensis DSM 13723^T	–/–/–	–/–	–	Seyfried et al. (2002)
Campylobacter fetus ssp. *fetus*, strain 84-32 (23D)	H/97-149/8.8	*sapA, sapA1-sapA8*/ AY211269	–	Blaser and Gotschlich (1990); Yang et al. (1992)
C. fetus ssp. *fetus*, strain 84-91	H/98/24	*sapB*/U25133	–	Dworkin et al. (1995)
C. fetus ssp. *fetus* CIP53.96^T	–/112/–	*sapB2*/AF048699	–	Casadémont et al. (1998)
C. fetus, several strains	S,H/97-149/8	*sapA*, *sapB*/AY211269	–	Thompson 2002
C. fetus ssp. *fetus*, several strains	–/–/–	–/–	–	Graham (2002)
C. fetus ssp. *fetus*	–/87 + 101/–	–/–	–	Gürtürk et al. (2007)

(*continued*)

Table 2.2 (continued)

Organism[a]	S-layer lattice type[b]/ molecular mass (kDa)/ dimensions (nm)	S-layer gene/GenBank accession no.	Glycosylation/ GenBank accession no.	Reference
Campylobacter rectus ATCC 33238	H/150/– –/150/–	*slp*/AB001876 –/–	– +	Miyamoto et al. (1998); Hinode et al. (2002)
C. rectus, strain 314	H/145/–	*crs*/AF010143	–	Wang et al. (1998); Thompson (2002)
C. rectus, several strains	–/150/–	–/–	–	Wyss (1995)
C. rectus, several strains	–/150-166/–	–/–	–	Nitta et al. (1997)
Carboxydobrachium pacificum DSM 12653^T	H/–/–	–/–	–	Sokolova et al. (2001)
Carboxydocella thermautotrophica DSM 12356^T	–/–/–	–/–	–	Sokolova et al. (2002)
Caulobacter vibrioides (*C. crescentus*), CB15 and several other strains	H/130/23.5 –/100-193/–	*rsaA*/AF062345 *rsaD*, *rsaE*/AY158231-AY158236	–	Smit and Agabian (1984) Iuga et al. (2004)
Clostridium acetireducens DSM 7310^T	P/–/–	–/–	–	Örlygsson et al. (1996)
Clostridium difficile, strain C253	S,H/36 + 47, 10.5 + 13.0	*slpA*/AJ291709	+	Karjalainen et al. (2001)
C. difficile, strains 167, Y	–/39 & 43, 37 & 41/–	*slpA*/AF478570, AF478571	+	Calabi et al. (2001); Calabi and Fairweather (2002)
C. difficile, several strains	–/20-40 + 45-64/–	–/–	–	Poxton et al. (1999)
C. difficile, 5 strains	S,H/36-43 + 46-56, 10.4-11.8 + 12.2-13.1	*slpA*/–	+	Cerquetti et al. (2000)
C. difficile, several strains	–/37-45 + 48-56 /–	–/–	–	McCoubrey and Poxton (2001); Poilane et al. (2007)
C. difficile, strains R8366, R7404, and several other strains	–/–/–	*slpA*/AF448119-448129, AF448365-AF448373, AF458877-AF458885, AJ300676, AJ300677	–	Karjalainen et al. (2002)
C. difficile, strains VPI 10463, 630	–/36 + 50, 35 + 48/–	–/–	–	Mukherjee et al. (2002)
C. difficile, strain 630	–/36 + 45/–	–/–	–	Wright et al. (2005)
C. difficile, strain R13537	–/–/–	–/–	–	Ní Eidhin et al. (2008)
Clostridium frigidicarnis, strain SPL77A^T	–/–/–	–/–	–	Broda et al. (1999)

Clostridium phytofermentans ATCC 700394^T	–/–/–	–/–	–	Warnick et al. (2002)
Clostridium tetani, strain E88	–/–/–	*CTC00462*, *CTC00491*/ AE015927	–	Brüggemann et al. (2003)
C. tetani, several strains	–/160–230/–	*slpA*/AE015927	–	Qazi et al. (2007)
Clostridium thermocellum ATCC 27405	–/–/–	*slp1*/Q06852 *slp2*/Q06853	–	Fujino et al. (1993)
C. thermocellum NCIMB 10682	P/140/–	*slpA*/U79117	–	Lemaire et al. (1998)
Clostridium thiosulfatireducens DSM 13105^T	–/–/–	–/–	–	Hernández-Eugenio et al. (2002)
Clostridium sp. strain LP1	P/–/–	–/–	–	McSweeney et al. (1999)
Comamonas-like organism	S/32/13	–/–	–	Wai et al. (1995)
Corynebacterium efficiens, strain YS-314	–/52/–	CE2690/BA000035	–	Hansmeier et al. (2006a)
Corynebacterium glutamicum, strain Bl 15	H/64/16	*csp2*/X69103	–	Peyret et al. (1993); Scheuring et al. (2002)
C. glutamicum, several strains	H/–/–	–/–	–	Sousal-Hoebeke et al. (1999)
C. glutamicum, several strains	H/55-66/15.2-17.4	*cspB*/AY524990-AY525017	–	Hansmeier et al. (2004)
C. glutamicum ATCC 13032	H/54/–	*cspB*/AY842007	–	Hansmeier et al. (2006b)
Corynebacterium melassecola ATCC 17965	H/–/–	–/–	–	Sousal-Hoebeke et al. (1999)
Cyanobacteria, several strains	P,O,S,H /–/2.5-22	–/–	–	Rachel et al. (1997); Šmarda et al. (1996; 2002)
Dehalobacter restrictus, strain TEA	H/–/–	–/–	–	Wild et al. (1996)
Dehalobacter restrictus DSM 9455^T	H/–/–	–/–	–	Holliger et al. (1998)
Dehalococcoides ethenogenes, strain 195	–/–/–	DET1470/CP000027	–	Morris et al. (2006)
Dehalococcoides sp., strain CBDB1	–/–/–	cbdbA1368/AJ965256	–	Adrian et al. (2007)
Deinococcus geothermalis DSM 11300^T	–/–/–	–/–	–	Ferreira et al. (1997)
Deinococcus murrayi DSM 11303^T	–/–/–	–/–	–	Ferreira et al. (1997)
Deinococcus radiodurans, strain Sark	H/107/18	*HPI gene*/M17895	+	Peters et al. (1987); Müller et al. (1996)
Deinococcus radiodurans, strain R1	H/99/– –/37/–	*hpi*/DR2508, NC_001263 *slpA*/DR1185	+	White et al. (1999); Rothfuss et al. (2006)

(*continued*)

Table 2.2 (continued)

Organism[a]	S-layer lattice type[b]/ molecular mass (kDa)/ dimensions (nm)	S-layer gene/GenBank accession no.	Glycosylation/ GenBank accession no.	Reference
Desulfotomaculum alkaliphilum DSM 12257^T	S/–/–	–/–	–	Pikuta et al. (2000b)
Desulfotomaculum auripigmentum ATCC 700205	H/–/–	–/–	–	Newman et al. (1997)
Desulfotomaculum halophilum DSM 11559^T	–/–/–	–/–	–	Tardy-Jacquenod et al. (1998)
Desulfurobacterium thermolithotrophicum DSM 11699^T	O/–/11.3 & 6.3	–/–	–	L'Haridon et al. (1998)
Flavobacterium denitrificans DSM 15936^T	–/–/–	–/–	–	Horn et al. 2005
Geobacillus stearothermophilus (*Bacillus stearothermophilus*), strain PV72/p6	H/130/22.5	*sbsA*/X71092	–	Kuen et al. (1994); Kuen et al. (1995)
G. stearothermophilus, strain PV72/p2	O/98/–	*sbsB*/X98095	–	Kuen et al. (1997)
G. stearothermophilus ATCC 12980^T	O/122/9.5 & 5.2	*sbsC*/AF055578	dTDP-L-Rha operon/ AY278519	Jarosch et al. (2000); Novotny et al. (2004b)
G. stearothermophilus ATCC 12980 variant	O/92[c]-175/–	*sbsD*/AF228338	+	Egelseer et al. (2001)
G. stearothermophilus, strain NRS 2004/3a	O/93[c]-170/11.6 & 9.4	*sgsE*/AF328862	*O*-glycan, *slg* cluster/ AF328862	Schäffer et al. (2002); Novotny et al. (2004b)
G. stearothermophilus, strain L32-65	O/96/11.9 & 8.6	*sgsF*/DQ414249	dTDP-L-Rha operon/ AY278518	Novotny et al. (2004b)
Geobacillus tepidamans, strain GS5-97^T	O/106-166/11.2 & 7.9	*sgtA*/AY883421	*O*-glycan, *slg* cluster/ AY883421	Schäffer et al. (2004); Kählig et al. (2005); Zayni et al. (2007)
Geosporobacter subterraneus DSM 17957^T	–/–/–	–/–	–	Klouche et al. (2007)
Gracilibacter thermotolerans DSM 17427^T	–/–/–	–/–	–	Lee et al. (2006c)
Hyphomonas jannaschiana ATCC 33884	–/29 + 116/–	–/–	+	Shen and Weiner (1998)
Lactobacillus acidophilus ATCC 4365^T	O/43/11.8 & 5.3	–/X71412 –/X89375, X89376	–	Boot et al. (1993); Boot et al. (1995)
Lactobacillus acidophilus ATCC 4365^T	–/20/–	*sbc*/	–	Yeh and Chou (2006)

L. acidophilus, strain NCFM	–/–/–	*slpA*/NC_006814	–	Altermann et al. (2005)
L. acidophilus, strain M92	–/45/–	*slpA*/–	–	Kos et al. (2003); Frece et al. (2005)
Lactobacillus brevis ATCC 8287	–/54/–	*slpA*/Z14250	–	Vidgrén et al. (1992)
L. brevis ATCC 14869	R type: O/43 + 50/8.5 & 5.0 S type: O/50/8.4 & 5.0	*slpB*/AY040846 *slpC*/AY040847 *slpD*/AY040846	–	Jakava-Viljanen et al. (2002)
L. brevis, several strains	–/38-55/–	–/–	–	Yasui et al. (1995)
L. brevis	–/54/– –/50/–	–/–	–	Garrote et al. (2004); Hollmann et al. (2007)
Lactobacillus crispatus JCM 5810	P/46/–	*cbsA*/AF001313 *cbsB*/AF079365	–	Sillanpää et al. (2000)
L. crispatus, strain LMG 12003	–/–/–	*slpNA*/AF253043 *slpNB*/AF253044	–	Sillanpää et al. (2000)
L. crispatus DSM 20584	–/–/–	–/–	–	Schär-Zammaretti and Ubbink (2003)
L. crispatus, strain M247	–/–/–	–/AJ007839	–	Ventura et al. (2000)
L. crispatus, strain MH315	–/–/–	*lbsA*, *lbsB*/AB110090	–	Mota et al. (2006)
L. crispatus, strain ZJ001	–/42/–	*slpA*, *slpB*/DQ923618	–	Chen et al. (2007)
Lactobacillus delbrueckii ssp. *bulgaricus*, several strains	–/31/–	–/–	–	Gatti et al. (2001)
L. delbrueckii ssp. *lactis*, several strains	–/18-21/–	–/–	–	Gatti et al. (2001)
Lactobacillus diolivorans DSM 14421^T	–/–/–	–/–	–	Krooneman et al. (2002)
Lactobacillus gallinarum, several strains	–/43-52/–	*lgsA–lgsI*/AY597259-AY597268	–	Hagen et al. (2005)
Lactobacillus helveticus ATCC 12046	O/43.5/9.6 & 4.5	–/–	+	Mozes and Lortal (1995)
L. helveticus, strain CNRZ 892	–/43/–	*slpH*/X91199, AJ001931	–	Callegari et al. (1998)
L. helveticus several strains	–/–/–	*slpH2*/X92752, AJ388558-AJ388564	–	Ventura et al. (2000)

(*continued*)

Table 2.2 (continued)

Organism[a]	S-layer lattice type[b]/ molecular mass (kDa)/ dimensions (nm)	S-layer gene/GenBank accession no.	Glycosylation/ GenBank accession no.	Reference
L. helveticus several strains	–/–/–	*slpH1*/X91199 *prtY*/AB026985.1	–	Gatti et al. (2005)
L. helveticus, strain R0052	–/48/–	*slp*/DQ123583	–	Naser et al. (2006a); Johnson-Henry et al. (2007)
L. helveticus, strain Zuc2	–/45/–	–/–	–	Scolari et al. (2006)
Lactobacillus kefir, JCM 5818 and several other strains	–/69/–	–/–	+/–	Garrote et al. (2004); Golowczyc et al. (2007); Hollmann et al. (2007); Mobili et al. (2009)
Lactobacillus kefirgranum, several strains	–/–/–	–/–	–	Vancanneyt et al. (2004)
Lactobacillus parakefir, several strains	–/66-69/–	–/–	–	Garrote et al. (2004)
Lactobacillus plantarum, several strains	–/–/–	–/–	–	Tallon et al. (2007)
Lactobacillus suntoryeus, strains SA^T, Y10, M4	–/–/–	*slp*/AY562189, AY641395, AY644397	–	Cachat and Priest (2005)
Lactobacillus sp., several strains	–/45-62/–	–/–	–	Jakava-Viljanen and Palva (2007)
Leptospira interrogans serovar Lai	–/–/–	*Lslp*/AF325807	–	Hu et al. (2004)
Lysinibacillus sphaericus (*Bacillus sphaericus*), strain 2362	S/125/14	*gene 125*/M28361 *gene 80* /–	–	Bowditch et al. (1989)
L. sphaericus, strain P1	S/140/13	*sequence 8*/A45814	–	Deblaere et al. (1999)
L. sphaericus NCTC 9602	S/142/12.5	–/– *slfA*/AJ849547	–	Mertig et al. (2001); Pollmann et al. (2005)
L. sphaericus, strain JG-A12	S/–/12.2	–/ AJ292965 *sflB*/AJ849549	–	Wahl et al. (2001); Pollmann et al. (2005)
L. sphaericus CCM 2177	S/127/13.1	*sbpA*/AF211170	–	Ilk et al. (2002)
L. sphaericus ATCC 4525	S/~120/13.3	*sbpA*/–	–	Norville et al. (2007)
magnetotactic cocci, several strains	H/–/–	–/–	–	Freitas et al. (2003)
Marinobacterium georgiense ATCC 700074	S/–/–	–/–	–	González et al. (1997)
Methylobacter alcaliphilus, strain 20Z	P/–/–	–/–	–	Khmelenina et al. (1999)
Methylobacter modestohalophilus, strain 10S	O/–/–	–/–	–	Kalyuzhnaya et al. (1998)
Methylomicrobium buryatense, strain 5B	–/–/–	–/–	–	Kaluzhnaya et al. (2001)

Methylomicrobium alcaliphilum	H/27/–	–/–	+	Trotsenko and Khmelenina (2002)
Methylothermus thermalis, strain MYHT[T]	O/–/–	–/–	–	Tsubota et al. (2005)
microbes from siliceous sediments (clostridia or *Desulfotomaculum* sp. strains)	H/–/9.7	–/–	–	Phoenix et al. (2005)
Microbulbifer hydrolyticus ATCC 700072	–/–/–	–/–	–	González et al. 1997
Mogibacterium diversum ATCC 700923[T]	–/–/–	–/–	–	Nakazawa et al. (2002)
Moorella glycerini DSM 11254	S/–/10	–/–	–	Slobodkin et al. (1997)
Nitrobacter alkalicus LMD 97.163	H/–/–	–/–	–	Sorokin et al. (1998)
Oceanicaulis alexandrii	P/–/–	–/–	–	Strömpl et al. (2003)
Oscillatoria sp.	Fibrils/–/25–30	–/–	–	Adams et al. (1999)
Oscillibacter valericigenes DSM 18026 [T]	–/–/–	–/–	–	Iino et al. (2007)
Paenibacillus anaericanus DSM 15890 [T]	–/–/–	–/–	–	Horn et al. (2005)
Paenibacillus terrae, srain MH72	–/–/–	–/–	–	Horn et al. (2005)
Paenibacillus sp. strain W–61	–/100/–	–/–	–	Ito et al. (2003)
Paenibacillus sp. strain JDR-2	–/80/–	–/–	–	St. John et al. (2006)
Parabacteroides distasonis (*Bacteroides distasonis*)	–/–/–	–/–	+	Fletcher et al. (2007)
Peptostreptococcus anaerobius, several strains	P/78/–	–/–	–	Kotiranta et al. (1995)
Phormidium unicatum, strain Baikal	O/–/10 & 9.6; fibrils	–/–	–	Hoiczyk and Hansel (2000)
Rickettsia prowazekii, strain Brein 1	S/120/13	*spaP*/M37647	–	Carl et al. (1990)
Rickettsia rickettsii, strain R	S/120/13	*p120*/X16353	–	Gilmore et al. (1989)
Rickettsia typhi, strain Wilmington	S/120/13	*slpT*/L04661	–	Hahn et al. (1993)
Selenomonas ruminantium, strain OB268	–/42/–		–	Kalmokoff et al. (2000)
Serratia marcescens, strains SM8000, SM176	–/100/–	*slaA*/AB007125	–	Kawai et al. (1998)
Sporobacter termitidis DSM 10068[T]	–/–/–	–/–	–	Grech-Mora et al. 1996
Sporosarcina ureae ATCC 13881	S/116/13.2	–/– *sslA*/AM293285	–	Mertig et al. (1999); Ryzhkov et al. (2007)
Sulfobacillus thermosulfidooxidans, two strains	–/–/–	–/–	+	Severina et al. (1995)
Symbiobacterium thermophilus, strain T[T]	–/–/–	–/–	–	Ohno et al. (2000)
Synechococcus sp. strain WH8113	O/–/12	–/–	–	Samuel et al. (2001)
Synechococcus sp. strain WH8102	O/–/12	*swmA*/AF056046	+	McCarren et al. (2005)
Synechocystis sp. strain PCC 6803	–/–/–	–/–	–	Mohamed et al. (2005)

(*continued*)

Table 2.2 (continued)

Organism[a]	S-layer lattice type[b]/ molecular mass (kDa)/ dimensions (nm)	S-layer gene/GenBank accession no.	Glycosylation/ GenBank accession no.	Reference
Tannerella forsythia (*Bacteroides forsythus*) ATCC 43037	–/270 + 230/–	–/–	?	Higuchi et al. (2000); Sabet et al. (2003); Lee et al. (2006b)
	–/200 + 210/–	*tfsA* + *tfsB*/AY423857	+	
T. forsythia, several strains	–/196-245/–	–/–	+	Honma et al. (2007)
Tepidimicrobium ferriphilum DSM 16624^T	–/–/–	–/–	–	Slobodkin et al. (2006)
Thermincola carboxydiphila DSM 17129^T	–/–/–	–/–	–	Sokolova et al. (2005)
Thermoanaerobacter kivui DSM 2030	H/~90/19	*slp*/M31069	+	Peters et al. (1989)
T. subterraneus DSM 13054^T	–/–/–	–/–	–	Fardeau et al. (2000)
T. tengcongensis JCM 11007^T	–/–/–	–/–	–	Xue et al. (2001)
T. wiegelii DSM 10319^T	–/–/–	–/–	–	Cook et al. (1996)
Thermoanaerobacterium aotearoense DSM 10170^T	H/–/~12	–/–	–	Liu et al. (1996)
T. polysaccharolyticum DSM 13641^T	–/–/–	–/–	–	Cann et al. (2001)
T. thermosaccharolyticum, strain E207-71	H/83[c]-210/~12	–/–	*O*-glycan, *slg* cluster/ AY422724	Altman et al. (1995); Novotny et al. (2004a)
T. thermosulfurigenes, strain EM1	–/83[c]-190/–		+	Brechtel et al. (1999)
T. zeae DSM 13642^T	–/–/–	–/–	–	Cann et al. (2001)
Thermobrachium celere, strain JW/YL-NZ35	H/145/8.5	–/–	–	Engle et al. (1996)
Thermotoga maritima, strain MSB8	T/42/12.4	*ompα*–/X68276	–	Engel et al. (1992)
Thermus thermophilus strain HB8	H/100/24	*slpA*/X57333	–	Faraldo et al. (1992); Olabarría et al. (1996)
	H/36 + 50 + 52/18.8	*slpM*/X90369	–	
Thiobaca trueperi DSM 13587^T	–/–/–	–/–	–	Rees et al. (2002)
Treponema ssp., several strains	H/43–62/–	*msp*/U66255, U66256	–	Fenno et al. (1997)
Weissella cibaria, several strains	–/~50/–	–/–	–	Kang et al. (2005)

[a]Bacterial names in parentheses (e.g. *Bacillus* sp.) refer to former designations of the organisms
[b]Abbreviations: H, hexagonal (p6); T, trimeric, (p3); S, square (p4); O, oblique (p1, p2); P, periodic
[c]Deglycosylated S-layer protein

In the following subsections, information has been summarized on those organisms that were the prime research targets within the last 5–6 years.

2.6.1 *Bacillus anthracis*

B. anthracis, a Gram-positive, spore-forming bacterium is the etiological agent of anthrax, a disease involving toxemia and septicemia. In 2001, its potential use in biological warfare was demonstrated in the US postal system.

In addition to virulence factors (Mock and Fouet 2001), *B. anthracis* synthesizes two S-layer proteins, termed Sap and EA1 (Etienne-Toumelin et al. 1995; Mesnage et al. 1997). Both proteins have the same modular organization, an N-terminal cell wall anchoring domain consisting of three S-layer homology (SLH) motifs followed by a putative crystallization domain (Mesnage et al. 1999). Electron microscopy and genetic analyses of Sap using a bacterial two-hybrid system confirmed that the region comprising 604 C-terminal amino acids represents the crystallization domain (Candela et al. 2005).

During the exponential growth phase, *B. anthracis* cells are surrounded by the Sap S-layer protein, which is replaced by the EA1 S-layer protein when the cells enter the stationary phase (Mignot et al. 2002). By using mutant strains for electron microscopical studies and image processing, the S-layer structural organization was elucidated (Couture-Tosi et al. 2002). The projection map of EA1 revealed that the protein consists of one very large domain forming the central body around which the three smaller domains are surrounded. EA1 subunits crystallize into a lattice with p1 symmetry. For the Sap S-layer protein, six or seven domains that repeat themselves along the two axes of the crystal were identified. The Sap protein is homogeneously distributed on the cell surface, whereas the EA1 protein displays a crazy-paving aspect (Couture-Tosi et al. 2002). For both S-layer proteins, a previously unrecognized ability to function as murein hydrolases has been demonstrated (Ahn et al. 2006).

The S-layer components are encoded by the contiguous chromosomal S-layer genes *sap* and *eag*, and the S-layer switch is controlled at the transcriptional level, with *sap* being transcribed during the exponential growth phase, and *eag* being only transcribed during the stationary phase. Transcription of both genes is ensured by distinct sigma factors and the phase-specific expression of *eag* is strictly dependent on Sap, because this protein can act as a direct repressor of *eag* (Mignot et al. 2002). Mignot et al. (2003, 2004) provided evidence that expression of the chromosomally located S-layer genes is also influenced by two genes located on the virulence plasmid pXO1 encoding the two transcriptional regulators PagR and AtxA (Mignot et al. 2003, 2004). The latter was found to be a master regulator that also controls the transcription of S-layer genes by controlling the synthesis of PagR.

In vitro translation of selected open reading frames (ORFs) on the virulence plasmid pXO1, followed by analysis of the reactivity of the ORF products with hyperimmune anti-*B. anthracis* antisera, led to the identification of two S-layer

proteins, both carrying three SLH-motifs (Ariel et al. 2002). Immunoreactivity studies using a truncated S-layer protein form devoid of the SLH moiety indicated that the C-terminal segment contributes significantly to S-layer immunogenicity (Ariel et al. 2002).

Cloning and sequencing of the DNA region upstream of the S-layer gene *sap* led to the identification of the genes *csaA* and *csaB* (cell surface anchoring), that are organized as an operon. CsaA revealed similarity to oligosaccharide transporters of Gram-negative bacteria, whereas CsaB was identified as pyruvyl transferase, which is supposed to be involved in pyruvylation of the peptidoglycan-associated cell wall polymer (Mesnage et al. 2000). Recently, Choudhury and coworkers (2006) described the structure of the polysaccharide released from *B. anthracis* vegetative cell walls by hydrogen fluoride (HF). It consists of Gal, ManNAc, and GlcNAc in a molar ratio of 3:1:2. Since it was speculated that the ability of neutrophils to kill vegetative *B. anthracis* cells depends on the binding of α-defensins to this carbohydrate by a lectin-like binding, the major cell wall polysaccharide could be considered as a target for development of specific antimicrobials against anthrax.

Chitlaru and coworkers identified a group of *B. anthracis* membrane proteins which could be useful for eliciting protective immune responses and serve as potential candidates for the development of an improved anthrax vaccine (Chitlaru et al. 2004, 2007). Among these, the S-layer proteins Sap and EA1 were found to represent more than 75% of the *B. anthracis* membrane fraction and undergo post-translational modifications. It was postulated that subpopulations of differently glycosylated Sap and EA1 may exist (Chitlaru et al. 2004). A comparative proteomic approach was employed to elucidate the differences among the extracellular proteomes (secretomes) of three isogenic strains under host-simulated conditions (Lamonica et al. 2005). In the fully virulent strain ($pXO1^+$ $pXO2^+$), while the partially cured derivative ($pXO1^+$ $pXO2^-$), the two chromosomally encoded S-layer proteins Sap and EA1 were found to be up-regulated whereas the pXO1-encoded S-layer protein A represented a unique protein in the derivative strain devoid of plasmid pXO2 (Chitlaru et al. 2004).

Proteome analysis was used to define the exact protein composition of the current UK anthrax vaccine (Whiting et al. 2004). In addition to the protective antigen PA, the key immunogen of the vaccine, the presence of the two other toxin components, as well as the S-layer proteins, EA1 and SAP, could be established. The S-layer proteins turned out to be also immunogenic in man but their presence did not adversely affect the protective immune response induced by PA (Baillie et al. 2003). Specific identification of *B. anthracis* is vital for the accurate treatment of afflicted personnel during biological warfare situations and civilian terrorist attacks. Unfortunately, this pathogenic *Bacillus* species shares so much genetic material with *B. cereus* and *B. thuringiensis* that its discrimination from the other species can be problematic. However, unique genomic differences could be identified and the S-layer gene sequence was found to be among these *B. anthracis*-specific "DNA signatures" which are capable of quickly detecting all six genetically

distinct groups of *B. anthracis* with any rapid DNA-based detection platform (Radnedge et al. 2003).

It was demonstrated that EA1, the abundant, highly antigenic S-layer protein of vegetative *B. anthracis* cells, is not a spore component as previously stated but a persistent contaminant in spore preparations. Thus, EA1 is not a suitable marker for spore detection and could result in failure to detect highly purified spores or to accurately estimate spore number, which would have catastrophic consequences (Redmond et al. 2004; Williams and Turnbough 2004). On the other hand, affinity purification of antigenic protein and tandem mass spectrometry revealed that EA1 represents a highly specific biomarker that enables simultaneous identification and verification of vegetative *B. anthracis* cells (Krishnamurthy et al. 2006).

2.6.2 *Clostridium difficile*

Clostridium difficile is a Gram-positive, spore-forming anaerobic bacterium, which is recognized as the etiological agent of antibiotic-associated diarrhea and pseudomembraneous colitis in humans and which can cause significant morbidity in hospitalized patients (Kelly and LaMont 1998). Its pathogenicity is mediated by two toxins, A and B, both of which damage the human colonic mucosa and are potent tissue-damaging enzymes (Borriello et al. 1990).

S-layer proteins are the most abundant extracellular *C. difficile* proteins found during both high and low toxin production (Mukherjee et al. 2002). This organism is unusual in expressing two S-layer proteins which associate to form the S-layer, one of high molecular weight (HMW) and the other of low molecular weight (LMW). Both subunits are encoded by the *slpA* gene and are produced from post-translational cleavage of a single precursor (Calabi et al. 2001; Karjalainen et al. 2001). The HMW peptide, derived from the C-terminal portion of the precursor, is highly conserved between strains, while the LMW peptide derived from the N-terminal portion of the precursor demonstrates considerable sequence diversity and appears to be the main serotyping antigen (Calabi and Fairweather 2002; Karjalainen et al. 2002). The HMW peptide has sequence similarity to the *N*-acetylmuramoyl-L-alanine amidase from *Bacillus subtilis*, and was shown to possess amidase activity (Calabi et al. 2001). By investigating the pattern of sequence conservation among different *C. difficile* isolates, a number of other genes encoding putative amidases, known as *slpA* paralogs, were found in the vicinity of *slpA* (Calabi and Fairweather 2002).

The *slpA* gene was identified in a genetic locus carrying 17 ORFs, 11 of which encode putative surface-anchored proteins with adhesive properties (Calabi et al. 2001; Karjalainen et al. 2001). Transcriptional analysis of this putative virulence cluster revealed that the *slpA* gene is strongly transcribed during the entire growth phase as a bicistronic transcript (Savariau-Lacomme et al. 2003). The isolation and investigation of this cluster is an important step in the characterization of the process of colonization by *C. difficile*.

McCoubrey and coworkers used a novel phenotypic typing method to determine the level of *Clostridium difficile* colonization and disease in a population of elderly patients (McCoubrey et al. 2003). This so-called "S-typing" is a simple method which utilizes the high degree in variation of the molecular masses of the two *C. difficile* S-layer proteins to compare the strains in the patients and in the ward environment. With the aid of this typing technique, the endemic nature of *C. difficile* in a geriatric population and the degree to which their environment is contaminated could be demonstrated (McCoubrey et al. 2003).

Karjalainen and coworkers found that the sequence of the variable regions of *slpA* were strictly identical in a given serogroup of *C. difficile* but divergent between serogroups (Karjalainen et al. 2002). Based on the strong correlation to serogroup designation, *slpA* sequence typing could constitute a reproducible and reliable alternative to *C. difficile* serotyping and seems to be especially useful when specimens contain only small numbers of *C. difficile* cells or are inappropriate for culturing (Kato et al. 2005). Sequence and phylogenetic analysis of the complete *slpA* gene sequence from 14 PCR-ribotypes of *C. difficile* also confirmed that the *slpA* sequence is strongly related to serogroup designation whereas PCR ribotyping that is based on the polymorphism of the intergenic rRNA region represents a more discriminatory typing method (Ní Eidhin et al. 2006). The results of a recent study suggest that the combination of PCR-ribotyping with PCR-RFLP analysis of *slpA* could be more suitable for studying *C. difficile* epidemiology (Poilane et al. 2007).

C. difficile S-layer proteins were shown to be involved in the mechanism of gut colonization and in the process of adhesion to the intestinal mucosa (Calabi et al. 2002; Cerquetti et al. 2002). It has been proposed that S-layer proteins could mediate the binding to both the intestinal epithelial cells and some components of their extracellular matrix fibers, contributing to further tissue damage (Calabi et al. 2002; Cerquetti et al. 2002). Microscopic and biochemical approaches provided evidence that the HMW peptide functions as an adhesin which mediates adherence of *C. difficile* to host cells (Calabi et al. 2002).

In a recent study, the ability of *C. difficile* S-layer proteins to modulate the function of human monocytes and dendritic cells (DC) and to induce inflammatory and regulatory cytokines was demonstrated (Ausiello et al. 2006). S-layer proteins may thus fine-tune the equilibrium of Th1/Th2 response and affect antibody responses. The fact that S-layer proteins are a target of antibody response in patients with *C. difficile*-associated diarrhea has already been demonstrated (Drudy et al. 2004). Host antibody response plays an important role in protection, in particular IgM anti S-layer proteins have been associated with a reduced risk of recurrent *C. difficile*-associated diarrhea in humans. In this context, a protective effect of anti-S-layer protein serum has also been observed in a lethal hamster challenge model (O'Brien et al. 2005). The potential mechanism of action of the antiserum was shown to be through enhancement of *C. difficile* phagocytosis. Taken together, these data are of interest in the light of the possible use of S-layer proteins in a multicomponent vaccine against *C. difficile* infections for high-risk patients.

2.6.3 *Lactobacillus* sp.

The lactobacilli are one of the industrially most important group of bacteria; this is reflected by the accumulation of genomic data on these bacteria (for review see Pfeiler and Klaenhammer 2007). Due to their GRAS (generally regarded as safe) status, these organisms are used in a variety of ways, including food production, health improvement, and production of macromolecules, enzymes, and metabolites. Lactobacilli are Gram-positive, non-sporulating rod-shaped bacteria, with many of them being confined by an S-layer. Several strains colonize important ecological niches, such as the oral cavity or the intestine of humans and higher animals. The application potential of lactobacilli is based on a profound characterization of these organisms; in particular, a detailed understanding of the properties linked to their S-layer proteins is necessary to improve the knowledge of the interactions between the bacterial cells and the surrounding environments. This is reflected by the fact that, besides a few surface enzymes, the S-layer proteins are the most frequently described components of the otherwise rather poorly understood cell wall of the lactobacilli. While the past five to six years have seen only a few reports on new lactobacterial isolates that possess an S-layer or on reclassifications, research has been focusing on the development of improved methods for identification: e.g., real-time PCR in combination with nested reverse transcription PCR for monitoring of the viable cell number of *L. helveticus* in human feces (Saito et al. 2004) and a simple and rapid antibody-based method for detection of *L. kefir* in fermented milk (Garrote et al. 2005), and for analysis of S-layer proteins, on functional aspects of S-layers, as well as on the application potential of *Lactobacillus* S-layers.

Lactobacilli with a confirmed S-layer include *L. acidophilus*, *L. crispatus*, *L. casei*, *L. plantarum*, *L. brevis*, *L. buchneri*, *L. fermentum*, *L. bulgaricus*, *L. amylovorus*, *L. gallinarum* (Sleytr et al. 1996b), and *L. helveticus*, which has been reclassified as *L. suntoryeus* (Cachat and Priest 2005; Naser et al. 2006a), as well as several strains of enteric lactobacilli (Reniero et al. 1990). Recently, S-layer encoding genes of 21 *L. helveticus* strains were characterized; phylogenetic analyses based on the identified S-layer genes revealed two main clusters, one of which includes a sequence similar to the *slpH1* gene of *L. helveticus* CNRZ 892 and a second cluster including genes with similarity to *prtY* (Gatti et al. 2005). Within *L. helveticus* species, there is a high degree of variability in relation to the presence of plasmid molecules, possibly representing different evolutionary lineages (Ricci et al. 2006). Among recent S-layer-carrying isolates of lactobacilli are *L. kefir* and *L. parakefir* (Garrote et al. 2004), the 1,2-propanediol-degrading bacterium *L. diolivorans* sp. nov. (Krooneman et al. 2002) and *L. kefiranofaciens*, which is a later synonym for *L. kefirgranum* (Vancanneyt et al. 2004), as well as *L. amylotrophicus* sp. nov. (formerly *L. amylophilus*) (Naser et al. 2006b). On *L. johnsonii* and *L. gasseri* cells, the presence of two surface proteins with compositional characteristics similar to S-layer proteins and functioning as aggregation promoting factors have been visualized by transmission electron microscopy (TEM) and SDS-PAGE (Ventura et al. 2002). Another S-layer-related protein according to in silico

analyses is the cell division protein CdpA from *L. acidophilus* NCFM; however, due to the low amount of this protein it is unlikely that it is involved in S-layer formation (Altermann et al. 2004).

So far, S-layer structural genes of many lactobacilli have been sequenced (see Table 2.2). The primary structure of the encoded S-layers predict proteins of 25–71 kDa with basic isoelectric points (pI > 9) due essentially to lysine-rich terminal regions that confer a positive charge to various S-layer proteins with rather conserved C-terminal amino acid sequences but otherwise limited amino acid sequence similarity. With regard to gene regulatory aspects, *L. acidophilus* ATCC 4356 is best investigated. To account for the high number of S-layer protein subunits that are required to ensure a complete coverage of the bacterial cell with a closed S-layer lattice during all stages of the growth cycle, the S-layer gene *slpA* was shown to be transcribed from a very strong promoter (with twice the strength of the lactate dehydrogenase promoter, which is considered one of the strongest bacterial promoters) (Pouwels et al. 1997). Additionally, the 5′-untranslated leader sequence (UTLS) of the *slpA* gene contributes to mRNA stabilization by producing a 5′ stem and loop structure (Narita et al. 2006). *L. acidophilus* ATCC 4356, which possesses two spontaneously interchangeable S-layer genes, *slpA* and *slpB*, was also one of the first organisms to study S-layer protein variation at the molecular level (Boot et al. 1996). Change of S-layer expression in response to environmental conditions has been found in *L. brevis* ATCC 14869 (Jakava-Vilijanen et al. 2002). For that organism, three *slp* genes (*slpB*, *slpC*, and *slpD*) were identified, whose differential expression could be linked to a reversible alteration of colony morphology under different growth conditions. Under aerobic conditions, R-colony type cells produce both SlpB and SlpD proteins, whereas under anaerobic conditions, S-colony-type cells synthesize essentially only SlpB. Northern blot analysis demonstrated that *slpB* and *slpD* form a monocistronic transcription unit and are effectively expressed, but *slpD* expression is induced under aerated conditions; *slpC* was silent under the tested conditions. Promoter analysis suggests that the variation of S-layer protein content involves activation of transcription by a soluble factor rather than DNA rearrangements, which are typical for most of the known S-layer phase variation mechanisms. The presence of silent S-layer genes, termed *lgsA* and *lgsB*, respectively, was also reported for different isolates of *Lactobacillus gallinarum* cultured from the crops of broiler chickens. In these organisms, only a second, strain-specific, S-layer gene is expressed both in vivo and in vitro (Hagen et al. 2005). It is hypothesized that gene duplication and S-layer sequence variation is a means for coexistence of the bacteria in the same habitat. It is important to note that in lactobacilli which contain more than one S-layer gene, the construction of S-layer knockout mutants has so far been unsuccessful. On the other hand, S-layer gene variability is not necessarily present in lactobacilli. The molecular background of strong *Lactobacillus* S-layer gene expression constituted the basis for the development of a high-level protein expression system in lactic acid bacteria by combining the UTLS of the *slpA* gene form *L. acidophilus* with the core promoter sequence of the heterologous protein to be produced (Narita et al. 2006). Another line of development utilized a cryptic plasmid pKC5b from *L. fermentum* for the

construction of a *Lactobacillus – E. coli* shuttle vector that was shown to be suitable for *slpA* gene expression in a heterologous *Lactobacillus* strain, which additionally demonstrated surface-bound expression of the S-layer protein (Pavlova et al. 2002). Lindholm and coworkers (2004) used the signal sequence of the *L. brevis* S-layer protein for heterologous protein secretion in *Lactococcus lactis*, which was shown to yield a significantly higher secretion rate than the signal peptide of *L. lactis* Usp45.

S-layer self-assembly is a key characteristic of *Lactobacillus* S-layer proteins that is also pivotal to many of the applications envisaged. Interestingly, all lactobacillar S-layer arrays that have been analyzed for their lattice properties exhibit a morphologically similar, oblique (p2) lattice structure (see Table 2.2). Based on the knowledge of the primary sequence, detailed structure–function analyses of *Lactobacillus* S-layer proteins have been performed. Sequence comparison of the 43 kDa SlpA protein of *L. acidophilus* ATCC 4356 with S-layer proteins from *L. helveticus*, *L. crispatus,* and the S-layer proteins encoded by silent genes suggested the presence of two domains, one comprising the N-terminal two-thirds (SAN), and another making up the C-terminal one-third (SAC) of the protein. While the N-terminal sequence is variable, the C-terminal domain is highly conserved in the S-layer proteins of these organisms and contains a tandem repeat (Smit et al. 2001). Analysis of the SAN domain by insertion and deletion mutagenesis in combination with proteolytic treatment identified this protein region as a crystallization domain, consisting of a ~12 kDa and a ~18 kDa C-terminal subdomain linked by a surface-exposed loop. Mutant SlpA protein synthesized in *E. coli* with 7–13 amino acid insertions of the c-*myc* epitope indicated that insertions in conserved regions or in regions with predicted secondary structure elements (positions 30, 67, 88 and 156) destroy the crystallization capacity (Smit et al. 2002). Thus, it is proposed that the regions of higher conservation are responsible for either intra- or intermolecular subunit interaction and, hence, are important for domain structure–function. SAC was identified as the cell wall binding domain of SlpA, with an acid-labile peptidoglycan-associated compound serving as a mediator for binding (Smit and Pouwels 2002). It consists of a tandemly repeated ~65 amino acid sequence with a conserved tyrosine doublet. The two repeats (the N-terminal repeat SAC1 and the C-terminal repeat SAC2) share 26% identical amino acids, most of which are basic and aromatic. SAC shows homology to carbohydrate-binding regions of *Clostridium difficile* toxins and cell wall-associated proteinases of lactic acid bacteria. Although the SAC repeats and SLH domains have similar sizes, they do not show amino acid sequence similarity. In addition, secondary structure determinations predict a β-stranded structure for SAC1 and SAC2 but a helix–loop–helix structure for SLH. Interestingly, it was shown that SAC2 can be deleted without compromising the cell wall binding capacity or proteolytic cleavage. This indicates that SAC1 is both a structural and a functional unit, whereas SAC might provide strength to the cell wall interaction of SAC without possessing direct binding capacity. The second functionally characterized *Lactobacillus* S-layer protein is the 410-amino-acid protein CbsA from *Lactobacillis crispatus* (Antikainen et al. 2002). Stepwise truncation of this S-layer protein from both

termini revealed that the region comprising amino acids 32–271 carries the information for self-assembly of CbsA into a periodic structure. Short deletions or substitutions in the border regions 30–34 and 269–274, which are conserved in valine-rich short sequences, affect the morphology of self-assembly products, which vary from sheet-like to tubular appearance. This observation is interpreted by increasing destabilization of the formed self-assembly structure through loss of intermolecular interactions. The basic C-terminal part of CbsA binds to lipoteichoic acid and teichoic acids and functions to anchor the S-layer to the lactobacillar cell wall. Summarizing, these studies confirmed that *Lactobacillus* S-layer proteins can be seen as composite molecules with two structurally and functionally independent domains (see Fig. 2.3c), whose additional adhesive properties are located in the N-terminal region. Recently, single molecule atomic force microscopy (AFM) was used to gain insight into the molecular forces driving the folding and assembly of the S-layer protein CbsA (Verbelen et al. 2007). To address the N-terminal and C-terminal regions of the protein, genetically engineered His-tagged entire mature CbsA as well as several peptides were coupled onto AFM tips, supports were modified with mixed self-assembled monolayers, and the surface morphology of the modified surfaces was characterized. This study revealed that secondary structures of the entire CbsA protein and of its N-terminal region can be unfolded using relatively small forces, suggesting that they consist of α-helices rather than β-sheets. By contrast, the C-terminal region cannot be unfolded but shows large, single adhesion events attributed to electrostatic intermolecular bridges involving cationic lysine residues.

The probiotic properties of lactobacilli have stimulated various types of research on the possible roles of S-layer proteins in adherence to specific host tissues. In this context, the specific surface properties mediated through the S-layer proteins are of major importance. A variety of strains from the genus *Lactobacillus* was investigated with respect to structure, softness, and interactions of their S-layers in order to construct structure–property relations (Schär-Zammaretti and Ubbink 2003). In this context it is important to note that for *L. acidophilus* it was demonstrated that its physicochemical surface properties are influenced by the composition of the fermentation medium (Schär-Zammaretti et al. 2005). In particular, in the absence of peptones, the expression of the S-layer protein is strongly enhanced, which suggests that the S-layer protein is preferentially expressed under conditions which are not optimal for bacterial growth; this is in line with a postulated protective effect whereby the S-layer is expressed in response to a stress factor. Many efforts were put into the improvement of microscopic and biophysical techniques for probing cell surface hydrophobicity and bacterial interactions with a host or a material surface (Ubbink and Schär-Zammaretti 2005). In particular, the flexibility of AFM in probing various types of physical interactions provides prospects for the elucidation of adhesion maps and their relationship to biological and structural data. Imaging of different *Lactobacillus* strains with AFM revealed major differences in the surface topography depending on the presence or absence of an S-layer. Force volume images calculated into elasticity and adhesion force maps showed that

L. crispatus and *L. helveticus* have a surface with a homogeneous stiffness without adhesion events, most likely caused by the S-layer. In contrast, for the S-layer-deficient strains *L. johnsonii* DSM 20533 and ATCC 33200 high adhesion forces were observed, which can be related to a surface rich in polysaccharides (Schär-Zammaretti and Ubbink 2003). While the S-layer has been known to convey hydrophobicity to the lactobacillar cell surface (van der Mei et al. 2003), it was interesting to find by AFM studies that S-layer-carrying strains do not necessarily adhere better to hydrophobic substrates than strains without an S-layer (Vadillo-Rodríguez et al. 2004, 2005). Moreover the tested strains exhibited a dynamic cell surface hydrophobicity in dependent on the ionic strength of the medium. *Lactobacillus* strains with an S-layer (e.g. *L. acidophilus* ATCC 4356) were found to be hydrophobic in 10 mmol/L KCl solution and became more hydrophilic in 100 mmol/L, while it was the opposite case for strains without an S-layer (e.g. *L. crispatus*). This observation suggests that cell surfaces of lactobacilli may adapt their hydrophobicity in response to environmental changes, like ionic strength or pH. The ability to adhere is thought to be important to lactobacilli in establishing or maintaining selective colonization. In general, S-layers have been shown to function as adhesins mediating the adherence of *Lactobacillus* cells to host epithelial cells and/or matrix proteins (Lorca et al. 2002; Åvall-Jääskeläinen and Palva 2005; Buck et al. 2005). In particular, the involvement of the S-layer in adhesion to erythrocytes has been demonstrated for many *Lactobacillus* species (Toba et al. 1995; Boris et al. 1997). The S-layer of an *L. acidophilus* isolate has been reported to act as an afimbrial adhesin in vitro, as it is involved in the interaction with avian epithelial cells (Schneitz et al. 1993; Edelman et al. 2002). The S-layer SlpA of *L. brevis* ATCC 8287 was identified as an adhesion with affinity for human epithelial cells and fibronectin. Using a flagellum display model of SlpA fragments on *E. coli* cells, the receptor-binding region was located within a fragment of 81 amino acids in the N-terminal part of the S-layer (Hynönen et al. 2002). Binding characteristics of this S-layer protein to extracellular matrix proteins were refined through surface plasmon resonance studies. SlpA was found to interact with high affinity with fibronectin and laminin with respective binding constants of 90 mM and 27 mM, while the interaction with collagen and fibrinogen was much lower with respective binding constants of 32 mM and 26 mM (de Leeuw et al. 2006). For *L. acidophilus* M92, which manifests a high degree of hydrophobicity, the 45 kDa S-layer protein was shown to be responsible for autoaggregation and adhesion to mouse ileal epithelial cells (Kos et al. 2003; Frece et al. 2005). Very recent studies have given further insight into the probiotic properties of S-layer-carrying lactobacilli against pathogens. For coaggregating *L. kefir* strains, it was demonstrated that the S-layer protein antagonizes the interaction of *Salmonella enterica* serovar Enteritidis with epithelial cells (Golowczyc et al. 2007). Similarly, the S-layer protein of *L. crispatus* ZJ001 is involved in the adhesion and competitive exclusion of pathogenic *Salmonella typhimurium* and *E. coli* O157:H7 to HeLa cells (Chen et al. 2007; Johnson-Henry et al. 2007). Decrease of pathogen adherence after pre-treatment of host epithelial cells with S-layer protein extracts

indicates that a non-viable constituent from a probiotic strain may prove effective in interrupting the infectious process of a pathogen. A similar effect has already been described for *L. crispatus* JCM 5810, which inhibited adhesion of diarrheagenic *E. coli* strains to reconstituted basement membrane preparations (Horie et al. 2002). In addition to the functions mentioned that are clearly attributable to the respective S-layer proteins, there were several reports on newly identified surface proteins that may or may not be associated with the S-layer. Among them is an aggregation-promoting factor for maintenance of cell shape in *L. gasseri* 4B2 (Jankovic et al. 2003), a surface protein Cpf of *L. coryniformis* DSM 20001^T mediating coaggregation with and aggregation among pathogens (Schachtsiek et al. 2004), and a surface protein from *L. fermentum* 104R that binds to porcine small intestinal mucus and gastric mucin (Rojas et al. 2002). Furthermore, a cell envelope protease was extracted from S-layer-carrying *L. helveticus* Zuc2 (Scolari et al. 2006).

The utilization of lactobacilli as in vivo delivery vectors for biologically active molecules has become increasingly attractive due to their non-pathogenicity and their ability to survive the gastrointestinal tract. In particular, strategies for obtaining periodic cell surface display of epitopes are being intensively investigated. It has already been demonstrated that S-layer protein subunits can be modified to carry foreign epitopes as a uniform recombinant S-layer on the *Lactobacillus* cell surface. The capacity of the SlpA S-layer protein of *L. acidophilus* ATCC 4356 to present epitopes, up to 19 amino acids residues in length, upon recrystallization of recombinant S-layer fusion protein on the surface of *Lactobacillus* cells that have been stripped off from their S-layer upon chemical treatment, makes this system suitable in principle as an oral vaccine vector (Smit et al. 2002). In vivo surface display of foreign epitopes was for the first time obtained via the *L. brevis* S-layer protein subunits and exemplified with the human 11-amino-acid c-*myc* proto-oncogene. For this purpose, a gene replacement system was optimized for replacement of the wild-type *slpA* gene with the *slpA-c-myc* construct (Åvall-Jääskeläinen et al. 2002). According to TEM evidence, a uniform S-layer was obtained displaying on its surface the desired antigen in all of the S-layer subunits. Based on the theoretically calculated presence of approximately 5×10^5 S-layer subunits on a *L. brevis* cell, with this cell surface display system it is possible to present at least such a large number of antigen epitope molecules on each cell. Surface display of vaccines as part of an S-layer would thus be a very effective way to present antigens to the mucosa-associated lymphoreticular tissue. In the context of vaccine development based on lactobacillar S-layer, in a recent study porcine-specific S-layer-carrying strains were analyzed for later use as vaccine vectors and/or probiotics (Jakava-Viljanen and Palva 2007). However, the amount of such organisms was rather low, and their adhesion affinities to various host tissues differed considerably. As a previous stage to the development of a vaccine vehicle for oral administration, positively charged liposomes were coated with the S-layer proteins from *L. brevis* and *L. kefir* (Hollmann et al. 2007). As already demonstrated with other S-layer proteins (Mader et al. 1999), the lactobacillar S-layers increased the stability of the liposomes when resuspended in bile salts and pancreatic extract and under thermal shock.

2.6.4 *Geobacillus stearothermophilus* strains

The genus *Geobacillus stearothermophilus* comprises Gram-positive, strictly aerobic species of endospore-forming bacteria. Many of them are covered with crystalline S-layers of different lattice geometry (Sleytr 1978; Messner et al. 1984; Sleytr et al. 2002). Among them are also strains with a glycosylated S-layer protein. Since prokaryotic protein glycosylation in general is now a rapidly expanding field and the most frequently observed modification of S-layer proteins (for reviews see Messner and Sleytr 1991, 1992; Sumper and Wieland 1995; Eichler and Adams 2005; Szymanski and Wren 2005; Messner et al. 2008), we have focused considerable efforts on the elucidation of this important post-translational protein modification. Currently, the best investigated bacterial S-layer glycoprotein is that of the Gram-positive, moderately thermophilic organism *G. stearothermophilus* NRS 2004/3a (Sleytr and Plohberger 1980; Küpcü et al. 1984; Messner et al. 1984; Messner et al. 1986b; Schäffer et al. 2002; Novotny et al. 2004a, b; Steiner et al. 2006, 2007; Bindila et al. 2007; Messner et al. 2008). The surface of this strain is covered by an oblique S-layer lattice composed of identical glycoprotein subunits. The S-layer glycan chains protrude from the cell surface and may create a hydrophilic coat around the bacterial cell comparable to LPS O-antigens of Gram-negative bacteria (Messner et al. 1986b; Raetz and Whitfield 2002; see also Chap. 4). After isolation and purification of the glycosylated S-layer protein SgsE of *G. stearothermophilus* NRS 2004/3a, the glycan chain structure was determined by straightforward one-and two-dimensional ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. To the S-layer protein subunits are attached elongated glycan chains that are composed of, on average, 15 trisaccharide repeats with the structure $[\rightarrow 2)$-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→2)-α-L-Rha*p*-$(1\rightarrow 3]_n$ and a 2-*O*-methyl modification of the terminal repeat at the non-reducing end of the glycan chain (Christian et al. 1986; Schäffer et al. 2002). The glycan chains are bound via core saccharides of, on average, two α1,3-linked α-L-Rha*p* residues to carbon-3 of β-D-galactose residues. The entire 903-amino-acid-residues-long S-layer glycans are then attached via *O*-glycosidic linkages to different serine and threonine residues of the S-layer protein subunits (Schäffer et al. 2002). On SDS-PA gels four bands appear, three of which represent glycosylated S-layer proteins. Due to the lack of adequate analytical techniques, a conclusive interpretation of the multiple banding pattern of this S-layer glycoprotein as observed by SDS-PAGE has been hampered in the past. Recently, straightforward mass spectrometry methods have allowed the accurate determination of the average masses of the three inherently heterogenic glycoprotein species of SgsE to be 101.66 kDa, 108.68 kDa, and 115.73 kDa, corresponding to SgsE with different numbers of attached glycan chains (Steiner et al. 2006). Each of the glycoforms revealed nanoheterogeneity with variation between 12 and 18 trisaccharide repeats and the possibility of extension of the already known di-rhamnose core region by one additional rhamnose residue (Steiner et al. 2006). On the 93-kDa SgsE S-layer protein, three glycosylation sites could be unequivocally identified, namely at positions threonine-590, threonine-620, and

serine-794. These data led to the logical interpretation that in the 101.66-kDa glycoform only one glycosylation site is occupied, in the 108.68-kDa glycoform two glycosylation sites are occupied, and in the 115.73-kDa glycoform all three glycosylation sites are occupied (Steiner et al. 2006). However, it is not yet known which of the sites are actually occupied and how long the attached glycans are in the in vivo situation. This task could only be approached by single molecule analysis of SgsE subunits, which is far beyond the resolution power of today's analytical techniques.

Recently, the structural gene *sgsE* encoding the S-layer protein of *G. stearothermophilus* NRS 2004/3a was identified by polymerase chain reaction-based techniques (Schäffer et al. 2002). The ORF codes for a protein of 903 amino acids, including a leader sequence of 30 amino acids. The mature S-layer protein has a calculated molecular mass of 93,684 Da and a pI of 6.1. In addition to *sgsE*, the structural genes coding for S-layer proteins of four other strains of *G. stearothermophilus* strains, termed *sbsA* – *sbsD*, have now been characterized (Egelseer et al. 2001; Schäffer et al. 2002; Novotny et al. 2004b). Comparison of the amino acid sequences of the *G. stearothermophilus* S-layer proteins SbsA–D (protein identification CAA50409, CAA66724, AAC12757, AAF34763, respectively) with SgsE (protein identification AAL46630) showed that they share similarities with respect to the following features: (1) they are synthesized with a typical N-terminal signal sequence consisting of 30 (SbsA, SbsC, SbsD, and SgsE) and 31 (SbsB) amino acids, respectively; (2) the signal peptide cleavage site is between the Ala–Ala motif, consistent with the proposed recognition sequence for signal peptidases; (3) all *G. stearothermophilus* S-layer proteins exhibit a weakly acidic isoelectric point. The highest sequence identities are found within the N-terminal regions of the S-layer proteins (Schäffer et al. 2002). In contrast, the N-terminus of SbsB does not reveal significant sequence homology; instead, it is the only one among the *G. stearothermophilus* S-layer proteins compared to possess an S-layer homology (SLH) domain (Lupas et al. 1994) between amino acids 31 and 168 (Sára et al. 1998).

Glycosylation of SgsE at the C-terminal region (Thr-590, Thr-620, and Ser-794) might be seen in the context of anchoring the S-layer glycoprotein to the bacterial cell wall (see also Sect. 2.5). Recently, a diacetamidodideoxymannuronic acid-containing glycan of *G. stearothermophilus* NRS 2004/3a with the repeating unit structure [→4)-β-D-Man*p*A2,3(NAc)$_2$-(1→6)-α-D-Glc*p*-(1→4)-β-D-Man*p*A2,3(NAc)$_2$-(1→3)-α-D-Glc*p*NAc-(1→]$_{n\sim5}$ was examined to identify its linkage to the bacterial cell wall. This glycan represents the secondary cell wall polymer (see Sect. 2.5) of *G. stearothermophilus* NRS 2004/3a (Schäffer et al. 1999). In the meantime, chemical characterization of secondary cell wall polymers from all *G. stearothermophilus* strains analysed so far that do not contain SLH domains in their S-layer protein, showed that they possess diacetamidodideoxyuronic acid-containing secondary cell wall polymers of identical structure (Schäffer and Messner 2005).

In the course of the genetic characterization of the *G. stearothermophilus* NRS 2004/3a S-layer glycoprotein glycosylation, the ~16.5 kb surface layer glycan biosynthesis (*slg*) gene cluster has been sequenced (GenBank AF328862) (Novotny et al. 2004a, b). The cluster is located immediately downstream of the S-layer

structural gene *sgsE* and consists of 13 ORFs that have been identified by database sequence comparisons. The cluster comprises genes encoding enzymes for dTDP-L-rhamnose biosynthesis (*rml* operon), required for building up the polyrhamnan S-layer glycan, as well as those for assembly and export of the elongated glycan chain, and its transfer to the S-layer protein. There is evidence that this cluster is transcribed as a polycistronic unit, whereas *sgsE* is transcribed monocistronically (Novotny et al. 2004b). Chromosomal DNA preparations of several *G. stearothermophilus* strains with glycosylated (NRS 2004/3a, GenBank AF328862) and without glycosylated S-layer proteins (L32-65, GenBank AY278518; ATCC 12980^T, GenBank AY278519) were screened for the presence of the *rml* operon, because L-rhamnose is a frequent constituent of S-layer glycans. The flanking regions of the operon were sequenced from *rml*-positive strains. Comparison with the *slg* gene cluster of *G. stearothermophilus* NRS 2004/3a (GenBank AF328862) revealed sequence homologies between adjacent genes. Cell-free extracts of the strains were capable of converting dTDP-D-glucose to dTDP-L-rhamnose. These results indicate that the *rml* locus is highly conserved among *G. stearothermophilus* strains, and that in the *rml*-containing strains investigated, dTDP-L-rhamnose is actively synthesized in vitro (Novotny et al. 2004b), even if the respective S-layer proteins are non-glycosylated.

The sequence determination of the genes of the *slg* gene cluster of *G. stearothermophilus* NRS 2004/3a was the basis for the functional characterization of the different enzyme proteins encoded. The biochemical characterization of WsaP showed this gene as a UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase that primes the S-layer glycoprotein glycan biosynthesis of *G. stearothermophilus* NRS 2004/3a and is therefore the initiation enzyme of the glycosylation reaction (Steiner et al. 2007). It was demonstrated that the enzyme transfers in vitro a galactose-1-phosphate from UDP-galactose to endogenous phosphoryl-polyprenol and that the C-terminal half of WsaP carries the galactosyltransferase function, capable of reconstituting polysaccharide biosynthesis in WbaP-deficient strains of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Steiner et al. 2007). For a better understanding of the general glycosylation process of bacterial S-layer glycoproteins, further studies of genes involved in S-layer protein glycosylation of different organisms are required.

Other important *G. stearothermophilus* species include *G. stearothermophilus* PV72 and *G. stearothermophilus* ATCC 12980^T. The first S-layer gene of this species which was sequenced and cloned was the S-layer gene *sbsA* (Kuen et al. 1994) of the wild-type strain *G. stearothermophilus* PV72/p6 exhibiting a hexagonally ordered S-layer lattice (Sára et al. 1996). Upon cultivation under unphysiologically high oxygen concentrations, the hexagonal S-layer lattice of the wild-type strain formed by SbsA was replaced by an oblique lattice type consisting of the S-layer protein SbsB of the variant designated PV72/p2 (Sára et al. 1996). Sequencing of the *sbsB* gene (Kuen et al. 1997) revealed that the S-layer protein SbsB carries three SLH motifs at the N-terminal part and showed only 25% identity to SbsA. On the molecular biological level, variant formation was found to depend on recombination events between a megaplasmid and the chromosome (Scholz et al.

2001). In addition to the change of the S-layer protein, the synthesis of a different type of SCWP was observed (Sára et al. 1996). In the variant strain, the non-pyruvylated, 2,3-diacetamido-2,3-dideoxymannuronic acid-containing SCWP of the wild-type strain was replaced by another type of SCWP which contained GlcNAc and ManNAc as the major components and pyruvate ketals as net negatively charged groups (Ries et al. 1997; Sára et al. 1998; Sára 2001). In a very recent study, the structure of this SCWP has been resolved by NMR (Petersen et al. 2008). Interestingly, the S-layer protein SbsA recognized both types of SCWPs as binding sites, guaranteeing complete coverage of the cell surface during the oxygen-induced switch. On the other hand, the S-layer protein SbsB showed only affinity for binding of the newly synthesized SCWP.

For biophysical characterization, SbsB was cloned and expressed as two separate but complementary parts, namely, the N-terminal part defined by the three consecutive S-layer homologous motifs and the remaining large C-terminal part (Rünzler et al. 2004). By applying this dissection approach, the SLH-domain of SbsB ($rSbsB_{32–208}$) was found to be exclusively responsible for SCWP binding whereas the larger C-terminal part represents the self-assembly domain. Furthermore, circular dichroism spectroscopy studies confirmed that most α-helical segments are arranged in the N-terminal SLH domain, whereas the middle and C-terminal part could be characterized as a β-sheet protein (Rünzler et al. 2004). The C-terminal part of SbsB was found to be highly sensitive against deletions since the removal of even less than 15 amino acids led to water-soluble S-layer protein forms (Howorka et al. 2000; Moll et al. 2002).

The interaction of the S-layer protein SbsB of *G. stearothermophilus* PV72/p2 and the corresponding SCWP was assessed by surface plasmon resonance (SPR) biosensor technology using native and chemically modified SCWPs devoid of pyruvic acid residues. The interaction proved to be highly specific for the carbohydrate component and glycan pyruvylation was found to be an essential requirement (Mader et al. 2004).

The cell surface of the type strain of that species, *G. stearothermophilus* $ATCC12980^T$, is completely covered with an oblique S-layer lattice formed by the S-layer protein SbsC, which was also found to be an adhesion site for a high-molecular-mass exoamylase (HMMA) (Egelseer et al. 1996). After elucidation of the *G. stearothermophilus* S-layer genes *sbsA* and *sbsB* of PV72/p6 and PV72/p2, respectively, *sbsC* was the third S-layer gene of this species to be sequenced and cloned (Jarosch et al. 2000). The entire *sbsC* sequence showed an ORF of 3,297 bp predicted to encode a protein of 1,099 amino acids with a theoretical molecular mass of 115,409 Da and an isoelectric point of 5.73. The elucidation of the S-layer gene sequence opened the possibility of investigating whether sequence identities and a common structure–function relationship exist in S-layer proteins of *G. stearothermophilus* wild-type strains.

Thus, in a first approach, different N- or C-terminally truncated S-layer proteins were produced heterologously and their self-assembly and recrystallization properties were investigated (Jarosch et al. 2001). Based on these results, the S-layer proteins could be characterized by two functionally and structurally separated parts, namely a

highly conserved N-terminal region which interacts with an SCWP composed of *N*-acetylglucosamine (GlcNAc), glucose, and 2,3-diacetamido-2,3-dideoxymannuronic acid, and the larger C-terminal part responsible for formation of the crystalline array (Jarosch et al. 2001). Interestingly, SbsC turned out to be highly tolerant against deletions, since significant portions at the N- or C-terminal part could be deleted without losing the capability of the subunits for lattice formation (Jarosch et al. 2001).

On the other hand, water-soluble N- or C-terminally truncated forms of SbsC were found to be well suited for first three-dimensional (3D) crystallization studies, thereby circumventing the intrinsic property of S-layer proteins to form two-dimensional lattices which prevent the formation of isotropic 3D crystals. For the C-terminally truncated form $rSbsC_{31–844}$, crystals which diffracted to a resolution of 3 Å using synchrotron radiation could be obtained (Pavkov et al. 2003). Native and heavy atom derivative data confirmed the results of the secondary structure prediction which indicated that the N-terminal region comprising the first 257 amino acids is mainly organized as α-helices, whereas the middle and C-terminal parts of SbsC consist of loops and β-sheets (Pavkov et al. 2003). In a very recent study, refinement of preliminary data led to the first high-resolution structure of the soluble N-terminal form $rSbsC_{31–844}$, showing a very elongated and flexible molecule, with strong and specific binding to the secondary cell wall polymer (SCWP) (Pavkov et al. 2008). The crystal structure of $rSbsC_{31–844}$ revealed a novel fold, consisting of six separate domains which are connected by short flexible linkers. Furthermore, SCWP binding induces considerable stabilization of the N-terminal domain.

Also recently, the basic interaction in the mechanism anchoring an S-layer protein devoid of S-layer-homologous (SLH) motifs to the rigid cell wall layer was systematically investigated by SPR biosensor technology, using the S-layer protein SbsC and the corresponding non-pyruvylated SCWP of *G. stearothermophilus* ATCC 12980^T as the model system (Ferner-Ortner et al. 2007). Two C-terminal truncations of SbsC ($rSbsC_{31–270}$ and $rSbsC_{31–443}$) carrying the SCWP binding domain as well as one N-terminal truncation ($rSbsC_{638–1099}$) comprising the residual part of SbsC were produced heterologously and used for affinity and SPR studies. The SPR data from both complementary experimental setups in which either the truncated rSbsC forms or the SCWP were immobilized on the sensor surface confirmed that the N-terminal region comprising the amino acid residues 31 to 270 was exclusively responsible for SCWP binding. Analysis of data from the setup in which SCWP was immobilized on a sensor chip and $rSbsC_{31–270}$ or $rSbsC_{31–443}$ represented the soluble analytes indicated a binding behaviour with low ($K_d = 9.32 \times 10^{-5}$ M and 2.95×10^{-6} M), medium ($K_d = 4.8 \times 10^{-9}$ M and 1.22×10^{-8} M), and high ($K_d = 1.94 \times 10^{-12}$ M and 2.05×10^{-12} M) affinity (Ferner-Ortner et al. 2007).

2.6.5 *Lysinibacillus sphaericus*

Lysinibacillus sphaericus (formerly *Bacillus sphaericus*) represents a strictly aerobic group of mesophilic endospore-forming bacteria. The S-layer lattice of

L. sphaericus CCM 2177 shows square symmetry and is composed of identical subunits with an estimated relative molecular weight of 127,000. The in vitro self-assembly of this S-layer protein was found to be dependent on the presence of bivalent cations (Pum and Sleytr 1995). This S-layer protein recognizes a negatively charged SCWP as the binding site in the rigid cell wall layer. The SCWP of *L. sphaericus* CCM 2177 is composed of disaccharide repeating units that consist of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmannosamine (ManNAc). Each second ManNAc residue carries a pyruvate ketal, which endows the polymer chains with a negative net charge (Ilk et al. 1999).

The gene encoding the S-layer protein of *L. sphaericus* CCM 2177 was sequenced, cloned, and expressed in *E. coli* (Ilk et al. 2002). The entire *sbpA* sequence indicated one ORF of 3,804 bp encoding a protein of 1,268 amino acids with a theoretical molecular mass of 132,062 Da and a calculated isoelectric point of 4.69. Prediction of the secondary structure from sequence data as well as by far-UV circular dichroism spectroscopy indicated the concentration of α-helices in the N-terminal part, whereas the middle and C-terminal parts are dominated by loops and short β-strands (Huber et al. 2005).

Comparative studies of the structure–function relationship as well as of the accessibility of the C-terminus in full-length rSbpA ($rSbpA_{31–1268}$) and the truncated form $rSbpA_{31–1068}$ revealed that the deletion of 200 C-terminal amino acids did not interfere with the self-assembly properties of the S-layer protein but significantly increased the accessibility of the C-terminal end (Ilk et al. 2002).

To identify the SCWP-binding domain of SbpA, seven C-terminal truncations and one N- and C-terminal truncation of this S-layer protein, as well as two chimeric proteins comprising either the SLH-domain of the S-layer protein SbsB of *G. stearothermophilus* PV72/p2 and the residual sequence of SbpA, and vice versa, were produced recombinantly and used for SPR studies (Huber et al. 2005). Surprisingly, $rSbpA_{31–202}$ solely comprising the three SLH motifs did not bind at all. The shortest C-terminal truncation with specific affinity to SCWP was $rSbpA_{31–318}$. Contrary to the SLH-domain-carrying S-layer protein SbsB (Mader et al. 2004), in SbpA the 3 SLH motifs and an additional 58 amino acids long SLH-like motif located behind the third SLH motif were required for reconstituting the functional SCWP domain. In the C-terminal part of this S-layer protein, up to 237 amino acids could be deleted without interfering with the square lattice structure. Most important, it was demonstrated that the deletion of a further 113 C-terminal amino acids led to a change from square (p4) to oblique (p1) lattice structure (Huber et al. 2005).

2.6.6 Tannerella and Bacteroides

Tannerella forsythia (formerly *Bacteroides forsythus*) is a filamentous, Gram-negative, oral anaerobe, which has been implicated in the development of periodontitis (Tran et al. 2001). *T. forsythia* possesses a distinctive cell envelope

ultrastructure, with an S-layer appearing as a regularly arrayed lattice formed of serrated structural subunits (Kerosuo 1988) and lipopolysaccharide but no capsule located outside of the outer membrane. Sabet and coworkers (2003) have reported on the small-scale isolation and purification of the *T. forsythia* S-layer using sodium deoxycholate followed by caesium chloride isopycnic gradient centrifugation. SDS-PAGE analysis of the S-layer preparation revealed two major protein bands, named TfsA and TfsB, exhibiting molecular masses of 200/230 kDa and 210/270 kDa, respectively (values for molecular masses vary in literature depending on the percentage of the PA gel), both of which were reactive with the periodic acid–Schiff reagent, which is indicative of glycosylation (Higuchi et al. 2000; Sabet et al. 2003). For identification of the *tfsA* and *tfsB* genes, partial amino acid sequences from electrophoretically purified S-layer proteins were compared with the genome of *T. forsythia* (Lee et al. 2006b; Sakakibara et al. 2007). Two genes, *tfsA*, encoding a ~135-kDa protein with an isoelectric point of 7.85, and *tfsB*, encoding a rather basic ~152-kDa S-layer protein with an isoelectric point of 9.1, are located in contigs TF2661–TF2662, and TF2663, respectively. The differences between the calculated molecular masses of the S-layer proteins and those estimated from the PA gels indicate a high degree of glycosylation of TfsA and TfsB. Interestingly, *tfsA* and *tfsB* are located in tandem and are transcribed as an operon (Lee et al. 2006b).

There are several indications that the S-layer functions as a virulence factor. The S-layer proteins were clearly recognized by sera from patients with adult and early-onset periodontitis, whereas the IgG response against the antigen was low in healthy control individuals (Yoneda et al. 2003). Functional tests have furthermore revealed that the S-layer is highly responsible for adherence/invasion of *T. forsythia* to human gingival epithelial cells, mediates hemagglutination, and is involved in coaggregation with *Porphyromonas gingivalis*, another crucial oral pathogen (Sabet et al. 2003; Sakakibara et al. 2007).

Comparison of the amino acid sequences of TfsA and TfsB with the protein database indicated that these S-layer proteins share no overall homology to any other deposited S-layer protein sequence. There is, however, profound similarity (between 63% and 75%) of the C-terminal domains of the *T. forsythia* S-layer proteins and nine recently described proteins of the related organism *Bacteroides distasonis*, named DgpA–I (Fletcher et al. 2007). Interestingly, there are strong indications that the Dgp proteins are also glycosylated S-layer proteins. They are highly abundant in the intestinal symbiont *B. distasonis* and their expression was shown to be phase-variable, which is taken as an indication for the importance of these S-layer glycoproteins to the bacterial–host symbiosis (Fletcher et al. 2007).

2.6.7 *Caulobacter vibrioides* (formerly *Caulobacter crescentus*)

Caulobacter vibrioides is a Gram-negative, non-pathogenic aquatic bacterium that is covered by a hexagonal S-layer lattice composed of a single 98 kDa

protein species termed RsaA (Smit et al. 1981, 1992). This S-layer protein is non-covalently bound to the cell surface via a smooth lipopolysaccharide (LPS) species whose O-side chain is composed, at least in part, of *N*-acetylperosamine. This LPS species is at least involved in S-layer anchoring, since strains deficient in O-side chain biogenesis were found to shed the S-layer (Walker et al. 1994; Awram and Smit 2001).

In contrast to Gram-positive bacteria, no general S-layer anchoring motifs have been identified in Gram-negative organisms. In a recent study using reattachment assays, the RsaA anchoring region was found to lie in the N-terminal region comprising approximately the first 225 amino acids (Ford et al. 2007). Assembly into the hexagonal array was revealed to require calcium, which may mediate RsaA crystallization via calcium bridging between RsaA monomers (Smit et al. 1992; Walker et al. 1992). A calcium-binding motif located near the C-terminus of RsaA, the so-called RTX (repeats in toxin) motif, is likely to mediate this process (Gilchrist et al. 1992).

The *rsaA* gene (Gilchrist et al. 1992), which encodes the RsaA protein comprising 1,026 amino acids with a predicted molecular weight of 98,132 Da, was exploited to display foreign peptides on the cell surface in a dense, highly ordered manner (Bingle et al. 1997). While most of the characterized S-layer transport systems from Gram-negative bacteria involve type II secretion, the S-layer protein of *C. vibrioides* is secreted by a type I mechanism which requires two outer membrane proteins, $RsaF_a$ and $RsaF_b$ (Toporowski et al. 2004). Interestingly, the S-layer-associated metalloprotease Sap (Umelo-Njaka et al. 2002) which primarily uses the S-layer type I secretion apparatus recognized a cleavage site in truncated RsaA mutants but not in full-length RsaA (Ford et al. 2007). This could be explained by inappropriate or slower folding of the RsaA mutants, thus exposing this weak cleavage site to the Sap protease. It can be speculated that this extracellular membrane-bound protease could be part of a "remove and replace" strategy for repairing S-layer damaged by various environmental causes (Ford et al. 2007).

Comparative studies between different display systems to identify peptides mimicking bovine herpes virus 1 (BoHV-1) envelope glycoprotein gE proved the feasibility of the commercially available PurePro™ *Caulobacter* expression system (Invitrogen) also as a display system (Lehmann et al. 2004). In the latter, the S-layer protein RsaA is used as a scaffold displaying the peptide of interest.

In the course of efforts to use *C. vibrioides* as a vector for immunotherapy by expressing cancer-associated peptides from genetic insertions in the S-layer gene, live and unmodified cells of wild-type *C. vibrioides* revealed unexpected tumor suppressive effects in mice (Bhatnagar et al. 2006). These results suggest that *C. vibrioides* may be a safe, bacterial immunomodulator for the treatment of tumors.

2.6.8 Campylobacter fetus

Campylobacter fetus, a microaerophilic spiral Gram-negative bacterium, has been recognized as an important pathogen in animals important for food production and

humans (Blaser 1998; Thompson and Blaser 2000). *C. fetus* has been isolated from numerous hosts including mammals, birds, and reptiles. *C. fetus* may be either type A or type B based on serotype, lipopolysaccharide structure, and S-layer protein type (Dubreuil et al. 1990; Blaser et al. 1994; Dworkin et al. 1995). *C. fetus* is covered by an S-layer composed of high-molecular-weight protein subunits (Dubreuil et al. 1988; Pei et al. 1988; Fujimoto et al. 1991). The S-layer proteins have been shown to play a critical role in *C. fetus* virulence (Blaser et al. 1987; Pei and Blaser 1990; Blaser and Pei 1993; Blaser et al. 1994; Grogono-Thomas et al. 2000, 2003) by protecting the bacterium from phagocytosis and serum killing (Blaser et al. 1988).

The *C. fetus* S-layer proteins are encoded by multiple *sap* genes and vary in size. Identification and characterization of the 53.8-kb chromosomal region containing the entire *sap* locus (termed the "sap island") in a wild-type strain showed that all eight complete *sapA* homologues share conserved regions at their 5′ regions, encode S-layer proteins from 96 kDa to 131 kDa that share similar characteristics, and can be divided into three phylogenetic groups based on their 3′ sequences (Tu et al. 2003). The extensive homologies in the *sap* island include both direct and inverted repeats, which allow DNA rearrangements, deletion, or duplication (Tu et al. 2004a). Thereby, each *sapA* homologue can reciprocally recombine with the others, with rearrangements permitting the creation of new homologs and their placement downstream of the unique *sapA* promoter. Genetic analyses of the *sap* islands and their boundaries in 18 different *C. fetus* strains suggest that the *sap* island was not acquired by recent horizontal gene transfer but is an ancient genomic constituent that has evolved differing genotypes that are plastic, perhaps enabling colonization of varied niches, in addition to antigenic variation (Tu et al. 2004a).

Genetic analyses of the *sap* locus were also found to be useful tools to determine the phylogenetic relationship of *C. fetus* strains of mammal or reptile origin (Tu et al. 2001). Although phenotypic testing did not definitively identify the organism in a patient suffering recurrent bacteremia, genotypic approaches utilizing PCRs specific for *sapA*, *sapB*, and reptile *sap* island insertion, as well as sequence analysis of *sapD* and 16 S rRNA in combination with RAPD, allowed identification of a *C. fetus* strain of reptile origin as a human pathogen (Tu et al. 2004b).

Data from challenge experiments using vaccinated ewes suggested that S-layer protein-expressing vaccines could protect animals from abortion and that this effect was independent of the S-layer protein expressed, indicating involvement of conserved epitopes in the S-layer protein. By epitope mapping of the conserved 184-amino-acid N-terminal region with rabbit anti-S-layer protein antisera by using overlapping synthetic 20-mer peptides, two putative epitopes were identified between amino acids 81 to 110 and 141 to 160. Conserved antigenic regions of the S-layer protein that induce protective immune responses may enable development of synthetic vaccine candidates for *C. fetus* ssp. *fetus*-associated ovine abortion (Grogono-Thomas et al. 2003).

Interestingly, the investigation of the antigenic property of the GroEL-like protein in *C. fetus* belonging to the heat shock protein (HSP) family HSP60 revealed the presence of a common immunodominant carbohydrate epitope between the S-layer

protein and the bacterial HSP (Hinode et al. 2002). Analyses of genotypic and phenotypic variation of *C. fetus* isolates from human patients with relapsing infections by comparing their *sap* type, *sap* island repertoire and organization, as well as the expressed S-layer proteins, point to at least three different mechanisms underlying recurrent *C. fetus* infections: (1) genetic rearrangement to up-regulate virulence, (2) rearrangement for antigenic variation, and (3) true latency of a fully virulent strain with later reactivation due to a change in the clinical status (Tu et al. 2005).

2.7 Conclusions

More than 50 years ago Houwink described the presence of a "macromolecular monolayer" in the cell wall of a *Spirillum* sp. (Houwink and Le Poole 1952; Houwink 1953). Soon after, similar arrays were reported in *Acinetobacter* and *Aquaspirillum* strains. Some 40 years ago for the first time "regular arrays of macromolecules" were identified on the cell surface of living *Bacillus* and *Clostridium* cells (Sleytr et al. 1967, 1968). Nevertheless, since the classical working strains in microbiology, molecular biology, and genetics, *Escherichia coli* and *Bacillus subtilis,* did not reveal such structures, it took a long time before the broad distribution and relevance of this cell wall structure was appreciated. In a previous review (Sleytr 1978) 80 species were already listed and currently about 800 bacterial and archaeal organisms are known to possess S-layers. In other words, S-layers can now be considered as one of the most commonly observed prokaryotic cell envelope structures. Moreover, composed of single subunits endowed with the ability to assemble into monomolecular arrays, they represent the simplest type of protein membrane developed during evolution (Sleytr and Plohberger 1980). Since S-layer-carrying organisms were demonstrated to be ubiquitous in the biosphere and S-layers are integral components of a variety of cell envelopes, it became obvious that they must provide the organisms with an advantage of selection in very difficult habitats.

Despite the significant increase in knowledge of the structure, chemistry, assembly, and genetics of S-layers over recent decades, relatively few firm data are available on the biological significance and selection advantage to organisms of producing such metabolically expensive layers (Murray 1993). Finally, it should be taken into consideration that a simple S-layer-type protein membrane capable of dynamic growth could have initiated a barrier membrane in the early stage of biological evolution (Sleytr and Plohberger 1980). The cell division process observed in lobed (e.g. *Methanocorpusculum sinense*) (Pum et al. 1991) or rod-shaped (e.g. *Thermoproteus tenax*) (Messner et al. 1986a) archaea clearly demonstrated such morphogenetic potentials of S-layers.

Finally, the increasing wealth of information on the general principles of S-layers will promote their use in life and non-life science applications (see Chap. 16).

Acknowledgements Financial support from the Austrian Science Fund [FWF, projects P18013-B10 (P.M.), P18510-B12 (E.M.E.), P19047-B12 (C.S), P20605-B12 (C.S.)], the

Erwin-Schrödinger Society for Nanosciences, the FP6 EC STREP NASSAP (project 13352; U.B. S.), the Hochschuljubiläumsstiftung der Stadt Wien, project H-1809/2006 (C.S.), and the Air Force Office of Scientific Research, USA [AFOSR, projects F49620-03-1-0222 and FA9550-07-1-0313 (U.B.S.)] is gratefully acknowledged.

References

Adams DG, Ashworth D, Nelmes B (1999) Fibrillar array in the cell wall of a gliding filamentous cyanobacterium. J Bacteriol 181:884–892

Adrian L, Rahnenführer J, Gobom J, Hölscher T (2007) Identification of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1. Appl Environ Microbiol 73:7717–7724

Ahn JS, Chandramohan L, Liou LE, Bayles KW (2006) Characterization of CidR-mediated regulation in *Bacillus anthracis* reveals a previously undetected role of S-layer proteins as murein hydrolases. Mol Microbiol 62:1158–1169

Altermann E, Buck LB, Cano R, Klaenhammer TR (2004) Identification and phenotypic characterization of the cell-division protein CdpA. Gene 342:189–197

Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR (2005) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA 102:3906–3912

Altman E, Schäffer C, Brisson J-R, Messner P (1995) Characterization of the glycan structure of a major glycopeptide from the surface layer glycoprotein of *Clostridium thermosaccharolyticum* E207–71. Eur J Biochem 229:308–315

Andersson M, Laukkanen M, Nurmiaho-Lassila E-L, Rainey FA, Niemelä SI, Salkinoja-Salonen M (1995) *Bacillus thermosphaericus* sp. nov. a new thermophilic ureolytic *Bacillus* isolated from air. Syst Appl Microbiol 18:203–220

Antikainen J, Anton L, Sillanpää J, Korhonen TK (2002) Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Mol Microbiol 46:381–394

Ariel N, Zvi A, Grosfeld H, Gat O, Inbar Y, Velan B, Cohen S, Shafferman A (2002) Search for potential vaccine candidate open reading frames in the *Bacillus anthracis* virulence plasmid pXO1: in silico and in vitro screening. Infect Immun 70:6817–6827

Ausiello CM, Cerquetti M, Fedele G, Spensieri F, Palazzo R, Nasso M, Frezza S, Mastrantonio P (2006) Surface layer proteins from *Clostridium difficile* induce inflammatory and regulatory cytokines in human monocytes and dendritic cells. Microbes Infect 8:2640–2646

Åvall-Jääskeläinen S, Palva A (2005) *Lactobacillus* surface layers and their applications. FEMS Microbiol Rev 29:511–529

Åvall-Jääskeläinen S, Kylä-Nikkilä K, Kahala M, Miikkulainen-Lahti T, Palva A (2002) Surface display of foreign epitopes on the *Lactobacillus brevis* S-layer. Appl Environ Microbiol 68:5943–5951

Awram P, Smit J (2001) Identification of lipopolysaccharide O antigen synthesis genes required for attachment of the S-layer of *Caulobacter crescentus*. Microbiology 147:1451–1460

Baillie L, Hebdon R, Flick-Smith H, Williamson D (2003) Characterisation of the immune response to the UK human anthrax vaccine. FEMS Immunol Med Microbiol 36:83–86

Baumeister W, Wildhaber I, Phipps BM (1989) Principles of organization in eubacterial and archaebacterial surface proteins. Can J Microbiol 35:215–227

Beveridge TJ (1994) Bacterial S-layers. Curr Opin Struct Biol 4:204–212

Beveridge TJ, Graham LL (1991) Surface layers of bacteria. Microbiol Rev 55:684–705

Bhatnagar PK, Awasthi A, Nomellini JF, Smit J, Suresh MR (2006) Anti-tumor effects of the bacterium *Caulobacter crescentus* in murine tumor models. Cancer Biol Ther 5:485–491

Bi ZX, Liu YJ, Lu CP (2007) Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res Vet Sci 83:150–156

Bindila L, Steiner K, Schäffer C, Messner P, Mormann M, Peter-Katalinić J (2007) Sequencing of O-glycopeptides derived from a S-layer glycoprotein of *Geobacillus stearothermophilus* NRS 2004/3a containing up to 51 monosaccharide residues at a single glycosylation site by Fourier transform ion cyclotron resonance infrared multiphoton dissociation mass spectrometry. Anal Chem 79:3271–3279

Bingle WH, Nomellini JF, Smit J (1997) Linker mutagenesis of the *Caulobacter crescentus* S-layer protein: toward a definition of an N-terminal anchoring region and a C-terminal secretion signal and the potential for heterologous protein secretion. J Bacteriol 179:601–611

Blaser MJ (1998) *Campylobacter fetus* - emerging infection and model system for bacterial pathogenesis at mucosal surfaces. Clin Infect Dis 27:256–258

Blaser MJ, Gotschlich EC (1990) Surface array protein of *Campylobacter fetus*. J Biol Chem 265:14529–14535

Blaser MJ, Pei Z (1993) Pathogenesis of *Campylobacter fetus* infections: critical role of high-molecular-weight S-layer proteins in virulence. J Infect Dis 167:372–377

Blaser MJ, Smith PF, Hopkins JA, Heinzer I, Bryner JH, Wang WL (1987) Pathogenesis of *Campylobacter fetus* infections: serum resistance associated with high-molecular-weight surface proteins. J Infect Dis 155:696–706

Blaser MJ, Smith PF, Repine JE, Joiner KA (1988) Pathogenesis of *Campylobacter fetus* infections. Failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. J Clin Invest 81:1434–1444

Blaser MJ, Wang E, Tummuru MK, Washburn R, Fujimoto S, Labigne A (1994) High-frequency S-layer protein variation in *Campylobacter fetus* revealed by *sapA* mutagenesis. Mol Microbiol 14:453–462

Boot HJ, Kolen CPAM, van Noort JM, Pouwels PH (1993) S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. J Bacteriol 175:6089–6096

Boot HJ, Kolen CPAM, Pouwels PH (1995) Identification, cloning and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J Bacteriol 177:7222–7230

Boot HJ, Kolen CPAM, Pouwels PH (1996) Interchange of the active and silent S-layer protein genes of *Lactobacillus acidophilus* by inversion of the chromosomal *slp* segment. Mol Microbiol 21:799–809

Boris S, Suárez JE, Barbés C (1997) Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. J Appl Microbiol 83:413–420

Borriello SP, Davies HA, Kamiya S, Reed PJ, Seddon S (1990) Virulence factors of *Clostridium difficile*. Rev Infect Dis 12(Suppl 2):S185–S191

Bowditch RD, Baumann P, Yousten AA (1989) Cloning and sequencing of the gene encoding a 125-kilodalton surface-layer protein from *Bacillus sphaericus* 2362 and of a related cryptic gene. J Bacteriol 171:4178–4188

Brechtel E, Matuschek M, Hellberg A, Egelseer EM, Schmid R, Bahl H (1999) Cell wall of *Thermoanaerobacterium thermosulfurigenes* EM1: isolation of its components and attachment of the xylanase XynA. Arch Microbiol 171:159–165

Broda DM, Lawson PA, Bell RG, Musgrave DR (1999) *Clostridium frigidicarnis* sp. nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats. Int J Syst Bacteriol 49:1539–1550

Brüggemann H, Bäumer S, Fricke WF, Wiezer A, Liesegang H, Decker I, Herzberg C, Martínez-Arias R, Merkl R, Henne A, Gottschalk G (2003) The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. Proc Natl Acad Sci USA 100:1316–1321

Buck BL, Altermann E, Svingerud T, Klaenhammer TR (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 71: 8344–8351

Cachat E, Priest FG (2005) *Lactobacillus suntoryeus* sp. nov., isolated from malt whisky distilleries. Int J Syst Evol Microbiol 55:31–34

Calabi E, Fairweather N (2002) Patterns of sequence conservation in the S-layer proteins and related sequences in *Clostridium difficile*. J Bacteriol 184:3886–3897

Calabi E, Ward S, Wren B, Paxton T, Panico M, Morris H, Dell A, Dougan G, Fairweather N (2001) Molecular characterization of the surface layer proteins from *Clostridium difficile*. Mol Microbiol 40:1187–1199

Calabi E, Calabi F, Phillips AD, Fairweather NF (2002) Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. Infect Immun 70:5770–5778

Callegari ML, Riboli B, Sanders JW, Cocconcelli PS, Kok J, Venema G, Morelli L (1998) The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression. Microbiology 144:719–726

Candela T, Mignot T, Hagnerelle X, Haustant M, Fouet A (2005) Genetic analysis of *Bacillus anthracis* Sap S-layer protein crystallization domain. Microbiology 151:1485–1490

Cann IKO, Stroot PG, Mackie KR, White BA, Mackie RI (2001) Characterization of two novel saccharolytic, anaerobic thermophiles, *Thermoanaerobacterium polysaccharolyticum* sp. nov. and *Thermoanaerobacterium zeae* sp. nov., and emendation of the genus *Thermoanaerobacterium*. Int J Syst Evol Microbiol 51:293–302

Carl M, Dobson ME, Ching W-M, Dasch GA (1990) Characterization of the gene encoding the protective paracrystalline-surface-layer protein of *Ricksettsia prowazekii*: presence of a truncated identical homolog in *Ricksettsia typhi*. Proc Natl Acad Sci USA 87:8237–8241

Casadémont I, Chevrier D, Guesdon J-L (1998) Cloning of a *sapB* homologue (*sapB2*) encoding a putative 112-kDa *Campylobacter fetus* S-layer protein and its use for identification and molecular genotyping. FEMS Immunol Med Microbiol 21:269–281

Cerquetti M, Molinari A, Sebastianelli A, Diociaiuti M, Petruzzelli R, Capo C, Mastrantonio P (2000) Characterization of surface layer proteins from different *Clostridium difficile* clinical isolates. Microb Pathol 28:363–372

Cerquetti M, Serafino A, Sebastianelli A, Mastrantonio P (2002) Binding of *Clostridium difficile* to Caco-2 epithelial cell line and to extracellular matrix proteins. FEMS Immunol Med Microbiol 32:211–218

Chen X, Xu J, Shuai J, Chen J, Zhang Z, Fang W (2007) The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157: H7 and *Salmonella typhimurium*. Int J Food Microbiol 115:307–312

Chitlaru T, Ariel N, Zvi A, Lion M, Velan B, Shafferman A, Elhanany E (2004) Identification of chromosomally encoded membranal polypeptides of *Bacillus anthracis* by a proteomic analysis: prevalence of proteins containing S-layer homology domains. Proteomics 4:677–691

Chitlaru T, Gat O, Grosfeld H, Inbar I, Gozlan Y, Shafferman A (2007) Identification of *in vivo*-expressed immunogenic proteins by serological proteome analysis of the *Bacillus anthracis* secretome. Infect Immun 75:2841–2852

Choudhury B, Leoff C, Saile E, Wilkins P, Quinn CP, Kannenberg EL, Carlson RW (2006) The structure of the major cell wall polysaccharide of *Bacillus anthracis* is species-specific. J Biol Chem 281:27932–27941

Christian R, Schulz G, Unger FM, Messner P, Küpcü Z, Sleytr UB (1986) Structure of a rhamnan from the surface layer glycoprotein of *Bacillus stearothermophilus* strain NRS 2004/3a. Carbohydr Res 150:265–272

Chu S, Cavaignac S, Feutrier J, Phipps BM, Kostrzynska M, Kay WW, Trust TJ (1991) Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. J Biol Chem 266:15258–15265

Claus H, Akça E, Debaerdemaeker T, Evrard C, Declercq J-P, Harris JR, Schlott B, König H (2005) Molecular organization of selected prokaryotic S-layer proteins. Can J Microbiol 51:731–743

Cook GM, Rainey FA, Patel BKC, Morgan HW (1996) Characterization of a new obligately anaerobic thermophile. *Thermoanaerobacter wiegelii* sp. nov. Int J Syst Bacteriol 46(123):127

Couture-Tosi E, Delacroix H, Mignot T, Mesnage S, Chami M, Fouet A, Mosser G (2002) Structural analysis and evidence for dynamic emergence of *Bacillus anthracis* S-layer networks. J Bacteriol 184:6448–6456

Davis ML, Thoden JB, Holden HM (2007) The X-ray structure of dTDP-4-keto-6-deoxy-D-glucose-3, 4-ketoisomerase. J Biol Chem 282:19227–19236

de Leeuw E, Li X, Lu W (2006) Binding characteristics of the *Lactobacillus brevis* ATCC 8287 surface layer to extracellular matrix proteins. FEMS Microbiol Lett 260:210–215

Deblaere RY, Desomer J, Dhaese P 1999. Expression of surface layer proteins. WO Patent No 9519371-A 8

Drudy D, Calabi E, Kyne L, Sougioultzis S, Kelly E, Fairweather N, Kelly CP (2004) Human antibody response to surface layer proteins in *Clostridium difficile* infection. FEMS Immunol Med Microbiol 41:237–242

Dubreuil JD, Logan SM, Cubbage S, Eidhin DN, McCubbin WD, Kay CM, Beveridge TJ, Ferris FG, Trust TJ (1988) Structural and biochemical analyses of a surface array protein of *Campylobacter fetus*. J Bacteriol 170:4165–4173

Dubreuil JD, Kostrzynska M, Austin JW, Trust TJ (1990) Antigenic differences among *Campylobacter fetus* S-layer proteins. J Bacteriol 172:5035–5043

Duda VI, Suzina NE, Severina LO, Bogdanova TI, Tsaplina IA, Karavaiko GI (2006) Ultrastructural organization of *Alicyclobacillus tolerans* strain $K1^T$ cells. Arch Microbiol 185:63–68

Dworkin J, Blaser MJ (1997) Molecular mechanisms of *Campylobacter fetus* surface layer protein expression. Mol Microbiol 26:433–440

Dworkin J, Tummuru MKR, Blaser MJ (1995) Segmental conversation of *sapA* sequences in type B *Campylobacter fetus* cells. J Biol Chem 270:15093–15101

Ebisu S, Tsuboi A, Takagi H, Naruse Y, Yamagata H, Tsukagoshi N, Udaka S (1990) Conserved structures of cell wall protein genes among protein-producing *Bacillus brevis* strains. J Bacteriol 172:1312–1320

Edelman S, Westerlund-Wikström B, Leskelä S, Kettunen H, Rautonen N, Apajalahti J, Korhonen TK (2002) In vitro adhesion specificity of indigenous lactobacilli within the avian intestinal tract. Appl Environ Microbiol 68:5155–5159

Egelseer EM, Schocher I, Sleytr UB, Sára M (1996) Evidence that an N-terminal S-layer protein fragment triggers the release of a cell-associated high-molecular-weight amylase in *Bacillus stearothermophilus* ATCC 12980. J Bacteriol 178:5602–5609

Egelseer E, Danhorn T, Pleschberger M, Hotzy C, Sleytr UB, Sára M (2001) Characterization of an S-layer glycoprotein produced in the course of S-layer variation of *Bacillus stearothermophilus* ATCC 12980 and sequencing and cloning of the *sbsD* gene encoding the protein moiety. Arch Microbiol 177:70–80

Egelseer E-M, Sára M, Pum D, Schuster B, Sleytr UB (2008) Genetically engineered S-layer proteins, S-layer-specific polysaccharides and lipids as components of a versatile molecular construction kit for application in nanobiotechnology. In: Shoseyov O, Levy I (eds) Nanobiotechnology: bioinspired devices and materials of the future. Science of Knowledge Press, Little Falls, NJ, pp 55–86

Eichler J, Adams MWW (2005) Posttranslational protein modification in Archaea. Microbiol Mol Biol Rev 69:393–425

Engel AM, Cejka Z, Lupas A, Lottspeich F, Baumeister W (1992) Isolation and cloning of Ompα a coiled-coil protein spanning the periplasmic space of the ancestral eubacterium *Thermotoga maritima*. EMBO J 11:4369–4378

Engelhardt H (2007) Are S-layers exoskeletons? The basic function of protein surface layers revisited. J Struct Biol 160:115–124

Engle M, Li Y, Rainey F, DeBlois S, Mai V, Reichert A, Mayer F, Messner P, Wiegel J (1996) *Thermobrachium celere*, gen. nov., sp. nov., a rapidly growing thermophilic, alkalitolerant, and proteolytic obligate anaerobe. Int J Syst Bacteriol 46:1025–1033

Esteve C, Alcaide E, Canals R, Merino S, Blasco D, Figueras MJ, Tomás JM (2004) Pathogenic *Aeromonas hydrophila* serogroup O:14 and O:81 strains with an S layer. Appl Environ Microbiol 70:5898–5904

Etienne-Toumelin I, Sirard J-C, Duflot E, Mock M, Fouet A (1995) Characterization of the *Bacillus anthracis* S-layer: cloning and sequencing of the structural gene. J Bacteriol 177:614–620

Faraldo MLM, de Pedro MA, Berenguer J (1992) Sequence of the S-layer gene of *Thermus thermophilus* HB8 and functionality of its promoter in *Escherichia coli*. J Bacteriol 174:7458–7462

Fardeau M-L, Magot M, Patel BKC, Thomas P, Garcia J-L, Ollivier B (2000) *Thermoanaerobacter subterraneus* sp. nov., a novel thermophile isolated from oilfield water. Int J Syst Evol Microbiol 50:2141–2149

Fenno JC, Wong GWK, Hannam PM, Müller K-H, Leung WK, McBride BC (1997) Conservation of *msp*, the gene encoding the major outer membrane protein of oral *Treponema* spp. J Bacteriol 179:1082–1089

Ferner-Ortner J, Mader C, Ilk N, Sleytr UB, Egelseer EM (2007) High-affinity interaction between the S-layer protein SbsC and the secondary cell wall polymer of *Geobacillus stearothermophilus* ATCC 12980 determined by surface plasmon resonance technology. J Bacteriol 189:7154–7158

Ferreira AC, Nobre MF, Rainey FA, Silva MT, Wait R, Burghardt J, Chung AP, da Costa MS (1997) *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. Int J Syst Bacteriol 47:939–947

Fletcher CM, Coyne MJ, Bentley DL, Villa OF, Comstock LE (2007) Phase-variable expression of a family of glycoproteins imparts a dynamic surface to a symbiont in its human intestinal ecosystem. Proc Natl Acad Sci USA 104:2413–2418

Ford MJ, Nomellini JF, Smit J (2007) S-layer anchoring and localization of an S-layer-associated protease in *Caulobacter crescentus*. J Bacteriol 189:2226–2237

Frece J, Kos B, Svetec IK, Zgaga Z, Mrša V, Šušković J (2005) Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J Appl Microbiol 98:285–292

Freitas F, Keim CN, Kachar B, Farina M, Lins U (2003) Envelope ultrastructure of uncultured naturally occurring magnetotactic cocci. FEMS Microbiol Lett 219:33–38

Fujimoto S, Takade A, Amako K, Blaser MJ (1991) Correlation between molecular size of the surface array protein and morphology and antigenicity of the *Campylobacter fetus* S-layer. Infect Immun 59:2017–2022

Fujino T, Béguin P, Aubert JP (1993) Organization of a *Clostridium thermocellum* gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in attachment of the cellulosome to the cell surface. J Bacteriol 175:1891–1899

Garrote GL, Delfederico L, Bibiloni R, Abraham AG, Pérez PF, Semorile L, de Antoni GL (2004) Lactobacilli isolated from kefir grains: evidence of the presence of S-layer proteins. J Dairy Res 71:222–230

Garrote GL, Serradell MA, Abraham AG, Añon MC, Fossati CA, de Antoni GL (2005) Development of an immunochemical method to detect *Lactobacillus kefir*. Food Agric Immunol 16:221–233

Gatti M, Fornasari ME, Neviani E (2001) Differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis* by SDS-PAGE of cell-wall proteins. Lett Appl Microbiol 32:352–356

Gatti M, Rossetti L, Fornasari ME, Lazzi C, Giraffa G, Neviani E (2005) Heterogeneity of putative surface layer proteins in *Lactobacillus helveticus*. Appl Environ Microbiol 71:7582–7588

Gilchrist A, Fisher JA, Smit J (1992) Nucleotide sequence analysis of the gene encoding the *Caulobacter crescentus* paracrystalline surface layer protein. Can J Microbiol 38:193–202

Gilmore RD Jr, Joste N, McDonald GA (1989) Cloning, expression and sequence analysis of the gene encoding the 120kD surface-exposed protein of *Rickettsia rickettsii*. Mol Microbiol 3:1579–1586

Gilmour R, Messner P, Guffanti AA, Kent R, Scheberl A, Kendrick N, Krulwich TA (2000) Two-dimensional gel electrophoresis analyses of pH-dependent protein expression in facultatively alkaliphilic *Bacillus pseudofirmus* OF4 lead to characterization of an S-layer protein with a role in alkaliphily. J Bacteriol 182:5969–5981

Golowczyc MA, Mobili P, Garrote GL, Abraham AG, de Antoni GL (2007) Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar Enteritidis. Int J Food Microbiol 118:264–273

González JM, Mayer F, Moran MA, Hodson RE, Whitman WB (1997) *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. Int J Syst Bacteriol 47:369–376

Graham LL (2002) *Campylobacter fetus* adheres to and enters INT 407 cells. Can J Microbiol 48:995–1007

Grech-Mora I, Fardeau M-L, Patel BKC, Ollivier B, Rimbault A, Prensier G, Garcia J-L, Garnier-Sillam E (1996) Isolation and characterization of *Sporobacter termitidis* gen. nov., sp. nov., from the digestive tract of the wood-feeding termite *Nasutitermes lujae*. Int J Syst Bacteriol 46:512–518

Grogono-Thomas R, Dworkin J, Blaser MJ, Newell DG (2000) Roles of the surface layer proteins of *Campylobacter fetus* subsp. *fetus* in ovine abortion. Infect Immun 68:1687–1691

Grogono-Thomas R, Blaser MJ, Ahmadi M, Newell DG (2003) Role of S-layer protein antigenic diversity in the immune responses of sheep experimentally challenged with *Campylobacter fetus* subsp. *fetus*. Infect Immun 71:147–154

Gürtürk K, Ekin IH, Arslan A (2007) Western blot analysis of the IgG-antibody response to acid-glycine-extracted antigens from *Campylobacter fetus* subsp. *fetus* and *C. jejuni* in naturally infected sheep. Acta Vet Brno 76:245–251

Hagen KE, Guan LL, Tannock GW, Korver DR, Allison GE (2005) Detection, characterization, and in vitro and in vivo expression of genes encoding S-proteins in *Lactobacillus gallinarum* strains isolated from chicken crops. Appl Environ Microbiol 71:6633–6643

Hahn M-J, Kim K-K, Kim I, Chang W-H (1993) Cloning and sequence analysis of the gene encoding the crystalline surface layer protein of *Rickettsia typhi*. Gene 133:129–133

Hansmeier N, Bartels FW, Ros R, Anselmetti D, Tauch A, Pühler A, Kalinowski J (2004) Classification of hyper-variable *Corynebacterium glutamicum* surface-layer proteins by sequence analyses and atomic force microscopy. J Biotechnol 112:177–193

Hansmeier N, Chao T-C, Pühler A, Tauch A, Kalinowski J (2006a) The cytosolic, cell surface and extracellular proteomes of the biotechnologically important soil bacterium *Corynebacterium efficiens* YS-314 in comparison to those of *Corynebacterium glutamicum* ATCC 13032. Proteomics 6:233–250

Hansmeier N, Albersmeier A, Tauch A, Damberg T, Ros R, Anselmetti D, Pühler A, Kalinowski J (2006b) The surface (S)-layer gene *cspB* of *Corynebacterium glutamicum* is transcriptionally activated by a LuxR-type regulator and located on a 6 kb genomic island absent from the type strain ATCC 13032. Microbiology 152:923–935

Hernández-Eugenio G, Fardeau M-L, Cayol J-L, Patel BKC, Thomas P, Macarie H, Garcia J-L, Ollivier B (2002) *Clostridium thiosulfatireducens* sp. nov., a proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor. Int J Syst Evol Microbiol 52:1461–1468

Heyndrickx M, Lebbe L, Vancanneyt M, Kersters K, De Vos P, Logan NA, Forsyth G, Nazli S, Ali N, Berkeley RCW (1997) A polyphasic reassessment of the genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* (Meier-Stauffer et al. 1996) as *Aneurinibacillus*

thermoaerophilus comb. nov., and emended descriptions of *A. aneurinilyticus* corrig., *A. migulanus*, and *A. thermoaerophilus*. Int J Syst Bacteriol 47:808–817

Higuchi N, Murakami Y, Moriguchi K, Ohno N, Nakamura H, Yoshimura F (2000) Localization of major, high molecular weight proteins in *Bacteroides forsythus*. Microbiol Immunol 44:777–780

Hinode D, Yokoyama M, Tanabe S, Yoshioka M, Nakamura R (2002) Antigenic properties of the GroEL-like protein of *Campylobacter rectus*. Oral Microbiol Immunol 17:16–21

Hoiczyk E, Hansel A (2000) Cyanobacterial cell walls: news from an unusual prokaryotic envelope. J Bacteriol 182:1191–1199

Holliger C, Hahn D, Harmsen H, Ludwig W, Schumacher W, Tindall B, Vazquez F, Weiss N, Zehnder AJB (1998) *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. Arch Microbiol 169:313–321

Hollmann A, Delfederico L, Glikmann G, de Antoni G, Semorile L, Disalvo EA (2007) Characterization of liposomes coated with S-layer proteins from lactobacilli. Biochim Biophys Acta 1768:393–400

Holt SC, Leadbetter ER (1969) Comparative ultrastructure of selected aerobic spore forming bacteria: a freeze-etching study. Bacteriol Rev 33:346–378

Honma K, Inagaki S, Okuda K, Kuramitsu HK, Sharma A (2007) Role of a *Tannerella forsythia* exopolysaccharide synthesis operon in biofilm development. Microb Pathog 42:156–166

Horejs C, Pum D, Sleytr UB, Tscheliessnig R (2008) Structure prediction of an S-layer protein by the mean force method. J Chem Phys 128:065106

Horie M, Kajikawa HS, Toba T (2002) Identification of *Lactobacillus crispatus* by polymerase chain reaction targeting S-layer protein gene. Lett Appl Microbiol 35:57–61

Horn MA, Ihssen J, Matthies C, Schramm A, Acker G, Drake HL (2005) *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. Int J Syst Evol Microbiol 55:1255–1265

Houwink AL (1953) A macromolecular monolayer in the cell wall of *Spirillum* spec. Biochim Biophys Acta 10:360–366

Houwink AL, Le Poole JB (1952) Eine Struktur in der Zellmembran einer Bakterie. Physikalische Verhandlungen 98

Hovmöller S (1993) Crystallographic image processing applications for S-layers. In: Beveridge TJ, Koval SF (eds) Advances in bacterial paracrystalline surface layers. Plenum Press, New York, pp 13–21

Howorka S, Sára M, Wang Y, Kuen B, Sleytr UB, Lubitz W, Bayley H (2000) Surface-accessible residues in the monomeric and assembled forms of a bacterial surface layer protein. J Biol Chem 275:37876–37886

Hu C-H, Bao L, Xie J-P, Zhang H-D (2004) Cloning, expression and immunogenicity of S-layer membrane protein gene (*Lslp*) of pathogenic *Leptospira interrogans*. Acta Microbiol Sin 44:500–503

Huber C, Ilk N, Rünzler D, Egelseer EM, Weigert S, Sleytr UB, Sára M (2005) The three S-layer-like homology motifs of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 are not sufficient for binding to the pyruvylated secondary cell wall polymer. Mol Microbiol 55:197–205

Huber R, Rossnagel P, Woese CR, Rachel R, Langworthy TA, Stetter KO (1996) Formation of ammonium from nitrate during chemolithoautotrophic growth of the extremely thermophilic bacterium *Ammonifex degensii* gen. nov. sp. nov. System Appl Microbiol 19:40–49

Hynönen U, Westerlund-Wikström B, Palva A, Korhonen TK (2002) Identification by flagellum display of an epithelial cell- and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. J Bacteriol 184:3360–3367

Iino T, Mori K, Tanaka K, K-i S, Harayama S (2007) *Oscillibacter valericigenes* gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam. Int J Syst Evol Microbiol 57:1840–1845

Ilk N, Kosma P, Puchberger M, Egelseer EM, Mayer HF, Sleytr UB, Sára M (1999) Structural and functional analyses of the secondary cell wall polymer of *Bacillus sphaericus* CCM 2177 that serves as an S-layer-specific anchor. J Bacteriol 181:7643–7646

Ilk N, Völlenkle C, Egelseer EM, Breitwieser A, Sleytr UB, Sára M (2002) Molecular characterization of the S-layer gene, *sbpA*, of *Bacillus sphaericus* CCM 2177 and production of a functional S-layer fusion protein with the ability to recrystallize in a defined orientation while presenting the fused allergen. Appl Environ Microbiol 68:3251–3260

Ito Y, Tomita T, Roy N, Nakano A, Sugawara-Tomita N, Watanabe S, Okai N, Abe N, Kamio Y (2003) Cloning, expression, and cell surface localization of *Paenibacillus* sp. strain W-61 xylanase 5, a multidomain xylanase. Appl Environ Microbiol 69:6969–6978

Iuga M, Awram P, Nomellini JF, Smit J (2004) Comparison of S-layer secretion genes in freshwater caulobacters. Can J Microbiol 50:751–766

Jakava-Viljanen M, Palva A (2007) Isolation of surface (S) layer protein carrying *Lactobacillus* species from porcine intestine and faeces and characterization of their adhesion properties to different host tissues. Vet Microbiol 124:264–273

Jakava-Viljanen M, Åvall-Jääskeläinen S, Messner P, Sleytr UB, Palva A (2002) Isolation of three new surface layer protein genes (*slp*) from *Lactobacillus brevis* ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions. J Bacteriol 184:6786–6795

Jankovic I, Ventura M, Meylan V, Rouvet M, Elli M, Zink R (2003) Contribution of aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri* 4B2. J Bacteriol 185:3288–3296

Jarosch M, Egelseer EM, Mattanovich D, Sleytr UB, Sára M (2000) S-layer gene *sbsC* of *Bacillus stearothermophilus* ATCC 12980: molecular characterization and heterologous expression in *Escherichia coli*. Microbiology 146:273–281

Jarosch M, Egelseer EM, Huber C, Moll D, Mattanovich D, Sleytr UB, Sára M (2001) Analysis of the structure-function relationship of the S-layer protein SbsC of *Bacillus stearothermophilus* ATCC 12980 by producing truncated forms. Microbiology 147:1353–1363

Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM (2007) Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. Cell Microbiol 9:356–367

Kählig H, Kolarich D, Zayni S, Scheberl A, Kosma P, Schäffer C, Messner P (2005) N-Acetylmuramic acid as capping element of α-D-fucose-containing S-layer glycoprotein glycans from *Geobacillus tepidamans* GS5-97 T. J Biol Chem 280:20292–20299

Kalmokoff M, Austin JW, Whitford MF, Teather RM (2000) Characterization of a major envelope protein from the rumen anaerobe *Selenomonas ruminantium* OB268. Can J Microbiol 46:295–303

Kalyuzhnaya MG, Khmelenina VN, Starostina NG, Baranova SV, Suzina NE, Trotsenko YA (1998) Newly moderately halophilic methanotroph of the genus *Methylobacter*. Microbiology (Moscow) 67:438–444

Kaluzhnaya M, Khmelenina V, Eshinimaev B, Suzina N, Nikitin D, Solonin A, Lin J-L, McDonald I, Murrell C, Trotsenko Y (2001) Taxonomic characterization of new alkaliphilic and alkalitolerant methanotrophs from soda lakes of the Southeastern Transbaikal Region and description of *Methylomicrobium buryatense* sp. nov. Syst Appl Microbiol 24:166–176

Kang M-S, Na H-S, Oh J-S (2005) Coaggregation ability of *Weissella cibaria* isolates with *Fusobacterium nucleatum* and their adhesiveness to epithelial cells. FEMS Microbiol Lett 253:323–329

Karjalainen T, Waligora-Dupriet AJ, Cerquetti M, Spigaglia P, Maggioni A, Mauri P, Mastrantonio P (2001) Molecular and genomic analysis of genes encoding surface-anchored proteins from *Clostridium difficile*. Infect Immun 69:3442–3446

Karjalainen T, Saumier N, Barc MC, Delmee M, Collignon A (2002) *Clostridium difficile* genotyping based on *slpA* variable region in S-layer gene sequence: an alternative to serotyping. J Clin Microbiol 40:2452–2458

Kato H, Yokoyama T, Arakawa Y (2005) Typing by sequencing the *slpA* gene of *Clostridium difficile* strains causing multiple outbreaks in Japan. J Med Microbiol 54:167–171

Kawai E, Akatsuka H, Idei A, Shibatani T, Mori K (1998) *Serratia marcescens* S-layer protein is secreted extracellularly *via* an ATP-binding cassete exporter, the Lip system. Mol Microbiol 27:941–952

Kelly CP, LaMont JT (1998) *Clostridium difficile* infection. Annu Rev Med 49:375–390

Kerosuo E (1988) Ultrastructure of the cell envelope of *Bacteroides forsythus* strain ATCC 43037 ^{T}. Oral Microbiol Immunol 3:134–137

Khmelenina VN, Kalyuzhnaya MG, Sakharovsky VG, Suzina NE, Trotsenko YA, Gottschalk G (1999) Osmoadaptation in halophilic and alkaliphilic methanotrophs. Arch Microbiol 172:321–329

Klouche N, Fardeau M-L, Lascourrèges J-F, Cayol J-L, Hacene H, Thomas P, Magot M (2007) *Geosporobacter subterraneus* gen. nov., sp. nov., a spore-forming bacterium isolated from a deep subsurface aquifer. Int J Syst Evol Microbiol 57:1757–1761

Kos B, Šušković J, Vuković S, Šimpraga M, Frece J, Matošić S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol 94:981–987

Kosma P, Neuninger C, Christian R, Schulz G, Messner P (1995a) Glycan structure of the S-layer glycoprotein of *Bacillus* sp. L420–91. Glycoconjugate J 12:99–107

Kosma P, Wugeditsch T, Christian R, Zayni S, Messner P (1995b) Glycan structure of a heptose-containing S-layer glycoprotein of *Bacillus thermoaerophilus*. Glycobiology 5:791-796; Erratum: Glycobiology (1996) 6:5

Kotiranta A, Haapasalo M, Lounatmaa K, Kari K (1995) Crystalline surface protein of *Peptostreptococcus anaerobius*. Microbiology 140:1065–1073

Kotiranta A, Haapasalo M, Kari K, Kerosuo E, Olson I, Sorsa T, Meurman JH, Lounatmaa K (1998) Surface structure, hydrophobicity, phagocytosis, and adherence to matrix proteins of *Bacillus cereus* cells with and without the crystalline surface protein layer. Infect Immun 66:4895–4902

Koval SF, Murray RGE (1984) The isolation of surface array proteins from bacteria. Can J Biochem Cell Biol 62:1181–1189

Krishnamurthy T, Hewel J, Bonzagni NJ, Dabbs J, Bull RL, Yates JR III (2006) Simultaneous identification and verification of *Bacillus anthracis*. Rapid Commun Mass Spectrom 20: 2053–2056

Krooneman J, Faber F, Alderkamp AC, Oude Elferink SJHW, Driehuis F, Cleenwerck I, Swings J, Gottschal JC, Vancanneyt M (2002) Lactobacillus diolivorans sp. nov., a 1, 2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int J Syst Evol Microbiol 52:639–646

Kuen B, Sleytr UB, Lubitz W (1994) Sequence analysis of the *sbsA* gene encoding the 130-kDa surface layer protein of *Bacillus stearothermophilus* strain PV72. Gene 154:115–120

Kuen B, Sára M, Lubitz W (1995) Heterologous expression and self-assembly of the S-layer protein SbsA of *Bacillus stearothermophilus* in *Escherichia coli*. Mol Microbiol 19:495–503

Kuen B, Koch A, Asenbauer E, Sára M, Lubitz W (1997) Molecular characterization of the *Bacillus stearothermophilus* PV72 S-layer gene *sbsB* induced by oxidative stress. J Bacteriol 179:1664–1670

Kuen B, Koch A, Asenbauer E, Sára M, Lubitz W (1997) Molecular characterization of the *Bacillus stearothermophilus* PV72 S-layer gene *sbsB* induced by oxidative stress. J Bacteriol 179:1664–1670

Küpcü Z, März L, Messner P, Sleytr UB (1984) Evidence for the glycoprotein nature of the crystalline cell wall surface layer of *Bacillus stearothermophilus* strain NRS 2004/3a. FEBS Lett 173:185–190

Lamonica JM, Wagner M, Eschenbrenner M, Williams LE, Miller TL, Patra G, DelVecchio VG (2005) Comparative secretome analyses of three *Bacillus anthracis* strains with variant plasmid contents. Infect Immun 73:3646–3658

Lee KY, Kang EY, Park S, Ahn SK, Yoo KH, Kim JY, Lee HH (2006a) Mass spectrometric sequencing of endotoxin proteins of *Bacillus thuringiensis* ssp. *konkukian* extracted from polyacrylamide gels. Proteomics 6:1512–1517

Lee S-W, Sabet M, Um H-S, Yang J, Kim HC, Zhu W (2006b) Identification and characterization of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. Gene 371:102–111

Lee YJ, Romanek CS, Mills GL, Davis RC, Whitman WB, Wiegel J (2006c) *Gracilibacter thermotolerans* gen. nov., sp. nov., an anaerobic, thermotolerant bacterium from a constructed wetland receiving acid sulfate water. Int J Syst Evol Microbiol 56:2089–2093

Lehmann D, Sodoyer R, Leterme S (2004) Characterization of BoHV-1 gE envelope glycoprotein mimotopes obtained by phage display. Vet Microbiol 104:1–17

Lemaire M, Miras I, Gounon P, Béguin P (1998) Identification of a region responsible for binding to the cell wall within the S-layer protein of *Clostridium thermocellum*. Microbiology 144:211–217

L'Haridon S, Cilia V, Messner P, Raguénès G, Gambacorta A, Sleytr UB, Prieur D, Jeanthon C (1998) *Desulfurobacterium thermolithotrophicum* gen. nov., sp. nov., a novel thermophilic anaerobic autotrophic sulfur-reducing bacterium isolated from a deep-sea hydrothermal vent. Int J Syst Bacteriol 48:701–711

Lindholm A, Smeds A, Palva A (2004) Receptor binding domain of *Escherichia coli* F18 fimbrial adhesin FedF can be both efficiently secreted and surface displayed in a functional form in *Lactococcus lactis*. Appl Environ Microbiol 70:2061–2071

Liu S-Y, Rainey FA, Morgan HW, Mayer F, Wiegel J (1996) *Thermoanaerobacterium aotearoense* sp. nov., a slightly acidophilic, anaerobic thermophile isolated from various hot springs in New Zealand, and emendation of the genus *Thermoanaerobacterium*. Int J Syst Bacteriol 46:388–396

Logan SM (2006) Flagellar glycosylation – a new component of the motility repertoire? Microbiology 152:1249–1262

Lorca G, Torino MI, de Valdez GF, Ljungh Å (2002) Lactobacilli express cell surface proteins which mediate binding of immobilized collagen and fibronectin. FEMS Microbiol Lett 206:31–37

Lupas A, Engelhardt H, Peters J, Santarius S, Volker S, Baumeister W (1994) Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. J Bacteriol 176:1224–1233

Mader C, Küpcü S, Sára M, Sleytr UB (1999) Stabilizing effect of an S-layer on liposomes towards thermal or mechanical stress. Biochim Biophys Acta 1418:106–116

Mader C, Huber C, Moll D, Sleytr UB, Sára M (2004) Interaction of the crystalline bacterial cell surface layer protein SbsB and the secondary cell wall polymer of *Geobacillus stearothermophilus* PV72 assessed by real-time surface plasmon resonance biosensor technology. J Bacteriol 186:1758–1768

Madetoja J, Pylkkö P, Pohjanvirta T, Schildt L, Pelkonen S (2003) Putative virulence factors of atypical *Aeromonas salmonicida* isolated from Arctic charr, *Salvelinus alpinus* (L.), and European grayling, *Thymallus thymallus* (L.). J Fish Dis 26:349–359

McCarren J, Heuser J, Roth R, Yamada N, Martone M, Brahamsha B (2005) Inactivation of *swmA* results in the loss of an outer cell layer in a swimming *Synechococcus* strain. J Bacteriol 187:224–230

McCoubrey J, Poxton IR (2001) Variation in the surface layer proteins of *Clostridium difficile*. FEMS Immunol Med Microbiol 31:131–135

McCoubrey J, Starr J, Martin H, Poxton IR (2003) *Clostridium difficile* in a geriatric unit: a prospective epidemiological study employing a novel S-layer typing method. J Med Microbiol 52:573–578

McSweeney CS, Dulieu A, Webb RI, Del Dot T, Blackall LL (1999) Isolation and characterization of a *Clostridium* sp. with cinnamoyl esterase activity and unusual cell envelope ultrastructure. Arch Microbiol 172:139–149

Meier-Stauffer K, Busse H-J, Rainey FA, Burghardt J, Scheberl A, Hollaus F, Kuen B, Makristathis A, Sleytr UB, Messner P (1996) Description of *Bacillus thermoaerophilus* sp. nov., to include sugar beet isolates and *Bacillus brevis* ATCC 12990. Int J Syst Bacteriol 46:532–541

Merino S, Rubires X, Aguilar A, Albertí S, Hernandez-Allés S, Benedí VJ, Tomás JM (1996) Mesophilic *Aeromonas* sp. serogroup O:11 resistance to complement-mediated killing. Infect Immun 64:5302–5309

Mertig M, Kirsch R, Pompe W, Engelhardt H (1999) Fabrication of highly oriented nanocluster arrays by biomolecular templating. Eur Phys J D 9:45–48

Mertig M, Wahl R, Lehmann M, Simon P, Pompe W (2001) Formation and manipulation of regular metallic nanoparticle arrays on bacterial surface layers: an advanced TEM study. Eur Phys J D 16:317–320

Mesnage S, Tosi-Couture E, Mock M, Gounon P, Fouet A (1997) Molecular characterization of the *Bacillus anthracis* main S-layer component: evidence that it is the major cell-associated antigen. Mol Microbiol 23:1147–1155

Mesnage S, Tosi-Couture E, Mock M, Fouet A (1999) The S-layer homology domain as a means for anchoring heterologous proteins on the cell surface of *Bacillus anthracis*. J Appl Microbiol 87:256–260

Mesnage S, Fontaine T, Mignot T, Delepierre M, Mock M, Fouet A (2000) Bacterial SLH domain proteins are non-covalently anchored to the cell surface *via* a conserved mechanism involving wall polysaccharide pyruvylation. EMBO J 19:4473–4484

Mesnage S, Haustant M, Fouet A (2001) A general strategy for identification of S-layer genes in the *Bacillus cereus* group: molecular characterization of such a gene in *Bacillus thuringiensis* subsp. *galleriae* NRRL 4045. Microbiology 147:1343–1351

Messner P (1996) Chemical composition and biosynthesis of S-layers. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Crystalline bacterial cell surface proteins. RG Landes/Academic Press, Austin, TX, pp 35–76

Messner P, Schäffer C (2000) Surface layer glycoproteins of bacteria and archaea. In: Doyle RJ (ed) Glycomicrobiology. Kluwer Academic/Plenum Publishers, New York, pp 93–125

Messner P, Schäffer C (2003) Prokaryotic glycoproteins. In: Herz W, Falk H, Kirby GW (eds) Progress in the chemistry of organic natural products, vol 85. Springer-Verlag, Wien, pp 51–124

Messner P, Sleytr UB (1988) Separation and purification of S layers from Gram-positive and Gram-negative bacteria. In: Hancock IC, Poxton I (eds) Bacterial cell surface techniques. John Wileys & Sons, Chichester, pp 97–104

Messner P, Sleytr UB (1991) Bacterial surface layer glycoproteins. Glycobiology 1:545–551

Messner P, Sleytr UB (1992) Crystalline bacterial cell-surface layers. In: Rose AH (ed) Advances in microbial physiology, vol 33. Academic Press, London, pp 213–275

Messner P, Hollaus F, Sleytr UB (1984) Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains. Int J Syst Bacteriol 34:202–210

Messner P, Pum D, Sára M, Stetter KO, Sleytr UB (1986a) Ultrastructure of the cell envelope of the archaebacteria *Thermoproteus tenax* and *Thermoproteus neutrophilus*. J Bacteriol 166:1046–1054

Messner P, Pum D, Sleytr UB (1986b) Characterization of the ultrastructure and the self-assembly of the surface layer of *Bacillus stearothermophilus* strain NRS 2004/3a. J Ultrastruct Mol Struct Res 97:73–88

Messner P, Scheberl A, Schweigkofler W, Hollaus F, Rainey FA, Burghardt J, Prillinger H (1997) Taxonomic comparison of different thermophilic sugar beet isolates with glycosylated surface layer (S-layer) proteins and their affiliation to *Bacillus smithii*. Syst Appl Microbiol 20:559–565

Messner P, Steiner K, Zarschler K, Schäffer C (2008) S-layer nanoglycobiology of bacteria. Carbohydr Res. 343:1934–1951

Mignot T, Denis B, Couture-Tosi E, Kolstø A-B, Mock M, Fouet A (2001) Distribution of S-layers on the surface of *Bacillus cereus* strains: phylogenetic origin and ecological pressure. Environ Microbiol 3:493–501

Mignot T, Mesnage S, Couture-Tosi E, Mock M, Fouet A (2002) Developmental switch of S-layer protein synthesis in *Bacillus anthracis*. Mol Microbiol 43:1615–1627

Mignot T, Mock M, Fouet A (2003) A plasmid-encoded regulator couples the synthesis of toxins and surface structures in *Bacillus anthracis*. Mol Microbiol 47:917–927

Mignot T, Couture-Tosi E, Mesnage S, Mock M, Fouet A (2004) In vivo *Bacillus anthracis* gene expression requires PagR as an intermediate effector of the AtxA signalling cascade. Int J Med Microbiol 293:619–624

Mikshis NI, Korsakova AI, Bolotnikova MF, Novikova LV, Popov IA (2006) Role of the components of the S-layer in immunogenicity of *Bacillus anthracis*. Zh Mikrobiol Epidemiol Immunobiol 2006(1):29–32

Miyamoto M, Maeda H, Kitanaka M, Kokeguchi S, Takashiba S, Murayama Y (1998) The S-layer protein gene of *Campylobacter rectus*: sequence determination and function of the recombinant protein. FEMS Microbiol Lett 166:275–281

Mock M, Fouet A (2001) Anthrax. Annu Rev Microbiol 55:647–671

Mohamed HE, van de Meene AML, Roberson RW, Vermaas WFJ (2005) Myxoxanthophyll is required for normal cell wall structure and thylakoid organization in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J Bacteriol 187:6883–6892

Mobili P, de los Ángeles Serradell M, Trejo SA, Puigvert FXA, Abraham AG, de Antoni GL (2009) Heterogeneity of S-layer proteins from aggregating and non-aggregating *Lactobacillus kefir* strains. Antonie van Leeuwenhoek 95:363–372

Moll D, Huber C, Schlegel B, Pum D, Sleytr UB, Sára M (2002) S-layer-streptavidin fusion proteins as template for nanopatterned molecular arrays. Proc Natl Acad Sci USA 99: 14646–14651

Morris RM, Sowell S, Barofsky D, Zinder S, Richardson R (2006) Transcription and mass-spectroscopic proteomic studies of electron transport oxidoreductases in *Dehalococcoides ethenogenes*. Environ Microbiol 8:1499–1509

Mota RM, Moreira JLS, Souza MR, Horta MF, Teixeira SMR, Neumann E, Nicoli JR, Nunes AC (2006) Genetic transformation of novel isolates of chicken *Lactobacillus* bearing probiotic features for expression of heterologous proteins: a tool to develop live oral vaccines. BMC Biotechnol 6:2

Mozes N, Lortal S (1995) X-ray photoelectron spectroscopy and biochemical analysis of the surface of *Lactobacillus helveticus* ATCC 12046. Microbiology 141:11–19

Mukherjee K, Karlsson S, Burman LG, Åkerlund T (2002) Proteins released during high toxin production in *Clostridium difficile*. Microbiology 148:2245–2253

Müller DJ, Baumeister W, Engel A (1996) Conformational change of the hexagonally packed intermediate layer of *Deinococcus radiodurans* monitored by atomic force microscopy. J Bacteriol 178:3025–3030

Müller DJ, Baumeister W, Engel A (1999) Controlled unzipping of a bacterial surface layer with atomic force microscopy. Proc Natl Acad Sci USA 96:13170–13174

Murray RGE (1993) A perspective on S-layer research. In: Beveridge TJ, Koval SF (eds) Advances in bacterial paracrystalline surface layers. Plenum Press, New York, pp 3–9

Nakazawa F, Poco SE Jr, Sato M, Ikeda T, Kalfas S, Sundqvist G, Hoshino E (2002) Taxonomic characterization of *Mogibacterium diversum* sp. nov. and *Mogibacterium neglectum* sp. nov., isolated from human oral cavities. Int J Syst Evol Microbiol 52:115–122

Narita J, Ispida S, Okano K, Rimura S, Fukuda H, Kondo A (2006) Improvement of protein production in lactic acid bacteria using 5′-untranslated leader sequence of *slpA* from *Lactobacillus acidophilus*: improvement in protein production using UTLS. Appl Microbiol Biotechnol 73:366–373

Naser SM, Hagen KE, Vancanneyt M, Cleenwerck I, Swings J, Tompkins TA (2006a) *Lactobacillus suntoryeus* Cachat and Priest 2005 is a later synonym of *Lactobacillus helveticus* (Orla-Jensen 1919) Bergey et al. 1925 (Approved Lists 1980). Int J Syst Evol Microbiol 56:355–360

Naser SM, Vancanneyt M, Snauwaert C, Vrancken G, Hoste B, de Vuyst L, Swings J (2006b) Reclassification of *Lactobacillus amylophilus* LMG 11400 and NRRL B-4435 as *Lactobacillus amylotrophicus* sp. nov. Int J Syst Evol Microbiol 56:2523–2527

Newman DK, Kennedy EK, Coates JD, Ahmann D, Ellis DJ, Lovley DR, Morel FMM (1997) Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. Arch Microbiol 168:380–388

Ní Eidhin D, Ryan AW, Doyle RM, Walsh JB, Kelleher D (2006) Sequence and phylogenetic analysis of the gene for surface layer protein, *slpA*, from 14 PCR ribotypes of *Clostridium difficile*. J Med Microbiol 55:69–83

Ní Eidhin DB, O'Brien JB, McCabe MS, Athié-Morales V, Kelleher DP (2008) Active immunization of hamsters against *Clostridium difficile* infection using surface-layer protein. FEMS Immunol Med Microbiol 52:207–218

Nitta H, Holt SC, Ebersole JL (1997) Purification and characterization of *Campylobacter rectus* surface layer proteins. Infect Immun 65:478–483

Norville JE, Kelly DF, Knight TF Jr, Belcher AM, Walz T (2007) 7 Å projection map of the S-layer protein SbpA obtained with trehalose-embedded monolayer crystals. J Struct Biol 160:313–323

Novotny R, Pfoestl A, Messner P, Schäffer C (2004a) Genetic organization of chromosomal S-layer glycan biosynthesis loci of *Bacillaceae*. Glycoconjugate J 20:435–447

Novotny R, Schäffer C, Strauss J, Messner P (2004b) S-layer glycan-specific loci on the chromosome of *Geobacillus stearothermophilus* NRS 2004/3a and dTDP-L-rhamnose biosynthesis potential of *G. stearothermophilus* strains. Microbiology 150:953–965

O'Brien JB, McCabe MS, Athié-Morales V, McDonald GSA, Ní Eidhin DB, Kelleher DP (2005) Passive immunisation of hamsters against *Clostridium difficile* infection using antibodies to surface layer proteins. FEMS Microbiol Lett 246:199–205

Ohno M, Shiratori H, Park M-J, Saitoh Y, Kumon Y, Yamashita N, Hirata A, Nishida H, Ueda K, Beppu T (2000) *Symbiobacterium thermophilus* gen. nov., sp. nov., a symbiotic thermophile that depends on co-culture with a *Bacillus* strain for growth. Int J Syst Evol Microbiol 50:1829–1832

Olabarría G, Fernández-Herrero LA, Carrascosa JL, Berenguer J (1996) *slpM*, a gene coding for an "S-layer-like array" overexpressed in S-layer mutants of *Thermus thermophilus* HB8. J Bacteriol 178:357–365

Örlygsson J, Krooneman J, Collins MD, Pascual C, Gottschal JC (1996) *Clostridium acetireducens* sp. nov., a novel amino acid-oxidizing, acetate-reducing anaerobic bacterium. Int J Syst Bacteriol 46:454–459

Pavkov T, Oberer M, Egelseer EM, Sára M, Sleytr UB, Keller W (2003) Crystallization and preliminary structure determination of the C-terminal truncated domain of the S-layer protein SbsC. Acta Crystallogr D Biol Crystallogr 59:1466–1468

Pavkov T, Egelseer EM, Tesarz M, Svergun DI, Sleytr UB, Keller W (2008) The crystal structure and binding behavior of the bacterial cell surface (S-)layer protein SbsC. Structure 16:1226–1237

Pavlova SI, Kiliç AO, Topisirovic L, Miladinov N, Hatzos C, Tao L (2002) Characterization of a cryptic plasmid from *Lactobacillus fermentum* KC5b and its use for constructing a stable *Lactobacillus* cloning vector. Plasmid 47:182–192

Pei Z, Blaser MJ (1990) Pathogenesis of *Campylobacter fetus* infections. Role of surface array proteins in virulence in a mouse model. J Clin Invest 85:1036–1043

Pei Z, Ellison RT III, Lewis RV, Blaser MJ (1988) Purification and characterization of a family of high molecular weight surface-array proteins from *Campylobacter fetus*. J Biol Chem 263:6416–6420

Peters J, Peters M, Lottspeich F, Schäfer W, Baumeister W (1987) Nucleotide sequence analysis of the gene encoding the *Deinococcus radiodurans* surface protein, derived amino acid sequence and complementary protein chemical studies. J Bacteriol 169:5216–5223

Peters J, Peters M, Lottspeich F, Baumeister W (1989) S-layer protein gene of *Acetogenium kivui*: cloning and expression in *Escherichia coli* and determination of the nucleotide sequence. J Bacteriol 171:6307–6315

Peyret JL, Bayan N, Joliff G, Gulik-Krzywicki T, Mathieu L, Shechter E, Leblon G (1993) Characterization of the *cspB* gene encoding PS2, an ordered surface-layer protein in *Corynebacterium glutamicum*. Mol Microbiol 9:97–109

Petersen BO, Sára M, Mader C, Mayer HF, Sleytr UB, Pabst M, Puchberger M, Krause E, Hofinger A, Duus JØ, Kosma P (2008) The structure of the secondary cell wall polymer of *Geobacillus stearothermophilus* PV72/p2. Carbohydr Res 343:1346–1358

Pfeiler EA, Klaenhammer TR (2007) The genomics of lactic acid bacteria. Trends Microbiol 15:546–553

Phoenix VR, Renaut RW, Jones B, Ferry FG (2005) Bacterial S-layer preservation and rare arsenic-antimony-sulphide bioimmobilization in siliceous sediments from Champagne Pool hot spring, Waiotapu, New Zealand. J Geol Soc 162:323–331

Pikuta E, Lysenko A, Chuvilskaya N, Mendrock U, Hippe H, Suzina N, Nikitin D, Osipov G, Laurinavichius K (2000a) *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic, alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. Int J Syst Evol Microbiol 50:2109–2117

Pikuta E, Lysenko A, Suzina N, Osipov G, Kusnetsov B, Tourova T, Akimenko V, Laurinavichius K (2000b) *Desulfotomaculum alkaliphilum* sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium. Int J Syst Evol Microbiol 50:25–33

Poilane I, Humeniuk-Ainouz C, Durand I, Janoir C, Cruaud P, Delmée M, Popoff MR, Collignon A (2007) Molecular characterization of *Clostridium difficile* clinical isolates in a geriatric hospital. J Med Microbiol 56:386–390

Pollmann K, Raff J, Schnorpfeil M, Radeva G, Selenska-Pobell S (2005) Novel surface layer protein genes in *Bacillus sphaericus* associated with unusual insertion elements. Microbiology 151:2961–2973

Pouwels PH, Kolen CPAM, Boot HJ (1997) S-layer protein genes of *Lactobacillus*. FEMS Microviol Rev 20:78–82

Poxton IR, Higgins PG, Currie CG, McCoubrey J (1999) Variation in the cell surface proteins of *Clostridium difficile*. Anaerobe 5:213–215

Prowe SG, Antranikian G (2001) *Anaerobranca gottschalkii* sp. nov., a novel thermoalkaliphilic bacterium that grows anaerobically at high pH and temperature. Int J Syst Evol Microbiol 51:457–465

Pum D, Sleytr UB (1995) Anisotropic crystal growth of the S-layer of *Bacillus sphaericus* CCM 2177 at the air/water interface. Colloids Surf A 102:99–104

Pum D, Messner P, Sleytr UB (1991) Role of the S layer in morphogenesis and cell division of the archaebacterium *Methanocorpusculum sinense*. J Bacteriol 173:6865–6873

Qazi O, Brailsford A, Wright A, Faraar J, Campbell J, Fairweather N (2007) Identification and characterization of the surface-layer protein of *Clostridium tetani*. FEMS Microbiol Lett 274:126–131

Rachel R, Pum D, Šmarda J, Šmajs D, Komrska J, Krzyzánek V, Rieger G, Stetter KO (1997) Fine structure of S-layers. FEMS Microbiol Rev 20:13–23

Radnedge L, Agron PG, Hill KK, Jackson PJ, Ticknor LO, Keim P, Andersen GL (2003) Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. Appl Environ Microbiol 69:2755–2764

Raetz CRH, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635–700

Redmond C, Baillie LW, Hibbs S, Moir AJ, Moir A (2004) Identification of proteins in the exosporium of *Bacillus anthracis*. Microbiology 150:355–363

Rees GN, Harfoot CG, Janssen PH, Schoenborn L, Kuever J, Lünsdorf H (2002) *Thiobaca trueperi* gen. nov., sp. nov., a phototrophic purple sulfur bacterium isolated from freshwater lake sediment. Int J Syst Evol Microbiol 52:671–678

Reniero R, Morelli L, Callegari ML, Sommi P, Bottazzi V (1990) Surface proteins in enteric lactobacilli. Ann Microbiol 40:83–91

Ricci G, Borgo F, Fortina MG (2006) Plasmids from *Lactobacillus helveticus*: distribution and diversity among natural isolates. Lett Appl Microbiol 42:254–258

Ries W, Hotzy C, Schocher I, Sleytr UB, Sára M (1997) Evidence that the N-terminal part of the S-layer protein from *Bacillus stearothermophilus* PV72/p2 recognizes a secondary cell wall polymer. J Bacteriol 179:3892–3898

Roberts K, Grief C, Hills GJ, Shaw PJ (1985) Cell wall glycoproteins: structure and function. J Cell Sci 2(Suppl):105–127

Rojas M, Ascencio F, Conway PL (2002) Purification and characterization of a surface protein from *Lactobacillus fermentum* 104R that binds to porcine small intestinal mucus and gastric mucin. Appl Environ Microbiol 68:2330–2336

Rothfuss H, Lara JC, Schmid AK, Lidstrom ME (2006) Involvement of the S-layer proteins Hpi and SlpA in the maintenance of cell envelope integrity in *Deinococcus radiodurans* R1. Microbiology 152:2779–2787

Rünzler D, Huber C, Moll D, Köhler G, Sára M (2004) Biophysical characterization of the entire bacterial surface layer protein SbsB and its two distinct functional domains. J Biol Chem 279:5207–5215

Ryzhkov PM, Ostermann K, Rödel G (2007) Isolation, gene structure, and comparative analysis of the S-layer gene *sslA* of *Sporosarcina ureae* ATCC 13881. Genetica 131:255–265

Sabet M, Lee S-W, Nauman RK, Sims T, Um H-S (2003) The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. Microbiology 149:3617–3627

Saito Y, Sakamoto M, Takizawa S, Benno Y (2004) Monitoring the cell number and viability of *Lactobacillus helveticus* GCL1001 in human feces by PCR methods. FEMS Microbiol Lett 231:125–130

Sakakibara J, Nagano K, Murakami Y, Higuchi N, Nakamura H, Shimozato K, Yoshimura F (2007) Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. Microbiology 153:866–876

Samuel ADT, Petersen JD, Reese TS (2001) Envelope structure of *Synechococcus* sp. WH8113, a nonflagellated swimming cyanobacterium. BMC Microbiol 1:4

Sára M (2001) Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria. Trends Microbiol 9:47–49

Sára M, Sleytr UB (2000) S-layer proteins. J Bacteriol 182:859–868

Sára M, Kuen B, Mayer HF, Mandl F, Schuster KC, Sleytr UB (1996) Dynamics in oxygen-induced changes in S-layer protein synthesis from *Bacillus stearothermophilus* PV72 and the S-layer-deficient variant T5 in continuous culture and studies of the cell wall composition. J Bacteriol 178:2108–2117

Sára M, Egelseer EM, Dekitsch C, Sleytr UB (1998) Identification of two binding domains, one for peptidoglycan and another for a secondary cell wall polymer on the N-terminal part of the S-layer protein SbsB from *Bacillus stearothermophilus* PV72/p2. J Bacteriol 180:6780–6783

Savariau-Lacomme MP, Lebarbier C, Karjalainen T, Collignon A, Janoir C (2003) Transcription and analysis of polymorphism in a cluster of genes encoding surface-associated proteins of *Clostridium difficile*. J Bacteriol 185:4461–4470

Schachtsiek M, Hammes WP, Hertel C (2004) Characterization of *Lactobacillus coryniformis* DSM 20001^T surface protein Cpf mediating coaggregation with and aggregation among pathogens. Appl Environ Microbiol 70:7078–7085

Schäffer C, Messner P (2004) Surface-layer glycoproteins: an example for the diversity of bacterial glycosylation with promising impacts on nanobiotechnology. Glycobiology 14:31R–42R

Schäffer C, Messner P (2005) The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. Microbiology 151:643–651

Schäffer C, Kählig H, Christian R, Schulz G, Zayni S, Messner P (1999) The diacetamidodideoxyuronic-acid-containing glycan chain of *Bacillus stearothermophilus* NRS 2004/3a represents the secondary cell wall polymer of wild-type *B. stearothermophilus* strains. Microbiology 145:1575–1583

Schäffer C, Wugeditsch T, Kählig H, Scheberl A, Zayni S, Messner P (2002) The surface layer (S-layer) glycoprotein of *Geobacillus stearothermophilus* NRS 2004/3a. Analysis of its glycosylation. J Biol Chem 277:6230–6239

Schäffer C, Franck WL, Scheberl A, Kosma P, McDermott TR, Messner P (2004) Classification of two isolates from locations in Austria and Yellowstone National Park as *Geobacillus tepidamans* sp. nov. Int J Syst Evol Microbiol 54:2361–2368

Schär-Zammaretti P, Ubbink J (2003) Imaging of lactic acid bacteria with AFM – elasticity and adhesion maps and their relationship to biological and structural data. Ultramicroscopy 97:199–208

Schär-Zammaretti P, Ubbink J (2003) The cell wall of lactic acid bacteria: surface constituents and macromolecular conformations. Biophys J 85:4076–4092

Schär-Zammaretti P, Dillmann M-L, D'Amico N, Affolter M, Ubbink J (2005) Influence of fermentation medium composition on physicochemical surface properties of *Lactobacillus acidophilus*. Appl Environ Microbiol 71:8165–8173

Scheuring S, Stahlberg H, Chami M, Houssin C, Rigaud J-L, Engel A (2002) Charting and unzipping the surface layer of *Corynebacterium glutamicum* with the atomic force microscope. Mol Microbiol 44:675–684

Schneitz C, Nuotio L, Lounatma K (1993) Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). J Appl Bacteriol 74:290–294

Scholz HC, Riedmann E, Witte A, Lubitz W, Kuen B (2001) S-layer variation in *Bacillus stearothermophilus* PV72 is based on DNA rearrangements between the chromosome and the naturally occurring megaplasmids. J Bacteriol 183:1672–1679

Schuster B, Györvary E, Pum D, Sleytr UB (2005) Nanotechnology with S-layer proteins. Methods Mol Biol 300:101–123

Scolari G, Vescovo M, Zacconi C, Vescovi F (2006) Extraction and partial characterization of proteolytic activities from the cell surface of *Lactobacillus helveticus* Zuc2. J Dairy Sci 89:3800–3809

Severina LO, Senyushkin AA, Karavaiko GI (1995) The structure and chemical composition of the S-layer in representatives of the genus *Sulfobacillus*. Microbiology (Moscow) 64:280–283

Seyfried M, Lyon D, Rainey FA, Wiegel J (2002) *Caloramator viterbensis* sp. nov., a novel thermophilic, glycerol-fermenting bacterium isolated from a hot spring in Italy. Int J Syst Evol Microbiol 52:1177–1184

Shen N, Weiner RM (1998) Isolation and characterization of S-layer proteins from a vent prosthecate bacterium. Microbios 93:7–16

Shida O, Takagi H, Kadowaki K, Yano H, Abe M, Udaka S, Komagata K (1994) *Bacillus aneurinolyticus* sp. nov., nom. rev. Int J Syst Bacteriol 44:143–150

Shida O, Takagi H, Kadowaki K, Udaka S, Nakamura LK, Komagata K (1995) Proposal of *Bacillus reuszeri* sp. nov., *Bacillus formosus* sp. nov., nom. rev., and *Bacillus borstelensis* sp. nov., nom. rev. Int J Syst Bacteriol 45:93–100

Sidhu MS, Olsen I (1997) S-layers of *Bacillus* species. Microbiology 143:1039–1052

Sillanpää J, Martínez B, Antikainen J, Toba T, Kalkkinen N, Tankka S, Lounatmaa K, Keränen J, Höök M, Westerlund-Wickström B, Pouwels PH, Korhonen TK (2000) Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. J Bacteriol 182:6440–6450

Sleytr UB (1975) Heterologous reattachement of regular arrays of glycoproteins on bacterial surfaces. Nature 257:400–402

Sleytr UB (1978) Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly and function. Int Rev Cytol 53:1–64

Sleytr UB (1981) Morphopoietic and functional aspects of regular protein membranes present on prokaryotic cell walls. In: Kiermayer O (ed) Cell biology monographs. Cytomorphogenesis in plants, vol 8. Springer-Verlag, Wien, pp 3–26

Sleytr U, Adam H, Klaushofer H (1967) Die elektronenmikroskopische Feinstruktur von Zellwand, Cytoplasmamembran und Geiseln von *Bacillus stearothermophilus*, dargestellt mit Hilfe der Gefrierätztechnik. Mikroskopie (Wien) 22:233–242

Sleytr U, Adam H, Klaushofer H (1968) Die Feinstruktur der Zellwandoberfläche von zwei thermophilen Clostridienarten, dargestellt mit Hilfe der Gefrierätztechnik. Mikroskopie (Wien) 23:1–10

Sleytr UB (1997) Basic and applied S-layer research: an overview. FEMS Microbiol Rev 20:5–12

Sleytr UB, Beveridge TJ (1999) Bacterial S-layers. Trends Microbiol 7:253–260

Sleytr UB, Glauert AM (1975) Analysis of regular arrays of subunits on bacterial surfaces; evidence for a dynamic process of assembly. J Ultrastruct Res 50:103–116

Sleytr UB, Messner P (1983) Crystalline surface layers on bacteria. Annu Rev Microbiol 37:311–339

Sleytr UB, Messner P (1989) Self-assembly of crystalline bacterial cell surface layers. In: Plattner H (ed) Electron microscopy of subcellular dynamics. CRC Press, Boca Raton, FL, pp 13–31

Sleytr UB, Messner P (2009) Crystalline cell surface layers (S layers). In: Schaechter M (ed) Encyclopedia of microbiology, 3rd edn. Elsevier, San Diego, CA, pp 89–98

Sleytr UB, Plohberger R (1980) The dynamic process of assembly of two-dimensional arrays of macromolecules on bacterial cell walls. In: Baumeister W, Vogell W (eds) Electron microscopy at molecular dimensions. Springer-Verlag, Berlin, pp 36–47

Sleytr UB, Messner P, Pum D, Sára M (eds) (1988) Crystalline bacterial cell surface layers. Springer-Verlag, Berlin, pp 1–193

Sleytr UB, Messner P, (1993) Crystalline bacterial cell surface layers. Pum D, Sára M. Mol Microbiol 10:911–916

Sleytr UB, Messner P, Pum D, Sára M (1996a) Occurence, location, ultrastructure and morphogenesis of S-layers. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Crystalline bacterial cell surface proteins. RG Landes/Academic Press, Austin, TX, pp 5–33

Sleytr UB, Messner P, Pum D, Sára M (1996b) Crystalline surface layers on eubacteria and archaeobacteria. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Crystalline bacterial cell surface proteins. RG Landes/Academic Press, Austin, TX, pp 211–225

Sleytr UB, Messner P, Pum D, Sára M (1999) Crystalline bacterial cell surface layers (S-layers): from supramolecular cell structure to biomimetics and nanotechnology. Angew Chem Int Ed 38:1034–1054

Sleytr UB, Sára M, Pum D, Schuster B (2001a) Characterization and use of crystalline bacterial cell surface layers. Progr Surf Sci 68:231–278

Sleytr UB, Sára M, Pum D, Schuster B (2001b) Molecular nanotechnology and nanobiotechnology with two-dimensional protein crystals (S-layers). In: Rosoff M (ed) Nano-surface chemistry. Marcel Dekker, New York, pp 333–389

Sleytr UB, Sára M, Pum D, Schuster B, Messner P, Schäffer C (2002) Self assembly protein systems: microbial S-layers. In: Steinbüchel A, Fahnestock S (eds) Biopolymers. Polyamides and complex proteinaceous matrices I, vol 7. Wiley-VCH, Weinheim, Germany, pp 285–338

Sleytr UB, Sára M, Pum D, Schuster B (2005) Crystalline bacterial cell surface layers (S-layers): a versatile self-assembly system. In: Ciferri A (ed) Supramolecular polymers, 2nd edn. Taylor & Francis, Boca Raton, FL, pp 583–616

Slobodkin A, Reysenbach A-L, Mayer F, Wiegel J (1997) Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. Int J Syst Bacteriol 47:969–974

Slobodkin AI, Tourova TP, Kostrikina NA, Lysenko AM, German KE, Bonch-Osmolovskaya EA, Birkeland N-K (2006) *Tepidimicrobium ferriphilum* gen. nov., sp. nov., a novel moderately thermophilic, Fe(III)-reducing bacterium of the order *Clostridiales*. Int J Syst Evol Microbiol 56:369–372

Šmarda J, Šmajs D, Komrska J (1996) Advances in cyanobacterial S-layers. Algol Stud 83:501–510

Šmarda J, Šmajs D, Komrska J, Krzyžánek V (2002) S-layers on cell walls of cyanobacteria. Micron 33:257–277

Smit E, Pouwels PH (2002) One repeat of the cell wall binding domain is sufficient for anchoring the *Lactobacillus acidophilus* surface layer protein. J Bacteriol 184:4617–4619

Smit E, Oling F, Demel R, Martinez B, Pouwels PH (2001) The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: identification and characterisation of domains responsible for S-protein assembly and cell wall binding. J Mol Biol 305:245–257

Smit E, Jager D, Martinez B, Thielen FJ, Pouwels PH (2002) Structural and functional analysis of the S-layer protein crystallization domain of *Lactobacillus acidophilus* ATCC 4356: evidence for protein–protein interaction of two subdomains. J Mol Biol 324:953–964

Smit J, Agabian N (1984) Cloning of the major protein of the *Caulobacter crescentus* periodic surface layer: detection and characterization of the cloned peptide by protein expression assays. J Bacteriol 160:1137–1145

Smit J, Grano DA, Glaeser RM, Agabian N (1981) Periodic surface array in *Caulobacter crescentus*: fine structure and chemical analysis. J Bacteriol 146:1135–1150

Smit J, Engelhardt H, Volker S, Smith SH, Baumeister W (1992) The S-layer of *Caulobacter crescentus*: three-dimensional image reconstruction and structure analysis by electron microscopy. J Bacteriol 174:6527–6538

Sokolova TG, González JM, Kostrikina NA, Chernyh NA, Tourova TP, Kato C, Bonch-Osmolovskaya EA, Robb FT (2001) *Carboxydobrachium pacificum* gen. nov., sp. nov., a new anaerobic, thermophilic, CO-utilizing marine bacterium from Okinawa trough. Int J Syst Evol Microbiol 51:141–149

Sokolova TG, Kostrikina NA, Chernyh NA, Tourova TP, Kolganova TV, Bonch-Osmolovskaya EA (2002) *Carboxydocella thermautotrophica* gen. nov., sp. nov., a novel anaerobic, CO-utilizing thermophile from a Kamchatkan hot spring. Int J Syst Evol Microbiol 52:1961–1967

Sokolova TG, Kostrikina NA, Chernyh NA, Kolganova TV, Tourova TP, Bonch-Osmolovskaya EA (2005) *Thermincola carboxydiphila* gen. nov., sp. nov., a novel anaerobic, carboxydotrophic, hydrogenogenic bacterium from a hot spring of the Lake Baikal area. Int J Syst Evol Microbiol 55:2069–2073

Sorokin DY, Muyzer G, Brinkhoff T, Kuenen JG, Jetten MSM (1998) Isolation and characterization of a novel facultatively alkaliphilic *Nitrobacter* species. N. alkalicus sp. nov. Arch Microbiol 170:345–352

Sousal-Hoebeke E, de Sousa-D'Auria C, Chami M, Baucher MF, Guyonvarch A, Bayan N, Salim K, Leblon G (1999) S-layer protein production by *Corynebacterium* strains is dependent on the carbon source. Microbiology 145:3399–3408

Steiner K, Pohlentz G, Dreisewerd K, Berkenkamp S, Messner P, Peter-Katalinić J, Schäffer C (2006) New insights into the glycosylation of the surface layer protein SgsE from *Geobacillus stearothermophilus* NRS 2004/3a. J Bacteriol 188:7914–7921

Steiner K, Novotny R, Patel K, Vinogradov E, Whitfield C, Valvano MA, Messner P, Schäffer C (2007) Functional characterization of the initiation enzyme of S-layer glycoprotein glycan biosynthesis in *Geobacillus stearothermophilus* NRS 2004/3a. J Bacteriol 189:2590–2598

St. John FJ, Rice JD, Preston JF (2006) *Paenibacillus* sp. strain JDR-2 and XynA1: a novel system for methylglucuronoxylan utilization. Appl Environ Microbiol 72:1496–1506

Strömpl C, Hold GL, Lünsdorf H, Graham J, Gallacher S, Abraham W-R, Moore ERB, Timmis KN (2003) *Oceanicaulis alexandrii* gen. nov., sp. nov., a novel stalked bacterium isolated from a culture of the dinoflagellate *Alexandrium tamarense* (Lebour) Balech. Int J Syst Evol Microbiol 53:1901–1906

Sumper M, Wieland FT (1995) Bacterial glycoproteins. In: Montreuil J, Vliegenthart JFG, Schachter H (eds) Glycoproteins. Elsevier, Amsterdam, pp 455–473

Sun M, Zhu C, Yu Z (2001) Cloning of parasporal body protein gene resembling to S-layer protein genes from *Bacillus thuringiensis* CTC strain. Acta Microbiol Sin 41:141–147

Szymanski CM, Wren BW (2005) Protein glycosylation in bacterial mucosal pathogens. Nature Rev Microbiol 3:225–237

Takacs CD, Ehringer M, Favre R, Cermola M, Eggertsson G, Palsdottir A, Reysenbach A-L (2001) Phylogenetic characterization of the blue filamentous bacterial community from an Icelandic geothermal spring. FEMS Microbiol Ecol 35:123–128

Tallon R, Arias S, Bressollier P, Urdaci MC (2007) Strain- and matrix-dependent adhesion of *Lactobacillus plantarum* is mediated by proteinaceous bacterial compounds. J Appl Microbiol 102:442–451

Tardy-Jacquenod C, Magot M, Patel BKC, Matheron R, Caumette P (1998) *Desulfotomaculum halophilum* sp. nov., a halophilic sulfate-reducing bacterium isolated from oil production facilities. Int J Syst Bacteriol 48:333–338

Thomas SR, Trust TJ (1995) Tyrosine phosphorylation of the tetragonal paracrystalline array of *Aeromonas hydrophila*: molecular cloning and high-level expression of the S-layer protein gene. J Mol Biol 245:568–581

Thompson SA (2002) *Campylobacter* surface-layers (S-layers) and immune evasion. Ann Periodontol 7:43–53

Thompson SA, Blaser MJ (2000) Pathogenesis of *Campylobacter fetus* infections. In: Nachamkin I, Blaser MJ (eds) Campylobacter. American Society for Microbiology, Washington, DC, pp 321–347

Toba T, Virkola R, Westerlund B, Bjorkman Y, Sillanpaa J, Vartio T, Kalkkinen N, Korhonen TK (1995) A collagen-binding S-layer protein in *Lactobacillus crispatus*. Appl Environ Microbiol 61:2467–2471

Toporowski MC, Nomellini JF, Awram P, Smit J (2004) Two outer membrane proteins are required for maximal type I secretion of the *Caulobacter crescentus* S-layer protein. J Bacteriol 186:8000–8009

Tran SD, Rudney JD, Sparks BS, Hodges JS (2001) Persistent presence of *Bacteroides forsythus* as a risk factor for attachment loss in a population with low prevalence and severity of adult periodontitis. J Periodontol 72:1–10

Trotsenko YA, Khmelenina VN (2002) Biology of extremophilic and extremotolerant methanotrophs. Arch Microbiol 177:123–131

Tsuboi A, Uchihi R, Tabata R, Takahashi Y, Hasiba H, Sasaki T, Yamagata H, Tsukagoshi N, Udaka S (1986) Characterization of the genes coding for two major cell wall proteins from protein-producing *Bacillus brevis* 47: complete nucleotide sequence of the outer wall protein gene. J Bacteriol 168:365–373

Tsuboi A, Uchihi R, Adachi T, Sasaki T, Hayakawa S, Yamagata H, Tsukagoshi N, Udaka S (1988) Characterization of the genes for the hexagonally arranged surface layer proteins in protein-producing *Bacillus brevis* 47: complete nucleotide sequence of the middle wall protein gene. J Bacteriol 170:935–945

Tsubota J, Eshinimaev BT, Khmelenina VN, Trotsenko YA (2005) *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. Int J Syst Evol Microbiol 55:1877–1884

Tu ZC, Dewhirst FE, Blaser MJ (2001) Evidence that the *Campylobacter fetus sap* locus is an ancient genomic constituent with origins before mammals and reptiles diverged. Infect Immun 69:2237–2244

Tu ZC, Wassenaar TM, Thompson SA, Blaser MJ (2003) Structure and genotypic plasticity of the *Campylobacter fetus sap* locus. Mol Microbiol 48:685–698

Tu ZC, Hui J, Blaser MJ (2004a) Conservation and diversity of sap homologues and their organization among *Campylobacter fetus* isolates. Infect Immun 72:1715–1724

Tu ZC, Zeitlin G, Gagner JP, Keo T, Hanna BA, Blaser MJ (2004b) *Campylobacter fetus* of reptile origin as a human pathogen. J Clin Microbiol 42:4405–4407

Tu ZC, Gaudreau C, Blaser MJ (2005) Mechanisms underlying *Campylobacter fetus* pathogenesis in humans: surface-layer protein variation in relapsing infections. J Infect Dis 191:2082–2089

Ubbink J, Schär-Zammaretti P (2005) Probing bacterial interactions: integrated approaches combining atomic force microscopy, electron microscopy and biophysical techniques. Micron 36:293–320

Umelo-Njaka E, Bingle WH, Borchani F, Le KD, Awram P, Blake T, Nomellini JF, Smit J (2002) *Caulobacter crescentus* synthesizes an S-layer-editing metalloprotease possessing a domain sharing sequence similarity with its paracrystalline S-layer protein. J Bacteriol 184:2709–2718

Vadillo-Rodríguez V, Busscher HJ, Norde W, de Vries J, van der Mei HC (2004) Dynamic cell surface hydrophobicity of *Lactobacillus* strains with and without surface layer proteins. J Bacteriol 186:6647–6650

Vadillo-Rodríguez V, Busscher HJ, van der Mei HC, de Vries J, Norde W (2005) Role of lactobacillus cell surface hydrophobicity as probed by AFM in adhesion to surfaces at low and high ionic strength. Colloids Surf B 41:33–41

van der Mei HC, van de Belt-Gritter B, Pouwels PH, Martinez B, Busscher HJ (2003) Cell surface hydrophobicity is conveyed by S-layer proteins - a study in recombinant lactobacilli. Colloids Surf B 28:127–134

Vancanneyt M, Mengaud J, Cleenwerck I, Vanhonacker K, Hoste B, Dawyndt P, Degivry MC, Ringuet D, Janssens D, Swings J (2004) Reclassification of *Lactobacillus kefirgranum* Takizawa *et al*. 1994 as *Lactobacillus kefiranofaciens* subsp. *kefirgranum* subsp. nov. and emended description of *L. kefiranofaciens* Fujisawa *et al*. 1988. Int J Syst Evol Microbiol 54:551–556

Ventura M, Callegari ML, Morelli L (2000) S-layer gene as a molecular marker for identification of *Lactobacillus helveticus*. FEMS Microbiol Lett 189:275–279

Ventura M, Jankovic I, Walker DC, Pridmore RD, Zink R (2002) Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. Appl Environ Microbiol 68:6172–6181

Verbelen C, Antikainen J, Korhonen TK, Dufrêne YF (2007) Exploring the molecular forces within and between CbsA S-layer proteins using single molecule force spectroscopy. Ultramicroscopy 107:1004–1011

Vidgrén G, Palva I, Pakkanen R, Lounatmaa K, Palva A (1992) S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. J Bacteriol 174:7419–7427

Wahl R, Raff J, Selenska-Pobell S, Mertig M, Pompe W (2001) A fast screening method for surface layers on Gram-positive bacteria. Biotechnol Lett 23:1485–1490

Wai SN, Takade A, Fujimoto S, Amako K (1995) The structure and characterization of the surface protein layer of a *Comamonas*-like organism. Microbiol Immunol 39:943–949

Walker SG, Smith SH, Smit J (1992) Isolation and comparison of the paracrystalline surface layer proteins of freshwater caulobacters. J Bacteriol 174:1783–1792

Walker SG, Karunaratne DN, Ravenscroft N, Smit J (1994) Characterization of mutants of *Caulobacter crescentus* defective in surface attachment of the paracrystalline surface layer. J Bacteriol 176:6312–6323

Wang B, Kraig E, Kolodrubetz D (1998) A new member of the S-layer protein family: characterization of the *crs* gene from *Campylobacter rectus*. Infect Immun 66:1521–1526

Wang L, Sun M, Yu Z (2004) Capacity of *Bacillus thuringiensis* S-layer protein displaying polyhistidine peptides on the cell surface. Appl Biochem Biotechnol 119:133–144

Warnick TA, Methé BA, Leschine SB (2002) *Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil. Int J Syst Evol Microbiol 52:1155–1160

White O, Eisen JA, Heidelberg JF, Hickey EK, Peterson JD, Dodson RJ, Haft DH, Gwinn ML, Nelson WC, Richardson DL, Moffat KS, Qin H, Jiang L, Pamphile W, Crosby M, Shen M, Vamathevan JJ, Lam P, McDonald L, Utterback T, Zalewski C, Makarova KS, Aravind L, Daly MJ, Minton KW, Fleischmann RD, Ketchum KA, Nelson KE, Salzberg S, Smith HO, Venter JC, Fraser CM (1999) Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science 286:1571–1577

Whiting GC, Rijpkema S, Adams T, Corbel MJ (2004) Characterisation of adsorbed anthrax vaccine by two-dimensional gel electrophoresis. Vaccine 22:4245–4251

Wild A, Hermann R, Leisinger T (1996) Isolation of an anaerobic bacterium which reductively dechlorinates tetrachloroethene and trichloroethene. Biodegradation 7:507–511

Williams DD, Turnbough CL Jr (2004) Surface layer protein EA1 is not a component of *Bacillus anthracis* spores but is a persistent contaminant in spore preparations. J Bacteriol 186:566–569

Wiwat C, Panbangred W, Mongkolsuk S, Pantuwatana S, Bhumiratana A (1995) Inhibition of a conjugation-like gene transfer process in *Bacillus thuringiensis* subsp. *israelensis* by the anti-S-layer protein antibody. Curr Microbiol 30:69–75

Wright A, Wait R, Begum S, Crossett B, Nagy J, Brown K, Fairweather N (2005) Proteomic analysis of cell surface proteins from *Clostridium difficile*. Proteomics 5:2443–2452

Wyss C (1995) Sticky, a novel phenotype of *Campylobacter rectus*. Microb Ecol Health Dis 8:175–179

Xu Z, Yao B, Sun M, Yu Z (2004) Protection of mice infected with *Plasmodium berghei* by *Bacillus thuringiensis* crystal proteins. Parasitol Res 92:53–57

Xue Y, Xu Y, Liu Y, Ma Y, Zhou P (2001) *Thermoanaerobacter tengcongensis* sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengcong, China. Int J Syst Evol Microbiol 51:1335–1341

Yan Y, Chen H, Lu C (1996) Purification and characterization of S-layer protein from *Aeromonas hydrophila*. Acta Microbiol Sin 36:144–150

Yang L, Pei Z, Fujimoto S, Blaser MJ (1992) Reattachment of surface array proteins to *Campylobacter fetus* cells. J Bacteriol 174:1258–1267

Yasui T, Yoda K, Kamiya T (1995) Analysis of S-layer proteins of *Lactobacillus brevis*. FEMS Microbiol Lett 133:181–186

Yeh C-M, Chou Y-T (2006) Study on the cell surface display system of lactic acid bacteria. Taiwanese J Agric Chem Food Sci 44:326–338

Yoneda M, Hirofuji T, Motooka N, Nozoe K, Shigenaga K, Anan H, Miura M, Kabashima H, Matsumoto A, Maeda K (2003) Humoral immune responses to S-layer-like proteins of *Bacteroides forsythus*. Clin Diag Lab Immunol 10:383–387

Zasada AA, Gierczynski R, Kaluzewski S, Jagielski M (2005) Virulotypes of *Bacillus anthracis* strains isolated in Poland. Med Dośw Mikrobiol 57:269–275

Zayni S, Steiner K, Pfoestl A, Hofinger A, Kosma P, Schäffer C, Messner P (2007) The dTDP-4-dehydro-6-deoxyglucose reductase encoding *fcd* gene is part of the surface layer glycoprotein glycosylation gene cluster of *Geobacillus tepidamans* GS5–97 T. Glycobiology 17:433–443

Zhang L, Ruan L, Hu C, Wu H, Chen S, Yu Z, Sun M (2007) Fusion of the genes for AHL-lactonase and S-layer protein in *Bacillus thuringiensis* increases its ability to inhibit soft rot caused by *Erwinia carotovora*. Appl Microbiol Biotechnol 74:767–775

Zhu X, McVeigh RR, Malathi P, Ghosh BK (1996) The complete nucleotide sequence of the *Bacillus licheniformis* NM 105S-layer-encoding gene. Gene 173:189–194

Chapter 3
Bacterial Polysaccharide Capsules

David Corbett, Thomas Hudson, and Ian S. Roberts

3.1 Introduction

The expression of extracellular polysaccharide (EPS) material is a common feature of many bacteria. This EPS coats the outside of the bacterial cell and as a consequence plays an intimate role in mediating interactions between the bacterium and its immediate environment. In essence, the expression of EPS results in the coating of the bacterium in a hydrated shell of high molecular weight polysaccharide molecules that in the majority of cases carry a net negative charge at physiological pH. The ubiquity of EPS expression across a diverse array of bacterial genera suggests that capsule expression is advantageous in a number of scenarios. A capsule is a discrete structure that is defined as a layer of polysaccharide that is either physically attached to, or remains tightly associated with, the cell surface of the bacterium. This is in contrast to slime, which has a loose association with the surface of the bacterium and is often shed in large amounts into the surrounding environment. This loss of EPS may have important implications in modifying the local environment and promoting the effective colonisation of a particular ecological niche. However, whilst this is a reasonable working dichotomy with which to classify EPS, one should appreciate that even capsular polysaccharides will be shed from the bacterial cell surface, though this may be on a smaller scale and usually in association with other cell surface material. This review will focus on a discussion of capsules rather than slime.

There is great potential for structural diversity amongst capsular polysaccharides both within and between bacterial species. The basis of this diversity is a consequence of differences in both the repeat monosaccharide components but also in the glycosidic linkage between the different repeating monosaccharide units. This is in contrast to proteins whose diversity is limited by the order and modification of their

I.S. Roberts (✉)
Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
e-mail: i.s.Roberts@manchester.ac.uk

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_3,

component amino acids. Capsule diversity is exemplified by *Streptococcus pneumoniae* with over 90 different capsule serotypes (Henrichsen 1995) and *Escherichia coli* with over 90 capsules or K antigens (Whitfield 2006). The diversity of capsular polysaccharides within a single bacterial species such as *S. pneumoniae,* which is a highly adapted human respiratory pathogen and will not have to grow outside the host, raises the obvious question regarding the function of this diversity. Whilst certain capsule serotypes are associated with invasive disease and it is possible to assign roles for capsules in this process, disease is really the atypical state reflecting a perturbation in the interaction between the bacterium and the host. The key question is, what role does the capsule fulfil in allowing these bacteria to colonise and maintain carriage and how is this related to capsule diversity? A consequence of capsule diversity is that there are a huge number of highly diverse microbial polysaccharides that have a range of physico-chemical properties germane to the environment in which the bacterium normally resides. This represents a reservoir of potentially useful polysaccharide molecules that could be exploited in a range of biotechnological applications. Underpinning this structural diversity is an array of glycosyl transferase enzymes that synthesise these diverse polysaccharides. Advances in our understanding of glycosyl transferase structure, coupled with the increasing availability of genome sequences, means that it is possible to clone and express in *E. coli* heterologous glycosyl tranferases to undertake polysaccharide engineering and generate polysaccharides with particular properties (Yavuz et al. 2008).

3.2 Functions of Bacterial Capsules

A number of functions have been assigned to capsules in different bacteria including adhesion, transmission, resistance to innate host defences, resistance to the host's adaptive immune response, chemokine and cytokine induction and intracellular survival (Table 3.1) (Roberts 1996; Corbett and Roberts 2008). Historically the capsule was perceived to function as a barrier, masking and coating cell surface

Table 3.1 Functions of bacterial capsules

Adhesion: Hyaluronic capsule in the adhesion of Group A streptococci to CD44. Inhibition of mucus clearance in *S. pneumoniae*
Transmission: In host-adapted respiratory pathogens, the polysaccharide capsule may provide an aqueous gel to prevent desiccation
Resistance to innate host defences: Masking of complement activators; capsules hinder the membrane attack complex reaching the outer membrane. Sialic acid containing polysaccharides bind factor H
Resistance to adaptive immune response: In the case of capsules that structurally resemble host molecules such as the sialic acid capsule of *N. menigitidis* B or *E. coli* K1
Intracellular survival: The *E. coli* K1 capsule is essential for surviving the crossing of the blood–brain barrier
Mediating host cell cytokine induction: Immunomodulation of host response

structures that might otherwise trigger innate host defences. While this is no doubt true, a picture is now emerging in which the polysaccharide molecule itself is capable of interacting directly with host cells to elicit a host response most likely to promote the continued colonisation of the bacteria in the host. The implications of these observations are that capsular polysaccharides may themselves function as signalling molecules in mediating the dialogue between the host and the bacterial cell.

3.3 Capsules and Adhesion

Generally the production of a capsule is known to inhibit the attachment of bacteria to both biotic and abiotic surfaces. However there are exceptions to this (see below). The inhibition of attachment is most likely through the masking of key cell surface appendages such as fimbriae, crucial for attachment of the bacterium to the host cell (Schembri et al. 2004). It has been shown in *Klebsiella pneumoniae* that in vitro the loss of capsule increased adhesion to non-mucus secreting cells but conversely reduced adhesion to cells capable of expressing mucus (Favre-Bonte et al. 1999a). The capsule minus mutant also colonised the mouse large intestine less well than the wild type, indicating a role for the capsule in colonisation by *K. pneumonia* (Favre-Bonté et al. 1999b). This increased colonisation probably reflects an increased ability of encapsulated cells to adhere to mucus-expressing cells in the large intestine, indicating an interaction between the polysaccharide capsule and mucus. Similarly, in the case of *S. pneumoniae* the expression of a capsule appears to reduce entrapment in luminal mucus, thereby reducing clearance and promoting adherence and colonisation (Nelson et al. 2007). A role for the *Escherichia coli* K5 capsule in the colonisation of the large intestine of the gnotobiotic rat has also been demonstrated (Hérias et al. 1997). Overall these data clearly implicate capsule expression and colonisation of host gut.

Impaired adhesion and biofilm production resulting from capsule expression has been observed in many other bacteria such as *Neisseria meningitidis*, *S. pneumoniae*, *Porphyromonas gingivalis* and *Vibrio fischeri* (Davey and Duncan 2006; Moscoso et al. 2006; Schembri et al. 2004; Yip et al. 2005). Interestingly, it has been demonstrated that treatment of abiotic surfaces with polysaccharide capsule derived from an *E. coli* Group 2 strain resulted in a drastic reduction of both initial adhesion and subsequent biofilm development (Valle et al. 2006). Paradoxically, the expression of EPS appears to be important in providing the matrix for the correct assembly of mature biofilms (Nakhamchik et al. 2008). The detrimental effects on adhesion as a consequence of capsule expression suggest that initial colonisation of surfaces may involve pioneer cells from within the population that show reduced capsule expression. Such pioneers may arise as a result of stochastic regulation of gene expression via mechanisms such as slip strand mispairing or other regulatory mechanisms that lead to bistable expression within the population (Dubnau and Losick 2006). Conversely there are examples where capsule expression is essential

for efficient colonisation. An excellent example is the adhesion of Group A streptococci to pharyngeal cells mediated via the interaction between the hyaluronic acid capsule and CD44, the hyaluronic acid binding protein (Cywes and Wessels 2001). This interaction between microbe and host induces a signalling pathway that promotes the efficient paracellular penetration of the mucosal epithelial layer and invasion of the underlying tissue (Cywes and Wessels 2001).

3.4 Capsules and Resistance to Host Defences

A major role played by bacterial capsules against the host's immune system is resistance to the complement system. The complement system is a cascade of proteins resulting in either the formation of a membrane attack complex (MAC) or opsono-phagocytosis by macrophages and neutrophils. MAC formation in the bacterium's membrane generates pores that result in cell lysis. There are three possible complement pathways; the classical, lectin and alternative pathway. The classical pathway involves proteins C1–C9 and is initiated by C1 binding to an antibody–antigen complex, known as complement fixation. Successive protein complexes are then formed, resulting in C3b, which is recognized for opsono-phagocytosis or used as the starting point for the formation of MAC. The two other complement pathways both result in the production of C3b but in an antibody-independent manner. The lectin pathway utilises mannose-binding protein within the serum, whereas the alternative pathway utilises free C3b within the serum binding to bacteria. Unlike host cells, invading bacteria are unable to inactivate C3b, allowing the innate immune system to distinguish self from non-self.

Production of a polysaccharide capsule is a key component in conferring complement resistance in a range of both Gram-positive (Cunnion et al. 2001; Neeleman et al. 1999) and Gram-negative (Roberts 1996) invasive bacterial pathogens. The capsule is thought to mask key complement binding sites on the bacterial cell surface, such as lipid A and outer membrane proteins. Therefore, complement pathways are inhibited, avoiding the potential for MAC formation or opsono-phagocytosis. Alternatively the MAC may be assembled in the capsule layer away from the cell surface, thereby avoiding cell lysis. Examples of complement resistance have been well documented for polysialic acid capsule producing strains (Cisowska and Jankowski 2004; Geoffroy et al. 2003; Pluschke et al. 1983; Uria et al. 2008; Vogel et al. 1996), although it seems that in *Neisseria meningitidis* sialylation of lipooligosaccharides is also crucial for serum resistance. It is known that C3b can be inactivated through the regulatory protein factor H, which converts C3b into iC3b, and that factor H can bind to heparin and sialic acid molecules (Pangburn et al. 1991). As such it was proposed that the complement resistance of strains expressing capsules containing sialic acid could be due to the deposition of factor H on the bacterial cell surface and its subsequent conversion of any bound C3b into iC3b. However, the observation that there was no detectable difference in the amount of bound C3b or inactivated iC3b between encapsulated and

unencapsulated *N. meningitidis* (Vogel et al. 1997) would suggest that the situation is more complex than first thought.

In the case of *E. coli* K1 strains, it was demonstrated that they have an increased binding ability for C4bp via the outer membrane protein OmpA (Wooster et al. 2006). The protein C4bp is an inhibitor of complement activation via the classical pathway through the action of factor I, which cleaves both C3b and C4b. This process is thought to be crucial for the serum resistance of *E. coli* K1 (Wooster et al. 2006). Conversely, encapsulated *N. meningitidis* serogroup B was shown to have 50% less C4bp bound compared to unencapsulated *N. meningitidis* (Jarva et al. 2005). This C4bp binding difference between *E. coli* K1 and *N. meningitidis* serogroup B, both of which express a chemically identical sialic acid capsule, would indicate that specific additional cell surface bacterial factors are important in contributing to complement resistance. Indeed, in the case of invasive *E. coli* strains, it has been shown that complement-mediated resistance is likely to involve a number of cell surface structures such as O and K antigen, both of which contribute to the overall effect (Burns and Hull 1998, 1999).

The ability of capsules to confer resistance to phagocytosis by polymorphonuclear cells (PMNL) has long been assigned to the negatively charged polysaccharide capsule and the repulsive effect on the negatively charged cell surface of the PMNL (Roberts 1996). However, it is likely that poor opsonisation with complement of encapsulated bacteria will also play a role in this protection (Roberts 1996). Recently it has been shown that the capsule of the Group B streptococcus (GBS) *Streptococcus agalactiae* inhibits the binding of macrophage scavenger receptor-A (SR-A), which plays a crucial role in the recognition and non-opsonic phagocytosis of pathogenic bacteria (Areschoug et al. 2008). The capsule blocks the binding of SR-A to the cell surface lipoprotein Blr, thereby reducing SR-A-mediated non-opsonic phagocytosis of *S. agalactiae* (Areschoug et al. 2008). This anti-phagocytic property will be of particular relevance in naive hosts such as neonates, which may lack specific antisera. In addition, the presence of a terminal sialic acid residue on the capsular polysaccharides of GBS has been shown to mediate interactions with sialic acid recognising lectins (Siglecs) on the surface of neutrophils and monocytes (Carlin et al. 2007). This interaction was dependent on the extent of *O*-acetylation of the sialic acid (Carlin et al. 2007). Engagement of Siglecs on the cell surface of leukocytes is believed to suppress T-cell signalling (Ikehara et al. 2004) and NK cell toxicity (Nicoll et al. 2003). As such, the interaction between sialic acids on the GBS capsule and Siglecs could represent an interaction between the microbe and the host that effectively dampens down the innate immune response.

The *E. coli* K1 capsule is vital for intracellular survival and crossing the blood brain barrier (Kim et al. 2003). Specifically, the K1 capsule moderates the maturation process of *E. coli* containing vacuoles inside endothelial cells, preventing fusion with lysosomes (Kim et al. 2003). As such, expression of the K1 capsule is critical to the pathology of the disease. The meningococcal group B capsule, which is chemically identical to the *E. coli* K1 antigen, has also been shown to be essential for intracellular survival, playing a key role in resistance to cationic antimicrobial peptides (Spinosa et al. 2007). The observation that capsule gene expression was

upregulated during intracellular growth supports a key role for capsule expression in intracellular survival (Spinosa et al. 2007).

Overall it is clear that encapsulation is an absolute requirement for effective systemic infection by invasive human and animal pathogens, and plays a number of roles in mitigating against and subverting the host's defences.

3.5 Capsules and Immunomodulation

Considering their location on the outermost surface of the bacterial cell, it is not surprising that capsules mediate interactions between the microbe and its immediate environment. There is increasing evidence that capsular polysaccharides may possess immunomodulatory activities and be able to moderate the local inflammatory response of epithelial cells to maximise their colonisation and survival within the host. In the case of *Staphyloccocus aureus* both capsular polysaccharide types 5 and 8 were able to bind to epithelial cells and induce IL-8 expression, in addition to inducing IL-8, IL-6, IL-1β and TNFα from monocytes (Soell et al. 1995). It was proposed that these capsular polysaccharides were acting as adhesins to promote attachment to epithelial cells whilst at the same time expressing immunomodulatory effects (Soell et al. 1995). It has been shown with the type 2 capsule of *Streptoccocus suis* that the expression of a capsule disrupts interactions between cell wall components and TLR2, probably through steric effects, thereby reducing cytokine responses to adherent *S. suis* (Graveline et al. 2007). However, the purified capsular polysaccharide was able to induce MCP-1 production by monocytes through a TLR2/MyD88-independent pathway (Graveline et al. 2007). The purified polysaccharide also induced late activation of CD14 and TLR2 but this was probably a consequence of MCP-1 downstream activation of NF-κB and AP-1 transcription factors (Graveline et al. 2007). In contrast, in *Salmonella enterica* serovar Typhi, the Vi capsular antigen reduces TLR-dependent IL-8 production from intestinal mucosa (Raffatellu et al. 2005) and IL-17 secretion (Raffatellu et al. 2007). This indicates that the Vi antigen is acting to reduce intestinal inflammation, possibly playing a role in reducing the influx of neutrophils to the site of infection and thereby promoting the increased survival of *S.* Typhi (Raffatellu et al. 2006). Most strikingly, the purified polysaccharide A (PSA) from the gut symbiont *Bacteroides fragilis* has been shown to have potent anti-inflammatory properties (Mazmanian et al. 2008). Administration of the polysaccharide prevented inflammatory disease in mice infected with *Helicobacter hepaticus* (Mazmanian et al. 2008). This anti-inflammatory response required IL-10-producing $CD4^+$ T cells and demonstrates how a cell surface polysaccharide could be playing key roles in mediating microbe–host interactions and preventing the induction of an inappropriate inflammatory response as a consequence of the colonisation by a gut symbiont (Mazmanian et al. 2008).

The potent anti-inflammatory properties of PSA are in contrast to the K1 capsular polysaccharide from the oral pathogen *Porphyromonas gingivalis* which

elicits MIP-2, RANTES and MCP-1 secretion from murine macrophages and also stimulates macrophage migration (d'Empaire et al. 2006). The ability of the purified K1 capsular polysaccharide to induce inflammatory chemokines would suggest that the capsular polysaccharide is involved in generating the inflammatory lesions typical of periodontal disease as a consequence of *P. ginivalis* infection (d'Empaire et al. 2006). Therefore it is clear that capsular polysaccharides themselves may function to elicit localised inflammatory responses that moderate the interactions between the microbe and the host. This interaction is above and beyond any steric effects the cell surface capsule may have in protecting the microbe from the host's defences.

3.6 Regulation of Capsule Gene Expression

The expression of a capsule represents a substantial investment of energy by the bacterial cell, involving the loss to the cell of valuable sugars that might otherwise be used for energy and growth. Capsule expression will also consume ATP, during, for example, transport of the polymer onto the cell surface. As such, one would expect capsule expression to be tightly regulated in order to avoid unnecessary expression. In essence, the bacterium should attempt to limit capsule expression to the environments in which it will confer a selective advantage. Hence, capsule expression will be regulated by environmental stimuli, allowing integration of local environmental conditions into the regulation of capsule expression. Alternatively, capsule expression may be stochastic with local selective pressures selecting for bacterial cells whose capsule expression is optimal for the environment they find themselves in. Both systems may operate in the same bacterium such that both fine and coarse tuning of capsule expression may take place. In the following sections we will describe the regulation of selected capsule gene clusters from a number of bacterial pathogens.

3.7 Regulation of *Escherichia coli* Capsule Gene Expression

Escherichia coli K antigens have been classified into four groups (Groups 1–4) on the basis of a number of biochemical and genetic criteria (Whitfield and Roberts 1999). In terms of regulation, Group 2 capsule gene clusters have been most studied, with some work on the regulation of those belonging to Group 1. The Group 1 capsule gene cluster and the *cps* gene cluster for the synthesis of colanic acid (M antigen) are allelic such that their expression is mutually exclusive (Whitfield 2006). This is not true for the other groups of K antigens that can express colanic acid in addition to a K antigen. Colanic acid is a polymer of glucose, galactose, fucose and glucuronic acid modified with acetyl and pyruvyl groups (Gottesman and Stout 1991). Colanic acid is expressed only at environmental temperatures

(below 30°C), and is not a virulence factor (Lopez-Torres and Stout 1996). It is readily shed from the cell surface and is akin to slime, with the principal roles of protecting the bacterium from the external environment, being required both for prevention of desiccation and biofilm formation (Ophir and Gutnick 1994; Sledjeski and Gottesman 1996). Since this review is focussing on capsules rather than slime, we will not discuss regulation of colanic acid expression in depth. A detailed discussion can be found in Corbett and Roberts (2008). Suffice it to say that regulation of colanic acid expression involves the Rcs phosphorelay, a highly conserved regulatory system amongst pathogenic or commensal enterobacteria (Erickson and Detweiler 2006; Hinchliffe et al. 2008; Laubacher and Ades 2008).

The Rcs phosphorelay is not directly involved in regulating expression of K antigens in *E. coli* (Jayaratne et al. 1993; Keenleyside et al. 1993). Unlike the *cps* operon, there are no RcsAB boxes, typical of genes regulated by the Rcs phosphorelay system, detectable in any of the four groups of capsule gene clusters in *E. coli*. However, in the case of the K30 antigen, a prototypical Group 1 K antigen, expression was affected by multi-copy RcsB, a key response regulator in the Rcs phosphorelay system (Rahn and Whitfield 2003). The explanation for this was that upstream of *galF*, whose gene product is involved in the synthesis of UDP-glucose, there is an RcsAB box. Multi-copy RcsB was found to induce the transcription of *galF*, which increased the levels of UDP-glucose and consequently caused an increase in K30 expression resulting in a mucoid phenotype (Rahn and Whitfield 2003). Thus, Group 1 capsule expression is in part regulated by the availability of activated sugar precursors, which in turn may be increased through activation of the Rcs phosphorelay system (Rahn and Whitfield 2003).

Both *E. coli* Group 1 and 2 capsule gene clusters are dependent upon a 39 bp sequence downstream of their promoters known as JUMPStart (Just Upstream of Many Polysaccharide gene Starts) (Hobbs and Reeves 1994). Within the JUMPStart sequence is a conserved GGCGGTAG motif known as *ops* (operon polarity suppressor) (Nieto et al. 1996). This sequence is essential for the function of the RfaH that plays a key role in reducing termination of transcription during the generation of long polycistronic messages. In the case of the K30 capsule gene cluster, the *ops* site is located between the *wzc* and *wbaP* genes such that transcription of the genes downstream of *wzc* and eventual expression of the K30 polysaccharide require RfaH (Rahn and Whitfield 2003). In the case of Group 2 capsule gene clusters the *ops* site is centred 28 bp 5′ to the *kpsM* gene (Fig. 3.1) (see below).

3.8 Group 2 Capsule Gene Clusters

Group 2 capsule gene clusters are organised into three regions (Fig. 3.1). Regions 1 and 3 contain the genes encoding proteins that transport the nascent capsular polysaccharide from the cytoplasm to the bacterial cell surface, and are conserved in all Group 2 capsule gene clusters. The central region 2 encodes the genes responsible for the synthesis of the polysaccharide and its precursors (Fig. 3.1)

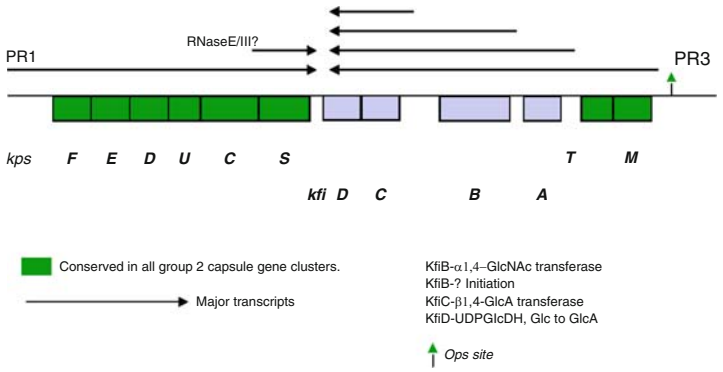

Fig. 3.1 Transcriptional organisation of the K5 capsule gene cluster, showing the conserved Group 2 capsule genes

and is serotype-specific (Whitfield 2006). Our understanding of the regulation of Group 2 capsule gene expression has come from studies on the K5 capsule gene cluster of *E. coli*.

Transcription of Group 2 capsule gene clusters originates from two convergent temperature-regulated promoters located upstream of regions 1 and 3 (Simpson et al. 1996; Stevens et al. 1997), designated PR1 and PR3 (Fig. 3.1). Transcription occurs at 37°C, but no transcription is detectable from either promoter at temperatures below 25°C (Cieslewicz and Vimr 1996; Rowe et al. 2000), Transcription from PR1, a σ^{70} promoter that is located 225 bp upstream of *kpsF*, generates an 8 kb polycistronic message, which is processed to yield a distinct 1.3 kb *kpsS*-specific transcript (Fig. 3.1) (Simpson et al. 1996). Region 3 (*kpsMT*) encodes an ABC transporter which translocates Group 2 capsular polysaccharide chains across the cytoplasmic membrane (Smith et al. 1990; Bliss and Silver 1996). PR3 is located 741 bp upstream of *kpsM* and transcription from this promoter continues through into region 2, past a Rho-dependent transcriptional terminator located between *kpsT* and *kfiA*, the first gene of region 2. Read-through transcription is dependent on RfaH and the *ops* site centred 28 bp 5′ to *kpsM* (Fig. 3.1). Region 2 (*kfiABCD*) is serotype-specific (Petit et al. 1995; Roberts 1996). Long intergenic regions of unknown function exist in region 2: *kfiA* and *kfiB* are separated by 340 bp, while *kfiB* is separated from *kfiC* by 1,293 bp (Petit et al. 1995). Expression of the *kfi* genes absolutely requires RfaH-mediated read-through transcription from PR3 (Stevens et al. 1997).

Temperature regulation of both PR1 and PR3 involves the global regulatory proteins H-NS and BipA; the function of BipA awaits elucidation and will not be discussed here. H-NS plays a curious regulatory role, being required for maximum transcription at 37°C, but also acting as a repressor of transcription at 20°C (Rowe et al. 2000). H-NS is a highly abundant protein that has been implicated in the temperature regulation of a number of genes and the silencing of horizontally acquired genetic material (Dorman 2007; Lucchini et al. 2006; Navarre et al. 2006). The transcriptional regulator, SlyA, is also involved in activating transcription from

PR1 and PR3 at 37°C (Corbett et al. 2007; Xue et al. 2009). SlyA is a regulator of the expression of a number of virulence genes in *S. typhimurium*, the majority of which encode proteins that localise in either the cell membrane, periplasm, or on the cell surface (Ellison and Miller 2006; Navarre et al. 2005; Spory et al. 2002). SlyA is essential for the virulence of *S. typhimurium* in mice, with *slyA* mutants showing reduced survival within murine macrophages with increased sensitivity to the oxidative burst (Buchmeier et al. 1997; Libby et al. 1994; Stapleton et al. 2002) SlyA is an H-NS anti-repressor (Heroven et al. 2004; Fang and Rimsky 2008), but at PR1 and PR3 the situation is more complicated. At PR1, H-NS and SlyA can bind to the promoter simultaneously. H-NS is required for maximal SlyA-mediated activation of transcription at this promoter, explaining the observation that *hns* mutants show reduced levels of capsule gene transcription at 37°C, and suggesting that at the capsule-permissive temperature, the two proteins function as co-activators of transcription (Corbett et al. 2007). Whether this is a result of direct protein–protein interactions or due to nucleoprotein remodelling which results in open complex formation is unclear. The complex interplay observed between H-NS, SlyA, and RNA polymerase has also been observed at the *E. coli hlyE* and *S. typhimurium pagC* and *ugtL* promoters (Lithgow et al. 2007; Perez et al. 2008).

At PR1 there is additional regulatory input through the activity of the IHF protein which is required for maximum transcription from PR1 at 37°C, and binds to a single site located 130 bp downstream of the transcription start point at PR1 (Rowe et al. 2000). IHF exerts its effects by bending DNA to an angle of more than 160° at the W base pair, and, in doing so, can potentially bringing other *cis*-acting regulatory proteins or functionally important regions of DNA into close apposition with RNA polymerase, and thus potentiate transcription (Engelhorn and Geiselmann 1998). In the absence of H-NS, IHF appears to be largely redundant, suggesting a role for IHF in H-NS anti-repression. Additionally, SlyA- and IHF-mediated regulation of this promoter are independent of each other (Corbett and Roberts unpublished).

Expression from PR3 also involves an interplay between H-NS and SlyA at 37°C (Xue et al. 2009). The requirement for H-NS and SlyA allows the co-ordinate regulation of PR1 and PR3 in response to temperature (Xue et al. 2009). A key difference between PR1 and PR3 is the large 741 bp UTR region between PR3 and the ATG of *kpsM* (Fig. 3.1). A number of roles have been assigned to the UTR; it moderates the extent of transcription that reaches *kpsM,* it contains a H-NS binding site 3′ to the transcriptional start that is essential for temperature regulation and contains the *ops* site essential for RfaH regulation (Xue et al. 2009).

In summary, *E. coli* Group 2 capsule gene clusters are regulated principally at the level of transcription. This involves the action of several global regulators acting at two convergent temperature-regulated promoters. Temperature is the only known environmental signal regulating transcription from these promoters and its effects are mediated through the H-NS, BipA and SlyA proteins. However it is likely that other, as yet identified host-induced stimuli will feed into this regulatory network.

3.9 *Salmonella enterica* serovar Typhi

The enteric Gram-negative bacterium *S*. Typhi is a facultative intracellular pathogen and the cause of typhoid fever in humans. It expresses a capsule that is comprised of a variably *O*-acetylated α-1,4-linked 2-deoxy-2-*N*-acetylgalactosamine (Szu et al. 1991). The capsule biosynthesis and export functions are encoded by the *viaB* locus that consists of ten ORFS, while the *viaA* locus contains two genes encoding homologues to the *E. coli* RcsB and RcsC proteins and is important in regulation of expression (Hashimoto et al. 1993; Virlogeux et al. 1995; Waxin et al. 1993).

The first gene of the *viaB* locus is *tviA,* that encodes the positive regulator TviA (Virlogeux et al. 1996). In concert with RcsB, TviA activates transcription of *tviB* from the *tviA* promoter enabling transcription to proceed past a proposed transcriptional terminator located between *tviA* and *tviB* (Virlogeux et al. 1996). RfaH is likely to enable transcription elongation through *viaB,* as a JUMPStart is located upstream of *tviB* (Hobbs and Reeves 1994). TviA is non-essential for capsule expression, but inactivation of *rcsB* abolishes capsule expression (Virlogeux et al. 1995, 1996). Based on the requirement for both TviA and RcsB for expression of the *viaB* locus, and extrapolating from the *E. coli* phosphorelay, it has been proposed that TviA is functionally homologous to RcsA (Virlogeux et al. 1996), However, TviA cannot complement an *E. coli rcsA* mutant, and TviA is not susceptible to degradation mediated by the Lon protease, as is the case for RcsA (Virlogeux et al. 1996). One possibility is that TviA may play a role in assisting RcsB–DNA interaction, as it is able to restore capsule expression to strains carrying mutations in the DNA-binding domain of RcsB, but it is unable to restore capsule expression to an *rcsB* knockout strain (Virlogeux et al. 1996). An RcsAB box has been identified upstream of the *tviA* gene that was bound by *E. coli* RcsAB in vitro (Wehland and Bernhard 2000). If TviA and RcsB do interact in vivo, this could represent a potential target for binding.

Environmental regulation of V_i capsule expression is complex. Growth in high levels of NaCl (>300 mmol/L), comparable to those found in the human gut, inhibits capsule gene transcription in wild-type cells and results in the secretion of invasion-promoting Sip proteins, which, whilst the bacterium is expressing a capsule, are not secreted but stockpiled in the cell (Arricau et al. 1998; Pickard et al. 1994; Santander et al. 2007). Elevated expression of the alternative sigma factor RpoS represses capsule expression. This might account for the fact that capsule expression is maximally expressed during exponential phase, i.e. when RpoS expression is minimal (Arricau et al. 1998; Lee et al. 2006; Santander et al. 2007). The expression of V_i capsule is further complicated by the observation that at low osmolarity there is a mixed population of V_i^+ and V_i^- cells, otherwise known as the VW phenotype (Santander et al. 2007). The basis for this phenotype is unclear. The OmpR-EnvZ two-component regulator system, as well as IHF and Fis proteins, have also been proposed to positively regulate V_i antigen synthesis (Pickard et al. 1994; Lee et al. 2006).

In summary, expression of the *S*. Typhi *viaB* capsule gene cluster is controlled by an Rcs-like system involving the proteins TviA and RcsB. The RcsB protein is essential for expression while TviA is not. RfaH, IHF, Fis and the OmpR-EnvZ system may also enhance transcription of the capsule gene cluster, although their exact roles have not been defined.

3.10 *Neisseria meningitidis*

The most common capsule serogroups of *N*. *meningitidis* are B, C, W-135, and Y, but serogroups B and C, with α-2,8 and α-2,9 linked polysialic acid respectively, cause more than 95% of meningococcal disease in the northern hemisphere (Jennings et al. 1977). Meningococcal capsule gene clusters have a segmental organisation reminiscent of *E*. *coli* Group 2 capsule gene clusters. Two regions termed B and C encode proteins involved in the translocation of the meningococcal polysaccharide onto the cell surface while a third region, A, encodes proteins for the synthesis of NeuNAc and its polymerisation to sialic acid (Roberts 1996). Expression of the meningococcal of serogroup B capsule is subject to phase variation with acapsular colonies occurring at a frequency of 10^{-4} (Hammerschmidt et al. 1996a). A number of mechanisms have been identified to account for this observed phase variation. One is the insertion of IS1301 into the *siaA* gene in approximately 20% of acapsular isolates (Hammerschmidt et al. 1996a). SiaA is responsible for the epimerisation of *N*-acetyl glucosamine to *N*-acetyl mannosamine, which is the substrate for the generation of *N*-acetyl neuraminic acid in a condensation reaction with phospho-enolpyruvate mediated by SiaC (Edwards et al. 1994). Phase variation may also result from the insertion or deletion of a cytidine residue in a run of seven cytidines within the coding region of *siaD*, encoding the α-2,8-polysialyl transferase (Edwards et al. 1994). This results in a premature stop codon downstream of this $(dC)_7$ box (Hammerschmidt et al. 1996b).

Superimposed on this phase-variable expression is transcriptional regulation. Following adhesion to epithelial cells, transcription of the *sia* genes is reduced (Deghmane et al. 2002). This reduction is brought about by CrgA, a LysR-type regulator that is induced upon host cell contact (Deghmane et al. 2000; Deghmane and Taha 2003). CrgA is an autorepressor that is expressed at low levels in the absence of host cell contact (Deghmane and Taha 2003; Ieva et al. 2005). Inhibition of transcription by CrgA is dependent upon the interaction of RNA polymerase with the C-terminal domain of CrgA and the two proteins display synergistic binding to the *crgA* promoter (P*crgA*) (Deghmane et al. 2004; Deghmane and Taha 2003). CrgA does not block open complex formation or transcription initiation; rather, it inhibits transcription elongation by RNA polymerase as it exits the transcription initiation complex (Deghmane et al. 2004). Other studies have challenged the involvement of CrgA in meningococcal capsule regulation (Von Loewenich et al. 2001; Ieva et al. 2005). One possibility is that these differences reflect different experimental approaches as well as the use of different strains of *N*. *meningitidis*.

It is clear, however, that the *N. meningitidis* capsule is phase-variable due to changes at the genetic level within *siaA* and *siaD* genes and that the capsule genes are also regulated at the transcriptional level by host epithelial cell contact.

3.11 *Streptococcus pneumoniae*

More than 90 capsule serotypes have been identified in *S. pneumoniae* and, with the exception of types 3 and 37, the first four genes (*cpsABCD*) involved in capsule biosynthesis are common to nearly all *S. pneumoniae* (Llull et al. 2001b). Capsule expression in *S. pneumoniae* is phase-variable with both encapsulated (opaque) and unencapsulated (transparent) colonies being detectable in clinical isolates of this bacterium. Phase variation from an opaque to a transparent phenotype occurs spontaneously with a frequency of 10^{-3} to 10^{-6} (Weiser et al. 1994) and opaque variants express 1.2–5.6-fold more capsule than transparent variants of the same strain (Kim and Weiser 1998). Invasive isolates have increased capsule production with elevated levels of *cspA* expression (Kim and Weiser 1998; Hathaway et al. 2007). One mechanism by which this phase variation is achieved appears to be as a result of tandem gene duplications. In the case of strains expressing the type 3 capsule, the cause of this phase variation was found to be the generation of perfect tandem duplications of varying size within the *cap3A* gene, which encodes a UDP-glucose dehydrogenase required for capsule biosynthesis (Waite et al. 2001). These duplications result in loss of function of Cap3A, and duplication-induced phase variation could be reproduced with artificial constructs (Waite et al. 2001). Similar observations involving tandem gene duplications have also been reported for expression of the type 8 and type 37 capsules (Llull et al. 2001a; Waite et al. 2003). Reversion to an encapsulated state occurred via a precise excision of the duplicated sequence, and the frequency of phase variation was found to increase with the size of the duplication (Waite et al. 2001, 2003).

Studies on the expression of the type 2 capsule gene cluster have identified the involvement of a transcription regulator RegM (Giammarinaro and Paton 2002). Different in vivo environments known to induce changes in capsule production, including the nasopharynx, lungs and blood, do not correlate with changes in transcription of the *cps2A* gene, indicating that transcriptional regulation is not the principal mechanism employed by this bacterium to regulate capsule expression (LeMessurier et al. 2006). Indeed, capsule expression appears to be regulated at the post-translational level, centred around the tyrosine phosphorylation of CpsD (LeMessurier et al. 2006).

CpsD is an auto-phosphorylating protein-tyrosine kinase that contains a C-terminal $[YGX]_4$ repeat domain (Morona et al. 2000), and promotes attachment of the capsular polysaccharide to teichoic acid in the bacterial cell wall (Morona et al. 2000, 2006). CpsD requires a partner protein CpsC for function (Morona et al. 2006). These proteins are the Gram-positive functional equivalents of the N-terminus and C-terminus respectively of Wzc in *E. coli* expressing Group 1 capsules.

It is proposed that CpsC interacts with unphosphorylated, active CpsD, enabling CpsD to bind ATP and interact with other biosynthetic enzymes to promote capsule biosynthesis. Upon tyrosine-phosphorylation of CpsD, interactions between the capsule biosynthetic proteins change such that capsule biosynthesis is reduced but attachment of capsular polysaccharide to the cell wall is increased. Mutation of the tyrosine residues results in continuous production of capsular polysaccharide, conferring a mucoid phenotype upon the bacterium. However, there is a concomitant reduction in attachment of the polysaccharide to the cell wall in these circumstances (Morona et al. 2000, 2003). CpsD is dephosphorylated by CpsB, a manganese-dependent phosphotyrosine-protein phosphatase, and the cycle begins again (Morona et al. 2002). Because CpsD cannot be cycled in the absence of CpsB, *cpsB* mutants display reduced capsule biosynthesis accompanied by an increase in the attachment of existing capsular polysaccharide to the cell wall (Morona et al. 2006). The observation that certain *cpsB* mutants exhibit a mucoid colony phenotype on blood agar is a consequence of secondary mutations in *cpsC*. These mutations demonstrate the importance of CpcC in addition to CpsD because they reduce by half the amount of capsular polysaccharide attached to the cell wall, despite producing wild-type levels of capsular polysaccharide (Morona et al. 2006).

Opaque variants of *S. pneumoniae* grown under microaerophilic conditions display elevated capsule expression concurrent with an increase in the level of phosphorylated CpsD in the cell. Therefore oxygen availability may be an environmental cue integrated into the regulation of capsule expression (Weiser et al. 2001), which might be expected given that *S. pneumoniae* colonises the naso-pharynx.

In summary, tandem duplications within the *S. pneumoniae capA* gene results in phase-variable capsule expression in this bacterium. Additional control is exerted at the post-translational level, through cyclic phosphorylation and dephosphorylation of CpsD by CpsC and CpsB.

3.12 *Streptococcus pyogenes*

Group A streptococci (GAS) express a capsule composed of hyaluronic acid identical to that found in human connective tissue (Dougherty and van de Rijn 1994). The genes responsible for hyaluronic acid synthesis are organised as a single transcriptional unit, *hasABC*, and capsule expression is upregulated during incubation in human blood, protecting the bacterium from opsono-phagocytosis (Dinkla et al. 2007).

The *hasABC* operon is part of the CovR-CovS (CsrR-CsrS) regulon which comprises approximately 15% of all of the genes in GAS including a large number of virulence genes (Federle et al. 1999; Heath et al. 1999; Levin and Wessels 1998; Graham et al. 2002). CovR and CovS form a two-component regulatory system in which CovS is the sensor showing homology to other histidine kinase proteins and CovR is the response regulator. Mutations in the *covR* gene result in a twofold increase in *has* operon transcription, corresponding to a sixfold increase in capsule

expression, increased resistance to serum-mediated killing and a 500-fold reduction of the LD_{50} of GAS in a mouse model of infection (Levin and Wessels 1998). Activation of CovR is achieved by CovS phosphorylation at an aspartate residue in its N-terminus, causing it to oligomerise (Dalton and Scott 2004; Gusa et al. 2006; Miller et al. 2001). The subsequent phosphorylation of CovR increases its binding efficiency to the *has* promoter region (P*has*) leading to repression of transcription (Federle and Scott 2002; Gao et al. 2005; Gusa et al. 2006; Miller et al. 2001). Phosphorylation is also essential for repression of transcription from P*has* in vivo, and enhances CovR-mediated repression of transcription from P*has* more than sixfold in vitro (Dalton and Scott 2004; Gusa et al. 2006). Therefore expression of the *S.pyogenes* capsule genes is primarily controlled at the level of transcription by the CovRS two-component global regulatory system, where CovR, dependent on its phosphorylation state, will represses transcription from P*has*.

3.13 Conclusions

The expression of bacterial capsules is a common theme that runs through a wide array of bacterial genera. We have described a number of possible functions for capsules with particular emphasis on their role in the lifestyle of pathogens and bacteria associated with humans. Clearly there are many more roles to be assigned to capsules when one extends the discussion to include environmental/rhizosphere isolates and bacteria from extreme environments. What is clear is that far from being the "sugar coating" that inertly protects the bacterium, capsular polysaccharides should be regarded as important signalling molecules in their own right with the ability to elicit and modify host responses to colonisation. This suggests that disrupting or enhancing such polysaccharide host interactions offers a route for both the treatment of infectious disease and the generation of new anti-inflammatory molecules to treat a range of inflammatory human disorders. In addition, when one considers the important uses of polysaccharides and glycoconjugates in biomedicine and the treatment and prevention of diseases, it is clear that the reservoir of diverse bacterial capsular polysaccharides is a valuable resource waiting to be mined and exploited to engineer novel polysaccharides with desired properties.

References

Areschoug T, Waldemarsson J, Gordon S (2008) Evasion of macrophage scavenger receptor A-mediated recognition by pathogenic streptococci. Eur J Immunol 38:3068–3079

Arricau N, Hermant D, Waxin H, Ecobichon C, Duffey PS, Popoff MY (1998) The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. Mol Microbiol 29:835–850

Bliss JM, Silver RP (1996) Coating the surface: a model for expression of capsular polysialic acid in *Escherichia coli* K1. Mol Microbiol 21:221–231

Buchmeier N, Bossie S, Chen CY, Fang FC, Guiney DG, Libby SJ (1997) SlyA, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. Infect Immun 65:3725–3730

Burns SM, Hull SI (1998) Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5. Infect Immun 66:4244–4253

Burns SM, Hull SI (1999) Loss of resistance to ingestion and phagocytic killing by O(−) and K(−) mutants of a uropathogenic *Escherichia coli* O75:K5 strain. Infect Immun 67:3757–3762

Carlin AF, Lewis AL, Varki A, Nizet V (2007) Group B streptococcal capsular sialic acids interact with siglecs (immunoglobulin-like lectins) on human leukocytes. J Bacteriol 189:1231–1237

Cieslewicz M, Vimr E (1996) Thermoregulation of *kpsF*, the first region 1 gene in the *kps* locus for polysialic acid biosynthesis in *Escherichia coli* K1. J Bacteriol 178:3212–3220

Cisowska A, Jankowski S (2004) The sensitivity of *Escherichia coli* strains with K1 surface antigen and rods without this antigen to the bactericidal effect of serum. Folia Microbiol (Praha) 49:471–478

Corbett D, Roberts IS (2008) Capsular polysaccharides in *Escherichia coli*. Adv Appl Microbiol 65:1–26

Corbett D, Bennett H, Askar H, Green J, Roberts IS (2007) SlyA and H-NS regulate transcription of the *Escherichia coli* K5 capsule gene cluster and expression of *slyA* in *Escherichia coli* is temperature dependent, positively autoregulated and independent of H-NS. J Biol Chem 282:33326–33335

Cunnion KM, Lee JC, Frank MM (2001) Capsule production and growth phase influence binding of complement to *Staphylococcus aureus*. Infect Immun 69:6796–6803

Cywes C, Wessels MR (2001) Group A *Streptococcus* tissue invasion by CD44-mediated cell signalling. Nature 414:648–652

Dalton TL, Scott JR (2004) CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*. J Bacteriol 186:3928–3937

Davey ME, Duncan MJ (2006) Enhanced biofilm formation and loss of capsule synthesis: Deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. J Bacteriol 188:5510–5523

Deghmane AE, Taha MK (2003) The *Neisseria meningitidis* adhesion regulatory protein CrgA acts through oligomerization and interaction with RNA polymerase. Mol Microbiol 47:135–143

Deghmane AE, Petit S, Topilko A, Pereira Y, Giorgini D, Larribe M, Taha MK (2000) Intimate adhesion of *Neisseria meningitidis* to human epithelial cells is under the control of the crgA gene, a novel LysR-type transcriptional regulator. EMBO J 19:1068–1078

Deghmane AE, Giorgini D, Larribe M, Alonso JM, Taha MK (2002) Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. Mol Microbiol 43:1555–1564

Deghmane AE, Giorgini D, Maigre L, Taha MK (2004) Analysis in vitro and in vivo of the transcriptional regulator CrgA of *Neisseria meningitidis* upon contact with target cells. Mol Microbiol 53:917–927

d'Empaire G, Baer MT, Gibson FC III (2006) The K1 serotype capsular polysaccharide of *Porphyromonas gingivalis* elicits chemokine production from murine macrophages that facilitates cell migration. Infect Immun 74:6236–6243

Dinkla K, Sastalla I, Godehardt AW, Janze N, Chhatwal GS, Rohde M, Medina E (2007) Upregulation of capsule enables *Streptococcus pyogenes* to evade immune recognition by antigen-specific antibodies directed to the G-related alpha2-macroglobulin-binding protein GRAB located on the bacterial surface. Microbes Infect 9:922–931

Dorman CJ (2007) H-NS, the genome sentinel. Nat Rev Microbiol 5:157–161

Dougherty BA, van de Rijn I (1994) Molecular characterization of *hasA* from an operon required for hyaluronic acid synthesis in group A streptococci. J Biol Chem 269:169–175

Dubnau D, Losick R (2006) Bistability in bacteria. Mol Microbiol 61:564–572
Edwards U, Muller A, Hammerschmidt S, Gerardy-Schahn R, Frosch M (1994) Molecular analysis of the biosynthesis pathway of the alpha-2, 8 polysialic acid capsule by *Neisseria meningitidis* serogroup B. Mol Microbiol 14:141–149
Ellison DW, Miller VL (2006) Regulation of virulence by members of the MarR/SlyA family. Curr Opin Microbiol 9:153–159
Engelhorn M, Geiselmann J (1998) Maximal transcriptional activation by the IHF protein of *Escherichia coli* depends on optimal DNA bending by the activator. Mol Microbiol 30:431–441
Erickson KD, Detweiler CS (2006) The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates ydeI, a gene important for persistent *Salmonella* infection of mice. Mol Microbiol 62:883–894
Fang FC, Rimsky S (2008) New insights into transcriptional regulation by H-NS. Curr Opin Microbiol 11:113–120
Favre-Bonte S, Joly B, Forestier C (1999) Consequences of reduction of *Klebsiella pneumoniae* capsule expression on interactions of this bacterium with epithelial cells. Infect Immun 67:554–561
Favre-Bonté S, Licht TR, Forestier C, Krogfelt KA (1999) *Klebsiella pneumoniae capsule* expression is necessary for colonization of large intestines of streptomycin-treated mice. Infect Immun 67:6152–6156
Federle MJ, Scott JR (2002) Identification of binding sites for the group A Streptococcal global regulator CovR. Mol Microbiol 43:1161–1172
Federle MJ, McIver KS, Scott JR (1999) A response regulator that represses transcription of several virulence operons in the group A streptococcus. J Bacteriol 181:3649–3657
Gao J, Gusa AA, Scott JR, Churchward G (2005) Binding of the global response regulator protein CovR to the sag promoter of *Streptococcus pyogenes* reveals a new mode of CovR-DNA interaction. J Biol Chem 280:38948–38956
Geoffroy MC, Floquet S, Métais A, Nassif X, Pelicic V (2003) Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. Genome Res 13:391–398
Giammarinaro P, Paton JC (2002) Role of RegM, a homologue of the catabolite repressor protein CcpA, in the virulence of *Streptococcus pneumoniae*. Infect Immun 70:5454–5461
Gottesman S, Stout V (1991) Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. Mol Microbiol 5:1599–1606
Graham MR, Smoot LM, Migliaccio CA, Virtaneva K, Sturdevant DE, Porcella SF, Federle MJ, Adams GJ, Scott JR, Musser JM (2002) Virulence control in group A *Streptococcus* by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. Proc Natl Acad Sci USA 99:13855–13860
Graveline R, Segura M, Radzioch D, Gottschalk M (2007) TLR2-dependent recognition of *Streptococcus suis* is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int Immunol 19:375–389
Gusa AA, Gao J, Stringer V, Churchward G, Scott JR (2006) Phosphorylation of the group A Streptococcal CovR response regulator causes dimerization and promoter-specific recruitment by RNA polymerase. J Bacteriol 188:4620–4626
Hammerschmidt S, Hilse R, van Putten JP, Gerardy-Schahn R, Unkmeir A, Frosch M (1996a) Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. EMBO J 15:192–198
Hammerschmidt S, Muller A, Sillmann H, Muhlenhoff M, Borrow R, Fox A, van Putten J, Zollinger WD, Gerardy-Schahn R, Frosch M (1996b) Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (*siaD*): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol 20:1211–1220
Hashimoto Y, Li N, Yokoyama H, Ezaki T (1993) Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. J Bacteriol 175:4456–4465

Hathaway LJ, Battig P, Muhlemann K (2007) In vitro expression of the first capsule gene of *Streptococcus pneumoniae*, *cpsA*, is associated with serotype-specific colonization prevalence and invasiveness. Microbiology 153:2465–2471

Heath A, DiRita VJ, Barg NL, Engleberg NC (1999) A two-component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect Immun 67:5298–5305

Henrichsen J (1995) Six newly recognized types of *Streptococcus pneumoniae*. J Clin Microbiol 33:2759–2762

Hérias MV, Midtvedt T, Hanson LA, Wold AE (1997) *Escherichia coli* K5 capsule expression enhances colonization of the large intestine in the gnotobiotic rat. Infect Immun 65:531–536

Heroven AK, Nagel G, Tran HJ, Parr S, Dersch P (2004) RovA is autoregulated and antagonizes H-NS-mediated silencing of invasin and rovA expression in *Yersinia pseudotuberculosis*. Mol Microbiol 53:871–888

Hinchliffe SJ, Howard SL, Huang YH, Clarke DJ, Wren BW (2008) The importance of the Rcs phosphorelay in the survival and pathogenesis of the enteropathogenic yersiniae. Microbiology 154:1117–1131

Hobbs M, Reeves PR (1994) The JUMPstart sequence: a 39 bp element common to several polysaccharide gene clusters. Mol Microbiol 12:855–856

Ieva R, Alaimo C, Delany I, Spohn G, Rappuoli R, Scarlato V (2005) CrgA is an inducible LysR-type regulator of *Neisseria meningitidis*, acting both as a repressor and as an activator of gene transcription. J Bacteriol 187:3421–3430

Ikehara Y, Ikehara SK, Paulson JC (2004) Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. J Biol Chem 279:43117–43125

Jarva H, Ram S, Vogel U, Blom AM, Meri S (2005) Binding of the complement inhibitor C4bp to serogroup B *Neisseria meningitidis*. J Immunol 174:6299–6307

Jayaratne P, Keenleyside WJ, MacLachlan PR, Dodgson C, Whitfield C (1993) Characterization of rcsB and rcsC from *Escherichia coli* O9:K30:H12 and examination of the role of the rcs regulatory system in expression of group I capsular polysaccharides. J Bacteriol 175:5384–5394

Jennings HJ, Bhattacharjee AK, Bundle DR, Kenny CP, Martin A, Smith IC (1977) Strucutres of the capsular polysaccharides of *Neisseria meningitidis* as determined by 13C-nuclear magnetic resonance spectroscopy. J Infect Dis 136(Suppl):S78–S83

Keenleyside WJ, Bronner D, Jann K, Jann B, Whitfield C (1993) Coexpression of colanic acid and serotype-specific capsular polysaccharides in *Escherichia coli* strains with group II K antigens. J Bacteriol 175:6725–6730

Kim JO, Weiser JN (1998) Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J Infect Dis 177:368–377

Kim KJ, Elliott SJ, Di Cello F, Stins MF, Kim KS (2003) The K1 capsule modulates trafficking of *E. coli*-containing vacuoles and enhances intracellular bacterial survival in human brain microvascular endothelial cells. Cell Microbiol 5:245–252

Laubacher ME, Ades SE (2008) The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J Bacteriol 190:2065–2074

Lee FK, Morris C, Hackett J (2006) The *Salmonella enterica* serovar *Typhi* Vi capsule and self-association pili share controls on expression. FEMS Microbiol Lett 261:41–46

LeMessurier KS, Ogunniyi AD, Paton JC (2006) Differential expression of key pneumococcal virulence genes in vivo. Microbiology 152:305–311

Levin JC, Wessels MR (1998) Identification of *csrR*/*csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A Streptococcus. Mol Microbiol 30:209–219

Libby SJ, Goebel W, Ludwig A, Buchmeier N, Bowe F, Fang FC, Guiney DG, Songer JG, Heffron F (1994) A cytolysin encoded by *Salmonella* is required for survival within macrophages. Proc Natl Acad Sci USA 91:489–493

Lithgow JK, Haider F, Roberts IS, Green J (2007) Alternate SlyA and H-NS nucleoprotein complexes control hlyE expression in *Escherichia coli* K-12. Mol Microbiol 66:685–698

Llull D, Garcia E, Lopez R (2001a) Tts, a processive beta-glucosyltransferase of *Streptococcus pneumoniae*, directs the synthesis of the branched type 37 capsular polysaccharide in Pneumococcus and other gram-positive species. J Biol Chem 276:21053–21061

Llull D, Lopez R, Garcia E (2001b) Genetic bases and medical relevance of capsular polysaccharide biosynthesis in pathogenic streptococci. Curr Mol Med 1:475–491

Lopez-Torres AJ, Stout V (1996) Role of colanic acid polysaccharide in serum resistance in vivo and in adherence. Curr Microbiol 33:383–389

Lucchini S, Rowley G, Goldberg MD, Hurd D, Harrison M, Hinton JC (2006) H-NS mediates the silencing of laterally acquired genes in bacteria. PLoS Pathog 8:e81

Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453:620–625

Miller AA, Engleberg NC, DiRita VJ (2001) Repression of virulence genes by phosphorylation-dependent oligomerization of CsrR at target promoters in *S. pyogenes*. Mol Microbiol 40:976–990

Morona JK, Paton JC, Miller DC, Morona R (2000) Tyrosine phosphorylation of CpsD negatively regulates capsular polysaccharide biosynthesis in *Streptococcus pneumoniae*. Mol Microbiol 35:1431–1442

Morona JK, Morona R, Miller DC, Paton JC (2002) *Streptococcus* pneumoniae capsule biosynthesis protein CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase. J Bacteriol 184:577–583

Morona JK, Morona R, Miller DC, Paton JC (2003) Mutational analysis of the carboxy-terminal (YGX)4 repeat domain of CpsD, an autophosphorylating tyrosine kinase required for capsule biosynthesis in *Streptococcus pneumoniae*. J Bacteriol 185:3009–3019

Morona JK, Morona R, Paton JC (2006) Attachment of capsular polysaccharide to the cell wall of *Streptococcus pneumoniae* type 2 is required for invasive disease. Proc Natl Acad Sci USA 103:8505–8510

Moscoso M, García E, López R (2006) Biofilm formation by *Streptococcus pneumoniae:* role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J Bacteriol 188:7785–7795

Nakhamchik A, Wilde C, Rowe-Magnus DA (2008) Cyclic-di-GMP regulates extracellular polysaccharide production, biofilm formation, and rugose colony development by *Vibrio vulnificus*. Appl Environ Microbiol 74:3068–3079

Navarre WW, Halsey TA, Walthers D, Frye J, McClelland M, Potter JL, Kenney LJ, Gunn JS, Fang FC, Libby SJ (2005) Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Mol Microbiol 56:492–508

Navarre WW, Porwollik S, Wang Y, McClelland M, Rosen H, Libby SJ, Fang FC (2006) Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. Science 313:236–238

Neeleman C, Geelen SP, Aerts PC, Daha MR, Mollnes TE, Roord JJ, Posthuma G, van Dijk H, Fleer A (1999) Resistance to both complement activation and phagocytosis in type 3 pneumococci is mediated by the binding of complement regulatory protein factor H. Infect Immun 67:4517–4524

Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN (2007) Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. Infect Immun 75:83–90

Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker PR (2003) Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. Eur J Imunol 33:1642–1648

Nieto JM, Bailey MJ, Hughes C, Koronakis V (1996) Suppression of transcription polarity in the *Escherichia coli* haemolysin operon by a short upstream element shared by polysaccharide and DNA transfer determinants. Mol Microbiol 19:705–713

Ophir T, Gutnick DL (1994) A role for exopolysaccharides in the protection of microorganisms from desiccation. Appl Environ Microbiol 60:740–745

Pangburn MK, Atkinson MA, Meri S (1991) Localization of the heparin-binding site on complement factor H. J Biol Chem 266:16847–16853

Perez JC, Latifi T, Groisman EA (2008) Overcoming H-NS-mediated transcriptional silencing of horizontally acquired genes by the PhoP and SlyA proteins in *Salmonella enterica*. J Biol Chem 283:10773–10783

Petit C, Rigg GP, Pazzani C, Smith A, Sieberth V, Stevens M, Boulnois G, Jann K, Roberts IS (1995) Region 2 of the *Escherichia coli* K5 capsule gene cluster encoding proteins for the biosynthesis of the K5 polysaccharide. Mol Microbiol 17:611–620

Pickard D, Li JL, Roberts M, Maskell D, Hone D, Levine M, Dougan G, Chatfield S (1994) Characterization of defined *ompR* mutants of *Salmonella typhi*: *ompR* is involved in the regulation of Vi polysaccharide expression. Infect Immun 62:3984–3993

Pluschke G, Mayden J, Achtman M, Levine RP (1983) Role of the capsule and the O antigen in resistance of O18:K1 *Escherichia coli* to complement-mediated killing. Infect Immun 42:907–913

Raffatellu M, Chessa D, Wilson RP, Dusold R, Rubino S, Bäumler AJ (2005) The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infect Immun 73:3367–3374

Raffatellu M, Chessa D, Wilson RP, Tükel C, Akçelik M, Bäumler AJ (2006) Capsule-mediated immune evasion: a new hypothesis explaining aspects of typhoid fever pathogenesis. Infect Immun 74:19–27

Raffatellu M, Santos RL, Chessa D, Wilson RP, Winter SE, Rossetti CA, Lawhon SD, Chu H, Lau T, Bevins CL, Adams LG, Bäumler AJ (2007) The capsule encoding the viaB locus reduces interleukin-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. Infect Immun 75:4342–4350

Rahn A, Whitfield C (2003) Transcriptional organization and regulation of the *Escherichia coli* K30 group 1 capsule biosynthesis (cps) gene cluster. Mol Microbiol 47:1045–1060

Roberts IS (1996) The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu Rev Microbiol 50:285–315

Rowe S, Hodson N, Griffiths G, Roberts IS (2000) Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli*. J Bacteriol 182:2741–2745

Santander J, Wanda SY, Nickerson CA, Curtiss R III (2007) Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in *Salmonella enterica* serotype *Typhi*. Infect Immun 75:1382–1392

Schembri MA, Dalsgaard D, Klemm P (2004) Capsule shields the function of short bacterial adhesins. J Bacteriol 186:1249–57

Simpson DA, Hammarton TC, Roberts IS (1996) Transcriptional organization and regulation of expression of region 1 of the *Escherichia coli* K5 capsule gene cluster. J Bacteriol 178:6466–6474

Sledjeski DD, Gottesman S (1996) Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J Bacteriol 178:1204–1206

Smith AN, Boulnois GJ, Roberts IS (1990) Molecular analysis of the *Escherichia coli* K5 *kps* locus: identification and characterization of an inner-membrane capsular polysaccharide transport system. Mol Microbiol 4:1863–1869

Soell M, Diab M, Haan-Archipoff G, Beretz A, Herbelin C, Poutrel B, Klein JP (1995) Capsular polysaccharide types 5 and 8 of *Staphylococcus aureus* bind specifically to human epithelial (KB) cells, endothelial cells, and monocytes and induce release of cytokines. Infect Immun 63:1380–1386

Spinosa MR, Progida C, Talà A, Cogli L, Alifano P, Bucci C (2007) The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. Infect Immun 75:3594–3603

Spory A, Bosserhoff A, von Rhein C, Goebel W, Ludwig A (2002) Differential regulation of multiple proteins of *Escherichia coli* and *Salmonella enterica* serovar *typhimurium* by the transcriptional regulator SlyA. J Bacteriol 184:3549–3559

Stapleton MR, Norte VA, Read RC, Green J (2002) Interaction of the *Salmonella typhimurium* transcription and virulence factor SlyA with target DNA and identification of members of the SlyA regulon. J Biol Chem 277:17630–17637

Stevens MP, Clarke BR, Roberts IS (1997) Regulation of the *Escherichia coli* K5 capsule gene cluster by transcription antitermination. Mol Microbiol 24:1001–1012

Szu SC, Li XR, Stone AL, Robbins JB (1991) Relation between structure and immunologic properties of the Vi capsular polysaccharide. Infect Immun 59:4555–4561

Uria MJ, Zhang Q, Li Y, Chan A, Exley RM, Gollan B, Chan H, Feavers I, Yarwood A, Abad R, Borrow R, Fleck RA, Mulloy B, Vazquez JA, Tang CM (2008) A generic mechanism in *Neisseria meningitidis* for enhanced resistance against bactericidal antibodies. J Exp Med 205:1423–1434

Valle J, Da Re S, Henry N, Fontaine T, Balestrino D, Latour-Lambert P, Ghigo JM (2006) Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. Proc Natl Acad Sci USA 103:12558–12563

Virlogeux I, Waxin H, Ecobichon C, Popoff MY (1995) Role of the viaB locus in synthesis, transport and expression of *Salmonella typhi* Vi antigen. Microbiology 141:3039–3047

Virlogeux I, Waxin H, Ecobichon C, Lee JO, Popoff MY (1996) Characterization of the rcsA and rcsB genes from *Salmonella typhi*: rcsB through tviA is involved in regulation of Vi antigen synthesis. J Bacteriol 178:1691–1698

Vogel U, Hammerschmidt S, Frosch M (1996) Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitis* serogroup B are prerequisites for virulence of meningococci in the infant rat. Med Microbiol Immunol 185:81–87

Vogel U, Weinberger A, Frank R, Müller A, Köhl J, Atkinson JP, Frosch M (1997) Complement factor C3 deposition and serum resistance in isogenic capsule and lipooligosaccharide sialic acid mutants of serogroup B *Neisseria meningitidis*. Infect Immun 65:4022–4029

Von Loewenich FD, Wintermeyer E, Dumig M, Frosch M (2001) Analysis of transcriptional control mechanisms of capsule expression in *Neisseria meningitidis*. Int J Med Microbiol 291:361–369

Waite RD, Struthers JK, Dowson CG (2001) Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. Mol Microbiol 42:1223–1232

Waite RD, Penfold DW, Struthers JK, Dowson CG (2003) Spontaneous sequence duplications within capsule genes cap8E and tts control phase variation in *Streptococcus pneumoniae* serotypes 8 and 37. Microbiology 149:497–504

Waxin H, Virlogeux I, Kolyva S, Popoff MY (1993) Identification of six open reading frames in the *Salmonella enterica* subsp. enterica ser. Typhi viaB locus involved in Vi antigen production. Res Microbiol 144:363–371

Wehland M, Bernhard F (2000) The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria. J Biol Chem 275:7013–7020

Weiser JN, Austrian R, Sreenivasan PK, Masure HR (1994) Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun 62:2582–2589

Weiser JN, Bae D, Epino H, Gordon SB, Kapoor M, Zenewicz LA, hchepetov M (2001) Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of *Streptococcus pneumoniae*. Infect Immun 69:5430–5439

Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem 75:39–68

Whitfield C, Roberts IS (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol 31:1307–1319

Wooster DG, Maruvada R, Blom AM, Prasadarao NV (2006) Logarithmic phase *Escherichia coli* K1 efficiently avoids serum killing by promoting C4bp-mediated C3b and C4b degradation. Immunology 117:482–493

Xue P, Corbett D, Goldrick M, Naylor C, Roberts IS (2009) Regulation of expression of the region 3 promoter of the Escherichia coli K5 capsule gene cluster involves H-NS, SlyA, and a large 5′ untranslated region. J Bacteriol 191:1838–1844

Yavuz E, Drouillard S, Roberts IS, Samain E, Priem B (2008) Glucuronylation in *Escherichia coli* for the bacterial synthesis of non-sulphated HNK-1 analogs. Glycobiology 18:152–157

Yip ES, Grublesky BT, Hussa EA, Visick KL (2005) A novel, conserved cluster of genes promotes symbiotic colonization and sigma-dependent biofilm formation by *Vibrio fischeri*. Mol Microbiol 57:1485–1498

Chapter 4
Lipopolysaccharides

Alba Silipo, Cristina De Castro, Rosa Lanzetta, Michelangelo Parrilli, and Antonio Molinaro

4.1 Introduction

Lipopolysaccharides (LPSs) were discovered, and named endotoxins, in 1892, by Richard Pfeiffer who, while working on cell lysates of *Vibrio cholerae,* identified a heat-resistant toxin that appeared to be attached to the bacterial body, unlike the exotoxins that instead were secreted by the bacteria. The term lipopolysaccharide was introduced in 1943 by Murray Shear to indicate the bacterial toxin, composed of polysaccharide and lipoidic material, used for the treatment of malignant tumors. Afterwards, this term was adopted by Otto Westphal and Otto Lüderlitz, two pioneer scientists of modern endotoxin science, to indicate the pyrogenic fractions arising from enterobacteria, mainly *Escherichia coli* and *Salmonella enterica*, which they were able to isolate in sufficiently pure amounts for structural studies. A very interesting and fascinating historical perspective of the endotoxin story by Prof. E. Th. Rietschel is available in the literature (Rietschel and Westphal 1999; Beutler and Rietschel 2003).

The LPSs are amphiphilic components present in the cell wall of almost all Gram-negative bacteria; in particular they are localized in the external leaflet of the lipid bilayer constituting the outer membrane (OM, Fig. 4.1) in which they represent approximately 75% of the outer surface. The OM is responsible for the low permeability of the Gram-negative surface compared with that of Gram-positive bacteria which lack this second membrane. In general the OM decreases the permeability of the barrier to hydrophobic compounds and higher molecular weight hydrophilic compounds owing to the very low fluidity of the highly ordered structure of the LPS monolayer. Furthermore, owing to their external location, LPSs are involved in all interactions with the external environment, in particular in the many

A. Molinaro (✉)
Department of Organic Chemistry and Biochemistry, University of Naples Federico II, via Cintia, 4 I-80126, Napoli, Italy
e-mail: molinaro@unina.it

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_4,

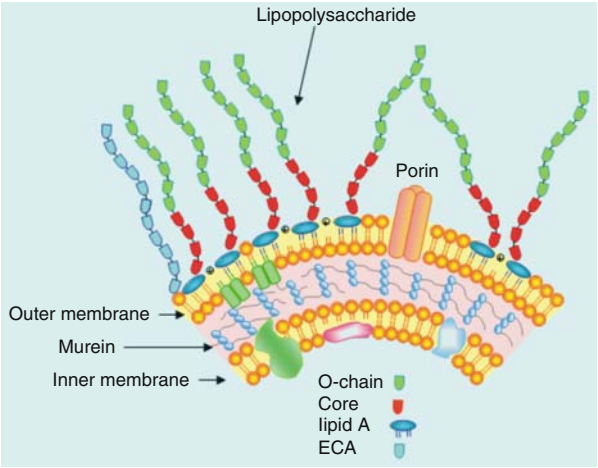

Fig. 4.1 The cell wall architecture of Gram-negative bacteria. It is basically built up of two lipid bilayers with a periplasmic space in between where murein is located. The outer membranes of almost all Gram-negative bacteria are asymmetrical and the outer side is essentially formed by the lipid A regions of the LPS molecules which make up about 75% of the total membrane surface. In addition to proteins like porins and LPSs, the outer membranes of enterobacteria present the enterobacterial common antigen (ECA) and, in some species and strains, also capsular polysaccharides. In a few cases, capsular polysaccharides such as colanic acid and ECA can be covalently linked to the core region of LPSs. As indicated by the charge symbols, divalent cations are tightly associated to the extremely anionic membrane proximal regions of the LPS molecules

aspects of host–bacterium interactions: recognition, adhesion, colonization, virulence in the case of pathogenic bacteria, symbiosis, tolerance for commensal bacteria and, in the case of extremophile bacteria, survival under harsh conditions.

4.2 General Structure of Lipopolysaccharides and Their Components

From the structural point of view, LPSs show the same general chemical architecture in all of the Gram-negative bacteria, that is, it depends neither on the bacterial activity (pathogenic, symbiontic, commensal), nor on its ecological niches (human, animal, soil, plant, water) nor on its growth conditions (Raetz and Whitfield 2002). From the biosynthetic and chemical points of view, three different parts are usually identified in the general structure of a LPS (Fig. 4.2): a polysaccharide (known as O-side chain, O-specific polysaccharide or O-antigen) which is covalently linked to an oligosaccharide part (core) which, in turn, is linked to a glycolipid portion (lipid A). The lipid A is part of the outer leaflet of the external membrane whereas the sugar moiety is oriented outwards (Raetz and Whitfield 2002). At the beginning of the 1900s, the bacterial colony was cataloged by morphology as rough or smooth;

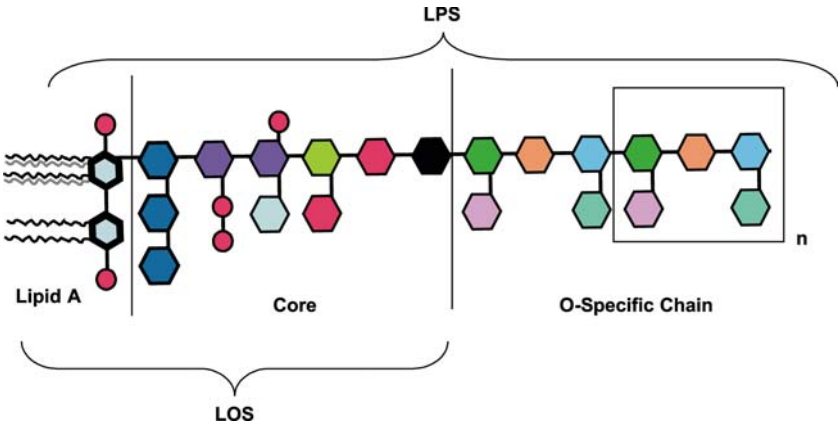

Fig. 4.2 General chemical structure of a LPS from Gram-negative bacteria. All forms of LPS known to date consist of a lipid A domain and a covalently linked polysaccharide or oligosaccharide portion (LPS or LOS, respectively). The polysaccharide domain is, in turn, composed of a core region and the O-specific chain., An inner and an outer core region are commonly distinguished according to preferential carbohydrate compositions in the core structure. Kdo residues proximal to lipid A are depicted in *blue* whereas heptose residues are in *violet*; phosphate residues are also visible as *pink* appendages

later it was understood that this was related to the occurrence of the O-side chain, being absent in the first case and present in the second. These observations led to the terminology currently used to designate the different types of LPS, namely with or without antigenic portion, as S-LPS or R-LPS (or lipo-oligosaccharide, LOS), respectively (Fig. 4.2).

However, some enteric bacteria in particular growth conditions covalently attach enterobacterial common antigen (ECA, a polysaccharide capsule) or colanic acid (CA, an exopolysaccharide) to the outer core region (Whitfield 2006; Meredith et al. 2007). Thus, the general architecture of smooth LPS must now be defined differently: polysaccharide, core region and lipid A, where by polysaccharide is meant the O-chain, a capsula such as ECA or an exopolysaccharide such as CA. In all cases, the LPS fraction of a bacterial strain is characterized by an intrinsic size and structural heterogeneity, and therefore it is more appropriate to describe them as a family of LPS molecules.

4.2.1 Lipid A

The first lipid A structure (Fig. 4.3), investigated early on in the *Enterobacteriaceae* by Westphal and Lüderlitz in 1954, was established in 1983 and consists of a β-(1-6)- linked 2-amino-2-deoxy-glucopyranose (GlcN, glucosamine) disaccharide that bears 3-(*R*)-hydroxy fatty acid residues, ester-linked at the 3 and 3′ positions, and amide-linked at 2 and 2′ positions, which are indicated as primary fatty acid

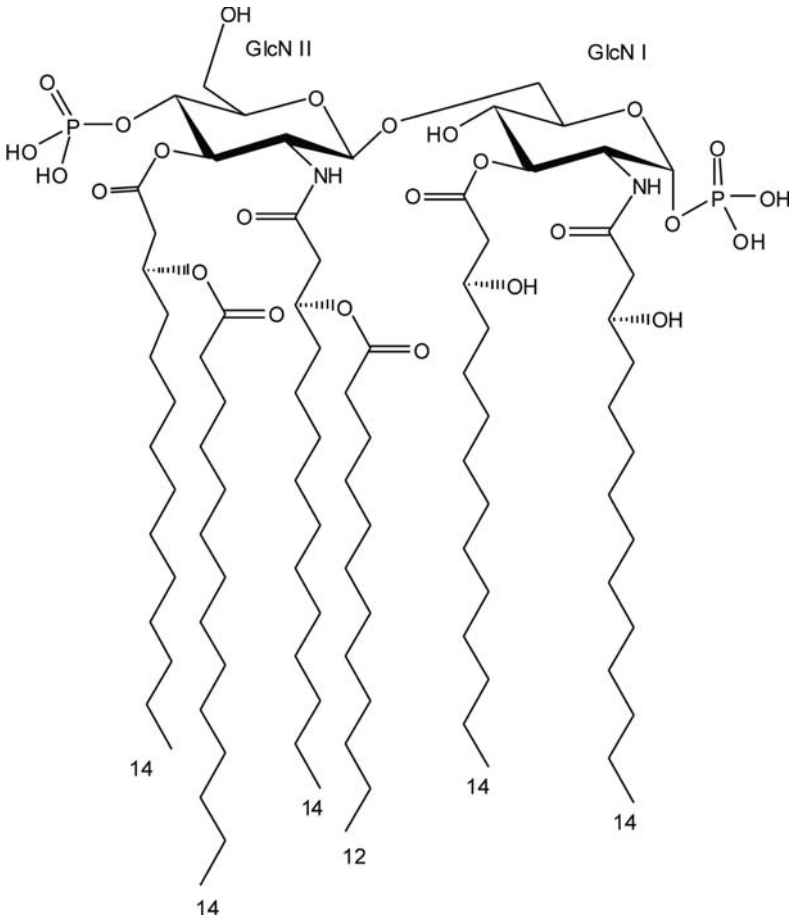

Fig. 4.3 The chemical structure of lipid A from *Escherichia coli* LPS. The β-(1-6)- 2-amino-2-deoxy-glucose (GlcN) disaccharide bears primary 3-(*R*)-hydroxy tetradecanoic acid residues either as ester (at the 3 and 3′ positions) or as amide-(at the 2 and 2′ positions). Both fatty acid residues on the $GlcN^{II}$ are further esterified by a dodecanoic and a tetradecanoic residue. The hydroxyl at position 4′ of the non-reducing $GlcN^{II}$ residue (*distal unit*) and that of the α-anomeric position of the reducing $GlcN^{I}$ residue (*proximal unit*) are both phosphorylated

residues. The 3-OH, in turn, can be further esterified by secondary fatty acids typically not carrying any other functional group. The hydroxyl at position 4′ of the non-reducing $GlcN^{II}$ residue (distal unit) and that of the α-anomeric position of the reducing $GlcN^{I}$ residue (proximal unit) are generally both linked to polar heads, phosphate groups (Fig. 4.3) (Zähringer et al. 1994). Despite this general chemical architecture conserved in most bacterial LPSs, there are many subtle chemical differences that are responsible for the lipid A variation among bacterial species (Fig. 4.4). Lipid A is the true endotoxic principle of LPSs of pathogenic bacteria able to elicit the cascade of events leading to the formation of pro-inflammatory

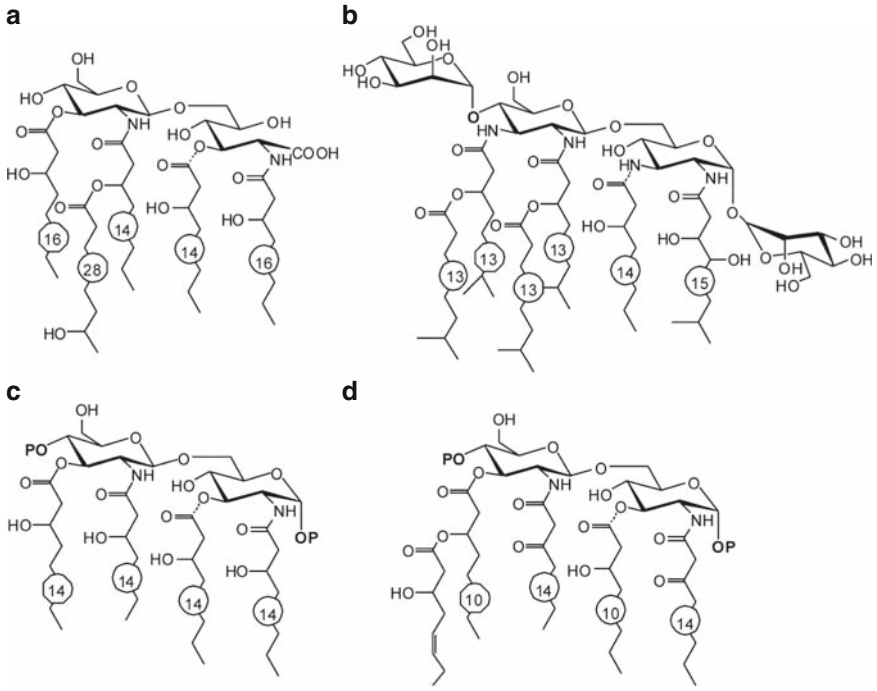

Fig. 4.4 The structures of some lipid A molecules that present remarkable chemical and biological differences with canonical *E. coli* lipid A. (**a**) the *Rhizobium etli* CE3 lipid A that carries 27-OH octacosanoic acid and the 2-amino-2-deoxy-gluconate residue that replaces $GlcN^{I}$; (**b**) the completely neutral *Bdellovibrio bacteriovorus* lipid A where the phosphates are replaced by two mannopyranose residues; (**c**) the classical form of endotoxin antagonist in human monocyte activation, the lipid IV_a, the biosynthetic precursor of hexacyl lipid A in *E.coli*; and (**d**) the major penta-acyl lipid A form from the phototrophic bacterium *Rhodobacter capsulatus* that is characterized by the presence of unsaturated and 3-keto acyl chains; *R. sphaeroides* lipid A differs only by the position of the secondary fatty acid that is on a ester-bound primary residue

products, and therefore the definition of its primary structure is of great importance (Zähringer et al. 1994; Raetz and Whitfield 2002; Raetz et al. 2007).

Phosphate groups can further link other phosphate groups to give a pyrophosphate or additionally be substituted by an 2-amino-ethanol group (EtN) or 4-amino-4-deoxy-L-arabinopyranose (arabinosamine, Ara4N). In a few cases, the phosphate group can be displaced by acid monosaccharides such as galacturonic acid (GalA) in the lipid A of *Aquifex pyrophilus* (Plötz et al. 2000) and *Rhizobium* (Bhat et al. 1994) or by neutral monosaccharide residues. In the case of the obligate predatory *Bdellovibrio bacteriovorus* LPS lipid A, no phosphate or other negatively charged group but two mannose residues are present, generating a totally neutral lipid A (Fig. 4.4) (Schwudke et al. 2003).

Acyl residues may vary in number, type and distribution. The more frequent primary fatty acid residues found in lipid A are 3-hydroxy-decanoic, dodecanoic,

tetradecanoic, esadecanoic, and octadecanoic acids and, as secondary, the corresponding 3-deoxy fatty acids; these latter can bear, as in *Pseudomonas*, the 2-OH (*S*) function (Zähringer et al. 1994; Raetz and Whitfield 2002; Raetz et al. 2007). Further "atypical" structural features less frequently found in the lipid A fatty acid residues are odd numbered carbon chains, methyl branches, unsaturation, different functional groups and hydroxyl substitutions, very long chains up to C28, as occurs for 27-OH octacosanoic acid in the *Rhizobiaceae* LPS (Bhat et al. 1991; Basu et al. 2002; Vedam et al. 2003, 2006; De Castro et al. 2008), but also in *Legionella* and *Brucella* LPSs (Fig. 4.4) (Zähringer et al. 1994; Raetz and Whitfield 2002; Raetz et al. 2007). The phototropic bacteria *Rhodobacter capsulatus* and *R. sphaeroides* both possess a penta-acylated lipid A in which the amide-linked fatty acids are further functionalized by an oxo group at C-3 and possess secondary fatty acids with a *cis* double bond (Fig. 4.4) (Salimath et al. 1983; Strittmatter et al. 1983). From these two lipid A molecules, a highly potent synthetic antagonistic compound (E5531) was synthesized, which carries ether-linked (instead of ester) chains at lipid A backbone and a further methoxy group at position 6′ (Christ et al. 1995). Among other factors, the grade of acylation and the fatty acid distribution between the GlcN units determines the three-dimensional structure, i.e., the conical or cylindrical molecular shape, of lipid A (Brandenburg et al. 1996; Seydel et al. 1993, 2000) which is correlated to its biological activity, i.e., the binding and recognition by proteins of the innate immune systems of both animals and plants (Medzhitov 2001; Akira et al. 2006).

Structural variations, even though rather infrequent, can be found on the GlcN disaccharide backbone as well. One or both GlcN residues can be replaced by a 2,3-diamino-2,3-dideoxy-D-glucopyranose (Zähringer et al. 1994; Raetz and Whitfield 2002; Raetz et al. 2007), whereas, in *Rhizobium etli* CE3, the proximal glucosamine unit is replaced by its aldonic acid derivative, the 2-amino-2-deoxy-gluconate residue (Fig. 4.4) (Bhat et al. 1994; Raetz et al. 2007; De Castro et al. 2008).

4.2.2 Core Oligosaccharide

The core region of LPSs consists of a complex oligosaccharide covalently linked to the lipid A (Holst 1999, 2007). It is also worth emphasizing once more that the core region is, in wild type R-LPS (LOS), the most external part of the molecule, thus playing a key role in the bacterial cell's interaction with its external environment (Fig. 4.2) (Holst 1999, 2007).

The core of LPSs can be built of several monosaccharides which can be arranged to give either a linear or a branched architecture. It is possible to identify an inner and an outer core moiety; the inner core links to lipid A and is composed of archetypal monosaccharides such as heptopyranoses and 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo, Fig. 4.5), whereas the outer core region usually consists of common hexose monosaccharides. In almost all Gram-negative bacteria the linkage

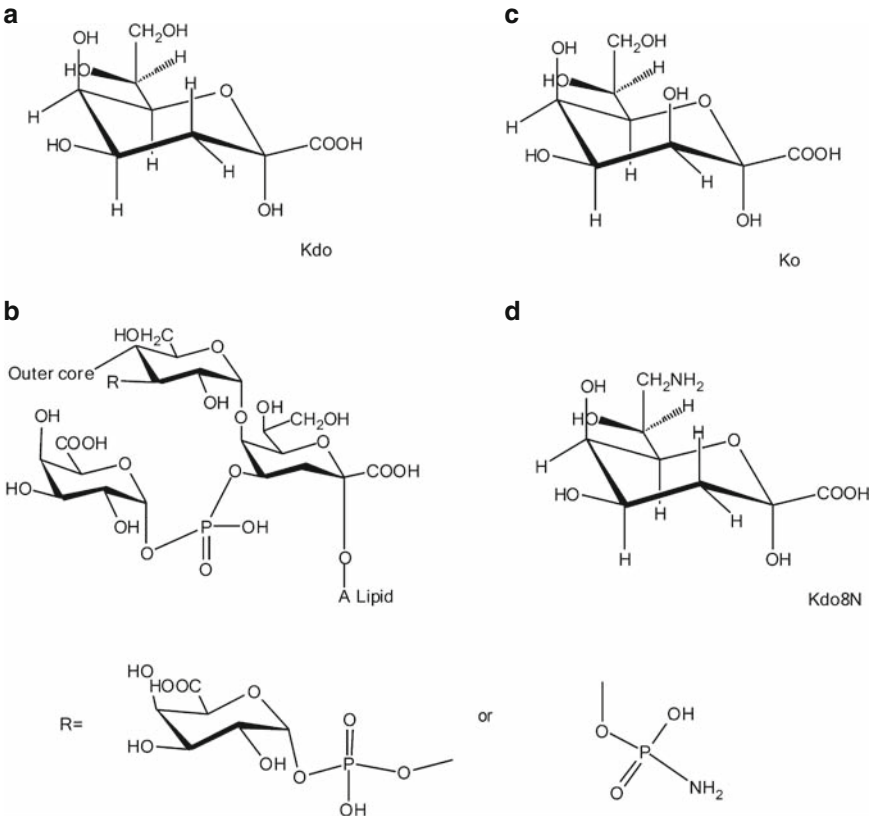

Fig. 4.5 (**a**) the chemical structure of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), the chemical hallmark of LPS from Gram-negative bacteria; (**b**) the inner core region of the LPS from *Xanthomonas campestris* in which is shown the presence of galacturonyl phosphate group and a phosphoramide group; (**c**) the D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko), a Kdo 3-hydroxy derivative frequently present in *Burkholderia* and *Acinetobacter* LPSs – in the latter case it interestingly replaces the Kdo^{I} as attachment point between lipid A and inner core region; and (**d**) the 8-amino-8-deoxy-*manno*-oct-2-ulosonic acid (Kdo8N) that replaces Kdo^{I} in several *Shewanella* LPSs

of core oligosaccharide to lipid A occurs between an α-Kdo residue and the 6′-hydroxymethylene of the $GlcN^{II}$ residue.

In the case of the enterobacterial LPS this first Kdo unit (Kdo^{I}) bears at its O-5 position a heptose trisaccharide fragment: α-L,D-Hep*p*^{III}-(1-7)-α-L,D-Hep*p*^{II}-(1-3)-α-L,D-Hep*p*^{I}; Kdo^{I} also carries another negatively-charged substituent at its O-4 position, generally a further α-Kdo unit/disaccharide or a phosphate group (Holst 1999, 2007). Substantial chemical variations in the Kdo inner core region are reported for both *Xanthomonas campestris* pv. *campestris* (Xcc) and *Arenibacter certesii* LPSs, in which a galacturonyl phosphate substituent is present at O-4 of Kdo^{I} (Silipo et al. 2005b, c); moreover, in Xcc LPS this can be replaced by a

phosphoramide group (Fig. 4.5). This weird chemical function was never described as a component of LPS, and was found only once as a component of a biomolecule (Silipo et al. 2005a, b, c). Interestingly, the phosphoramide moiety is highly sensitive to the acid or alkaline conditions commonly used to work-up LOS and LPS molecules, and therefore it cannot be excluded that this chemical group is more widely present.

Even if Kdo^{I} almost always occurs as the first residue of LPS inner core, there are two main exceptions: *Acinetobacter* (Vinogradov et al. 1997) and a few *Shewanella* strains (Fig. 4.5) (Vinogradov et al. 2003). In the first case, Kdo is replaced by its C-3 hydroxy-derivative, D-*glycero*-D-*talo*-oct-2-ulopyranosic acid (Ko), whereas in the second case it is replaced by its C-8 amino-derivative, 8-amino-8-deoxy-*manno*-oct-2-ulosonic acid (Kdo8N).

Beside lipid A, Ara4N residue is also present in the inner core region of LPSs; in *Proteus* and *Serratia* LPSs it is attached to the Kdo^{II} residue while in *Burkholderia* LPS, it is always present as terminal non-reducing residue of the trisaccharide Ara4N-(1-8)-α-Ko-(2-4)-α-Kdo^{I}. In particular, the finding of Ara4N in such a chemical arrangement in *Burkholderia* LPS represents a chemo-taxonomic marker for this genus, shared by all *Burkholderia* strains, both animal and plant pathogens (Molinaro et al. 2003; Silipo et al. 2005a, b, c). It is speculated that Ara4N, which bears a positively charged free amino group, might play a key role in pathogenesis, since it reduces the net negative charge on the surface of the external membrane, rendering it positively charged or in an isoelectric state. In turn, this variation confers resistance to antibiotic compounds and host cationic antimicrobial peptides as demonstrated in studies of polymyxin B resistant strains (Trent et al. 2001; Raetz and Whitfield 2002).

As for the heptose region, these residues can be substituted by any carbohydrate residue and by other appendages such as phosphate, pyrophosphate, and PEtN. Such substituents are often present in non-stoichiometric amounts, thus contributing to the core structural heterogeneity. In *P. aeruginosa* inner core LPS, a number of chemical peculiarities were found, such as the presence of the unique carbamoyl phosphate group substituting O-6 of the second heptose and also the presence of a triphosphate group attached to Hep^{I} (Knirel et al. 2006). In a few cases, the biosynthetic precursor of L,D-Hep*p*, D-*glycero*-D-*manno*-heptose (D,D-Hep), can be also found in the outer core region (Holst 1999, 2007).

Some genera, such as *Francisella, Legionella, Acinetobacter, Chlamydia, Moraxella, Rhizobium, Xanthomonas,* and *Agrobacterium*, produce core oligosaccharides devoid of heptoses in which an array of monose residues can sit at O-5 of Kdo^{I}, such as Man (largely), but also Glc, Gal or GalA (Holst 1999, 2007). In the outer core region there are no very typical chemical hallmarks, however in *Acinetobacter*, *Rhizobium,* and *Agrobacterium* strains 3-deoxy-D-*lyxo*-hept-2-ulosaric acid has been found (Vinogradov et al. 1997; Russa et al. 1991; De Castro et al. 2008), whereas in *Proteus* LPS and subsequently in *Shewanella* LPS a new kind of glycosydic linkage was discovered that involves an open chain acetal linkage of a GlcN residue that is present as the non-cyclic carbonyl form (Vinogradov and Bock 1999; Vinogradov et al. 2003).

4.2.3 O-chain Polysaccharide

The O-chain polysaccharides, which typify smooth-type Gram-negative bacteria, are usually characterized by regular polysaccharide with repeating units consisting of up to eight monosaccharides. The regularity of the majority of polymers is caused by their biosynthesis; in fact, the oligosaccharide repeating unit is typically pre-assembled and then added to a growing chain polymer (Raetz and Whitfield 2002). The size heterogeneity due to their different degrees of polymerization is responsible for the characteristic ladder appearance of the electrophoretic profiles of S-LPSs that is absent in the case of rough-type R-LPSs. In the mammalian immune system, the O-chain polymers are the target of the adaptive immune system, which is the reason they are called O-antigen polysaccharides, and their reaction with specific antibodies is the basis for serotype classification among the different bacterial species. From the structural point of view, the O-chains are the most variable part of LPSs; their huge variety is determined first of all by the large collection of monosaccharides constituting the repeating unit. Over a hundred types of monosaccharides have been found in the O-chain polysaccharides, and, in several cases, possess a very peculiar chemical structure (Jansson 1999). Moreover, a plethora of non-carbohydrate substituents can be present as further chemical decorations that enormously increase the potential chemical variety of the O-chain structures. These decorations, which can also be present in non-stoichiometric amounts, range from acetate, sulfate, and phosphate groups to aminoacid, hydroxyl-acid, and acid residues (Jansson 1999; Stenutz et al. 2006). The function of these further substitutions on the sugar rings can be very case-specific and in some cases is still unknown, even when the hypothesis of a chemical camouflage useful for escaping host detection is considered (Jansson 1999; Whitfield 2006).

Remarkable examples of interesting and weird chemical structures are, in our opinion, the C_{12} branched-chain sugar caryophyllose which has been characterized as 3,6,10-trideoxy-4-*C*-[(*R*)-1-hydroxy-ethyl]-D-*erythro*-D-*gulo*-decose, or the caryose, a C_9 sugar, the only carbocyclic sugar found in nature so far, named as 4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose (Fig. 4.6). They were both fully identified in our laboratories as components of two homopolymeric O-chain polysaccharides from *Burkholderia caryophylli* LPS, a phytopathogenic bacterium responsible for wilting of carnations (Adinolfi et al. 1995, 1996). Both these polysaccharides, built up of these exceptional sugar residues, are further complicated by an almost stochastic and very complex acetylation pattern (Fig. 4.6) (Molinaro et al. 2000).

Amino sugars, diamino sugars and their 6-deoxy derivatives, uronic acids, and aminouronic acids, in addition to the neutral hexoses, pentoses, heptoses, and their deoxy derivatives, have been found in the O-chain polysaccharides. Both pyranose and furanose rings, anomeric and absolute configurations have been reported (Jansson 1999). Even though some monosaccharides can be found more frequently in the same bacterial genera, no chemotype classification based on the O-chain monosaccharide composition has been created. However, a very high frequency of the rhamnose residue in the O-chain backbones of plant pathogen bacterial LPSs,

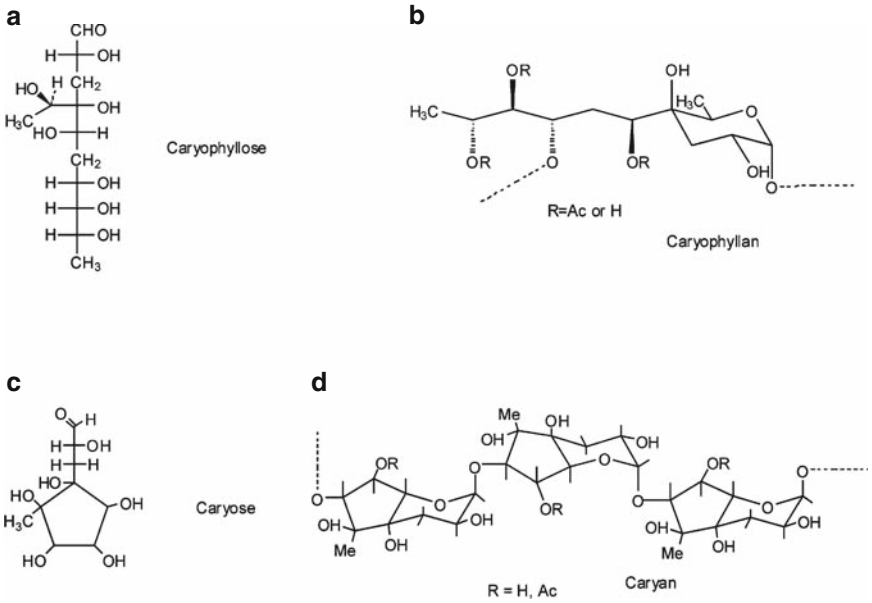

Fig. 4.6 The chemical structure of caryophyllose and caryose. (**a**) Caryophyllose, 3,6,10-trideoxy-4-*C*-[(*R*)-1-hydroxy-ethyl]-D-*erythro*-D-*gulo*-decose, is a twelve carbon atom sugar, branched at C-4 with a six-carbon lateral chain. It constitutes the homopolymer caryophyllan (**b**) one of the two O-chain polysaccharides from *Burkholderia caryophylli* – the polymer is non stoichiometrically acetylated; (**c**) Caryose monosaccharide, 4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose, is a carbocyclic monosaccharide, the only one found in nature to date. It constitutes the homopolymer caryan (**d**) the second O-chain polysaccharide from *B. caryophylli* LPS in which caryose is present as bicyclic form and is acetylated blockwise to yield a very long repeating unit of 19 residues

together with a limited assortment of the other monosaccharides, has been reported and seems to be a peculiarity of most phytobacteria (Corsaro et al. 2001).

A different masking strategy is adopted by some human pathogenic bacteria such as *Campylobacter*, *Helicobacter*, *Haemophilus*, *Neisseria* and other related genera which mimic animal surface glycolipids and glycoproteins. In fact, the terminal oligosaccharide structures of their LPS O-chains or core regions possess the same carbohydrate oligosaccharides antigen present in host gangliosides, glycosphingolipids or blood groups (Moran et al. 1996; Estabrook et al. 1997; Holst 1999, 2007; Moran 2008). This mimicry contributes to increased resistance of the particular bacterial species or strain to host immune defense mechanisms, including opsonization and cell damage.

4.2.4 Further Reading

A complete overview of LPS function, structure, biosynthesis, and medicine up to 1999 is presented in the book "Endotoxin in health and disease" edited by Brade, H.,

Morrison, D. C., Opal, S., and Vogel, S., Marcel Dekker Inc., New York. We also suggest other related review articles by Raetz (2002, 2007), that are true keystones and contain all the necessary information and further references on the subject of LPSs, especially in the section on mammalian/animal pathogens.

We also have recently published two review articles on the structures of LPSs from plant-associated bacteria (Corsaro et al. 2001; De Castro et al. 2008) and another on the structure–function relationship of LPSs and plant defense response (Newman et al. 2007).

4.3 Structural Investigation of Glycolipids (Lipopolysaccharides or Lipo-oligosaccharides)

Lipopolysaccharides (or LOSs) may be extracted from intact cells by a variety of procedures, but nowadays those commonly used exploit the phenol/chloroform/light ether and/or phenol/water extractions (Galanos et al. 1969; Westphal and Jahn 1965). Depending on the polarity of the glycolipid, it can be isolated from the aqueous, phenol or organic phase.

Enzymatic treatments to remove proteins and nucleic acids and chloroform washings to remove lipid and phospholipid components are usually employed. The amphiphilic nature of these molecules is responsible for their low solubility in both aqueous and organic solvents and renders purification by any chromatographic technique very hard. Electrophoretic analysis using a denaturing agent is highly informative and allows ascertaining of the typology of the extracted material: a ladder-like profile is diagnostic of LPS molecules whereas the presence of material at the bottom of the gel profile is related to LOS material. This information is valuable and affects the subsequent procedures for the structural investigation. However, in both cases, the study of the lipid A moiety is carried out after its cleavage from the rest of the molecule by mild acid hydrolysis of the acid-labile glycosydic linkage of Kdo. During this treatment, the lipid A part precipitates whereas the saccharide moiety remains in solution in the supernatant.

The primary structure determination of very complex molecules such as the LPSs (LOSs) currently exploits state-of-the-art 1D and 2D NMR experiments and soft-ionization mass spectrometry techniques, together with compositional and linkage chemical analyses, usually carried out on intact products (when possible) and *ad hoc* selectively degraded derivatives.

4.3.1 Lipid A Structure Elucidation

The lipid fraction obtained by precipitation after mild acid treatment of LPS (or LOS) is composed of a mixture of intrinsically heterogeneous lipid A molecules.

The structural approach makes extensive use of mass spectrometry analysis by MALDI-TOF and ESI MS techniques on both the native and selectively degraded lipid A fractions. The MALDI-TOF and ESI-MS data allow insights to be gained into the number of lipid A species present in the fraction, the presence of polar heads, and the distribution of acyl residues on each GlcN units of the disaccharide backbone (Que et al. 2000). This latter issue is of particular importance given that fatty acid composition and distribution strongly affects lipid A/LPS endotoxic activity, i.e., agonist/antagonist activity. Through chemical and spectroscopic study of several lipid As and LPSs in our laboratory, novel general approaches for gaining structural insights into such molecules have been developed. In particular, by combining MALDI-MS or ESI-MS and selective chemical degradation by ammonium hydroxide hydrolysis, we have developed a general and simple methodology for obtaining the secondary fatty acid distribution, which is one of most demanding issues in the structural determination of lipid A (Silipo et al. 2002; Sforza et al. 2004; Sturiale et al. 2005). These approaches must be supported by classical chemical analyses of lipid A, which imply the removal of the ester-linked fatty acid moiety by mild alkaline treatment, or complete removal by strong alkaline treatment and GLC-MS analyses of fatty acids as methyl ester derivatives (Rietschel 1976; Zähringer et al. 1994)

NMR investigation is less used but equally important (Ribeiro et al. 1999; Brecker 2003; Silipo et al. 2004). The amphiphilic nature of lipid A entails a very poor solubility of the sample in whichever solvent system is used; however, when possible it allows one to obtain diagnostic information mainly about the saccharide backbone, i.e., the anomeric configuration of the carbohydrate residues and their sequence, and the occurrence and position of the polar heads.

4.3.2 O-chain Polysaccharide Structure

The O-chain of LPSs, recovered in the supernatant after mild acid treatment, usually consists of a regular long-chain polysaccharide with the core oligosaccharide still at the reducing end of the chain; however, since this oligosaccharide moiety represents a small percentage of the whole polymer, it is generally barely detectable from the chemical and spectroscopic point of view. The O-chain structure determination aims to define the composition of monosaccharides, the relative, the anomeric and the absolute configurations of each monosaccharide, their ring sizes, the linkage of each residue and their sequence within the repeating unit. In some cases, the non-stoichiometric presence of some substituents can determine heterogeneity and hide the structural regularity (Jansson 1999).

Determination of monosaccharide composition is achieved by the classical analytic approaches, consisting of acid hydrolysis and/or methanolysis followed by further chemical modification (such acetylation) to perform GLC-MS analyses. The reaction sequence methylation, acid hydrolysis, reduction and acetylation

is carried out to find the glycoside substitution sites and the ring sizes of each monosaccharide (Ciucanu and Kerek 1984). For the absolute configurations, GLC analysis of convenient glycoside derivatives is usually exploited (Leontein and Lönngren 1978). The anomeric configurations and the sequence of the monosaccharide residues are determined by 1D and 2D ^{1}H and ^{13}C-NMR experiments (COSY, TOCSY, NOESY, ROESY, HSQC, HMBC). The complete assignment of all proton and carbon signals confirms the monosaccharide identification as well.

4.3.3 Core Oligosaccharide Structure

The target of the structural elucidation of a LOS core oligosaccharide is generally much more complex than that of the O-chain because of the higher monosaccharide number, commonly up to 15, with respect to the components of one O-chain repeating unit, and the presence of labile groups and other substituent that are often present in non-stoichiometric amounts. In addition, the acid hydrolysis used to remove the lipid A from LOS occurs by hydrolysis of Kdo that in its reducing form usually appears in different anomeric and cyclic forms, thus causing an "artificial" increase in the core heterogeneity. Therefore, the LOS sample is generally treated under alkaline conditions that do not cleave the Kdo linkage and allow study of the complete sugar backbone including the two GlcN of lipid A. In particular, the LOS sample is first treated under mild alkaline conditions in order to selectively remove the lipid A ester-bound fatty acids. This more soluble *O*-deacylated LOS is *N*-deacylated under stronger alkaline conditions, affording completely deacylated backbone oligosaccharide, which represents the intact sugar skeleton of LOS suitable for sequence determination by NMR analysis (Holst 2000). The direct strong de-*O*- and de-*N*-acylation is usually not performed in order to avoid the formation of alkali-resistant unsaturated *N*-linked fatty acids. In order to assign phosphate groups and their possible further attached substituents, 1D and 2D ^{31}P-NMR experiments are carried out as well.

Since alkaline treatment frequently causes the loss of base-labile functions, the oligosaccharide moiety released under acid conditions is also studied as a complimentary and informative approach. More recently, the full NMR study of the *O*-deacylated sample has been carried out in an aqueous solution containing a denaturing agent (Vinogradov et al. 2003). Every step of the selective degradation is usually accompanied by MALDI MS spectrometry analysis, which is a very valuable and complementary approach in the study of such molecules. Our collaborators have also contributed strongly to new MALDI MS approaches by developing new methods of preparation and execution of the experiments. It is worth underlining that such experiments carried out on the intact sample are of very high value since they yield a "picture" of the intact endotoxin without any loss of structural information (Sturiale et al. 2005).

4.4 Endotoxins as Elicitors of Mammalian Innate Immune System

The endotoxic properties of LPS principally reside in the lipid A component, the primary immunostimulator that acts as a strong elicitor of the innate immune system by the induction of inflammatory cytokines in mammalian cells (Zähringer et al. 1994; Medzhitov 2001; Beutler and Rietschel 2003; Akira et al. 2006). Mammals respond to microbial invasion with innate and adaptive immune responses. The adaptive immune system is triggered by the innate immune system, which is evolutionarily ancient and is common to mammals, insects and plants (eukaryotic organisms) which, through this non-specific and universal response, detect the presence of an organism regardless of its pathogenic properties. Cells of the immune system, including macrophages and monocytes, constitutively express or secrete the receptor targets of the antimicrobial compounds. The Toll-like receptor (TLR) family is a class of trans-membrane receptors with a central role in the induction of several antimicrobial responses and in the control of the adaptive immune system. In mammalian species, there are at least ten TLRs, each having a distinct role, namely a different ligand specificity (Medzhitov 2001; Akira et al. 2006). The primary and essential requisite of TLRs in the activation of the innate immune response is the detection of microbial components that are constitutively expressed, highly conserved, and invariant through different species, i.e., essential for their survival. Such molecular targets are called Pathogen Associated Molecular Patterns (PAMPs). The receptors that recognize PAMPs are called Pattern Recognition Receptors (PRR). The recognition of those PAMPs, produced only by microbes, allows to the host to unequivocally signal the presence of infection. Lipopolysaccharides, lipoproteins, and peptidoglycans are motifs not present in eukaryotic cells; they are all molecular signatures for a given pathogen class (Medzhitov 2001; Akira et al. 2006). TLR4 is the signal-transducing receptor of LPSs; lipid A is the real effector required to activate the TLR4 signaling pathway in conjunction with a soluble co-receptor protein MD2, which directly and physically binds to LPS (Fig. 4.7) (Poltorak et al. 2000; Ohto et al. 2007). The activation of the TLR4–MD2 complex triggers the induction of inflammatory cytokines such as TNF-α, IL-1, and IL-6, acting as endogenous mediators of the infection, as well as the superoxide anion (O_2^-), hydroxyl radicals ($^{\cdot}OH$), nitric oxide, and antimicrobial peptides. The low and balanced concentrations of these mediators and soluble immune response modulators leads to a resulting inflammation that is one of the most important and ubiquitous aspects of the immune host defense against invading microorganisms. Beside these positive effects, an uncontrolled and massive immune response, due to the circulation of large amount of endotoxins, leads to symptoms of sepsis and of septic shock.

The toxicity of lipid A is strongly influenced by its primary structure, whose chemical variations strongly influence their capacity to interact and activate receptors of the immune system. A number of factors influence the biological activity of lipid A. Its possession of a *bis*-phosphorylated disaccharide backbone with an

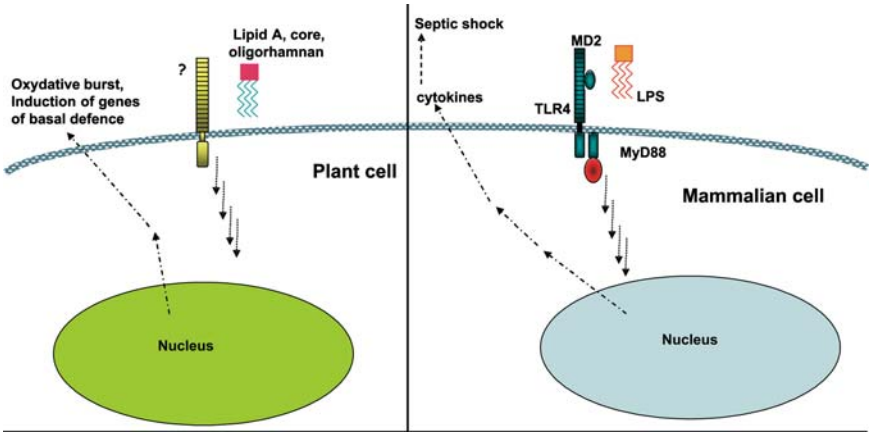

Fig. 4.7 Schematic picture of animal and plant recognition of bacterial LPSs. In animal cells (*right-hand side*), the surface-associated Toll-like receptor protein TLR4 perceives the pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). These leucine-rich repeat (LRR) proteins have a cytoplasmic Toll-Interleukin-1 receptor domain (TIR) that initiates signal transduction by MyD88 protein or other effectors. In plant cells (*left-hand side*), even though it is known that either LPS lipid A or LPS core region are recognized and the signal is transduced, the receptor(s) for this molecule are not known. However, receptor-like kinases (RLKs) recognize a diverse range of signals including bacterial components flagellin (through *Arabidopsis* FLS2) and the EF-Tu elongation factor (through *Arabidopsis* EFR) These RLK proteins resemble the animal TLR receptors except that they have an intracellular serine/threonine kinase domain, rather than a TIR domain

asymmetric (4 + 2) distribution of six acyl residues represents the most agonist structure for innate immunity in human (Zähringer et al. 1994; Raetz and Whitfield 2002; Beutler and Rietschel 2003), such as the classical case of the lipid A of *E. coli* LPS (Fig. 4.3). Different primary structures with respect to this latter lipid A are less or not agonistically active. The major determinants that influence the toxicity are the number and the distribution of acyl chains, the phosphorylation pattern, and the presence of charged groups on the polar heads (Raetz et al. 2007). Increasing acylation of lipid A correlates with a greater cytokine induction capacity, as previously reported in several bacterial LPSs (Munford and Varley 2006), with hexa-acylated species being the most active ones. In this context, lipid A species with low or absent endotoxic activity have been identified and operate as "antagonists," reducing or, in a dose-dependent manner, completely inhibiting the cytokine production otherwise induced by strongly endotoxic lipid A species. It has been proposed that the inhibition of the immune cell occurs in a competitive way: the antagonist lipid A is able to compete with toxic species for the interaction with the receptor complex on the immune cells but does not exert the necessary stress for activation. The variation of the primary structures of lipid A obviously dictates its physicochemical and biological behavior and likewise the structure of their supramolecular aggregates. The classical antagonistic lipid A in human cells is the tetra-acyl lipid IV_a structure: it is the biosynthetic precursor of the agonistic

E. coli hexa-acylated lipid A and differs from this last for the absence of both secondary fatty acids (Zähringer et al. 1994; Raetz and Whitfield 2002; Beutler and Rietschel 2003). Strong antagonistic effects have also been demonstrated for the penta-acylated LPS lipid A from two phototropic species, *Rhodobacter sphaeroides* and *R. capsulatus,* and their chemical derivatives (Fig. 4.4) (Christ et al. 1995). It has been demonstrated that the intrinsic conformation of lipid A is responsible for its agonistic and antagonistic activity (Brandenburg et al. 1996; Seydel et al. 1993, 2000).

Lipid A's capacity to stimulate the innate immune response is beneficial for the host, because it leads to a complex series of events that culminate in the priming of the adaptive immune system and thence to resistance to the infection. A substantial step further has been the discovery of monophosphoryl lipid A as vaccine adjuvant, this compound has a poor toxicity and has proven to be safe and effective (Mata-Haro et al. 2007; Fitzgerald and Golenbock 2007).

4.5 Endotoxins as Elicitors of Plant Innate Immune System

The mechanisms of plants' interaction with invading pathogens display parallels with animals (Zipfel and Felix 2005; Jones and Dangl 2006; Newman et al. 2007). Plants have evolved and maintained competence to recognize several general elicitors that are pathogen surface molecules and which can be considered as PAMPs. They bind to plant PRR and trigger the expression of immune response genes and the production of antimicrobial compounds. LPSs are surface compounds that also act as general elicitors of plant innate immunity and can be considered as PAMPs (Dow et al. 2000; Newman et al. 2002, 2007). The activation of innate responses upon recognition of PAMPs recalls the mechanisms of activation of the innate responses in mammals and insects (Fig. 4.7) (Medzhitov 2001). The delivery of an invading bacterium triggers the activation of a signal transduction pathway that can lead to the Hypersensitive Response (HR). HR is a programmed cell death response, triggered by live bacteria, that is associated with plant host resistance. It is associated with a decline in the number of viable bacteria recovered in the tissue and follows a rapid necrosis of plant tissue representing the final stage of resistance, when stress signals induce strong defensive responses. One of the most widely studied effects of LPSs on plant cells is their ability to retard or completely block the HR induced by avirulent bacteria. Pre-inoculation of leaves with heat-killed bacterial cells delays or prevents the disease symptoms expected with a subsequent inoculation with living bacteria (Dow et al. 2000; Newman et al. 2002).

Recent findings have shed light on the molecular aspect of these biological events. The lipid A moiety may be at least partially responsible for LPS perception by *Arabidopsis thaliana* leading to a rapid burst of NO, a hallmark of innate immunity in animals (Zeidler et al. 2004). The minimal structural requirements for the elicitor activity can be different from that in mammalian hosts. In the case of LOS, both lipid A and core oligosaccharide from a pathogenic bacterium can be

recognized by plant receptors (Silipo et al. 2005a, b, c) and are potent inducers of immune responses. Using synthetic O-antigen polysaccharides (oligorhamnans) it has been shown that the O-chain of LPS is recognized by *Arabidopsis*, and that this recognition leads to elicitation of a specific gene transcription response associated with defense (Bedini et al. 2005). A single pathogen-associated compound can consequently originate multiple signals, indicating the existence of multiple receptors of several general elicitors. Intact LOS, the lipid A and core oligosaccharide derived from *X. campestris* pv. *campestris* are all able to induce defense-related genes *PR1* and *PR2* in *Arabidopsis* and to prevent the HR caused by avirulent bacteria; furthermore, these defensive cellular responses occur via a distinctive recognition event that occurs in different temporal phases (Silipo et al. 2005a, b, c). As in the case of mammalian pathogen bacteria, structural variation in the lipid A region can strongly influence the ability of LPS to induce defense-related genes and then to be recognized as PAMPs (Silipo et al. 2008). The alteration of the acylation pattern and the modification of the polar heads seem to play a central role. Thus far, the receptor for LPS in plant cells has not been identified and cloning and characterization of LPS receptors in plants is currently a major goal in this area.

4.6 Conclusions

Lipopolysaccharides represent the most abundant type of molecules involved in the social behavior of Gram-negative bacteria, in addition to being essential for bacterial viability. In fact, they are engaged in the early mechanisms of host recognition and adhesion which then determine the cascade of immunological or tolerance responses which are important for both host resistance and colonization. The knowledge of the structure of the single LPS components, O-chain, core, and lipid A, remains one of the essential goals for in-depth comprehension of their biological role, which could provide a spin-off for the fields of both medicine and agriculture.

References

Adinolfi A, Corsaro MM, De Castro C, Evidente A, Lanzetta R, Mangoni L, Parrilli M (1995) Analysis of the polysaccharide components of the lipopolysaccharide fraction of *Pseudomonas caryophylli*. Carbohydr Res 274:223–232

Adinolfi A, Corsaro MM, De Castro C, Evidente A, Lanzetta R, Molinaro A, Parrilli M (1996) Caryose: a carbocyclic monosaccharide from *Pseudomonas caryophylli*. Carbohydr Res 284:111–118

Akira S, Uematsu S, Osamu T (2006) Pathogen recognition and innate immunity. Cell 124:783–801

Basu SS, Karbarz MJ, Raetz CHR (2002) Expression cloning and characterization of the C28 acyltransferase of Lipid A biosynthesis in *Rhizobium leguminosarum*. J Biol Chem 277:28959–28971

Bedini E, De Castro C, Erbs G, Mangoni L, Dow JM, Newman MA, Parrilli M, Unverzagt C (2005) Structure-dependent modulation of a pathogen response in plants by synthetic O-antigen polysaccharides. J Am Chem Soc 127:2414–2416

Beutler B, Rietschel ETh (2003) Innate immune sensing and its roots: the story of endotoxin. Nature Rev Immunol 3:169–176

Bhat UR, Carlson RW, Busch M, Mayer H (1991) Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipopolysaccharides from bacteria belonging to the alpha-2 subgroup of Proteobacteria. Int J Syst Bacteriol 41:213–217

Bhat UR, Forsberg LS, Carlson RW (1994) Structure of lipid A component of *Rhizobium leguminosarum* pv. *phaseoli* lipopolysaccharide. Unique nonphosphorylated lipid A containing 2-amino-2-deoxygluconate, galacturonate, and glucosamine. J Biol Chem 269:14402–14410

Brandenburg K, Seydel U, Schromm AB, Loppnow H, Koch MHJ (1996) Rietschel ETh Conformation of lipid A, the endotoxic center of bacterial lipopolysaccharides. J Endotoxin Res 3:173–178

Brecker L (2003) Nuclear magnetic resonance of lipid A: the influence of solvents on spin relaxation and spectral quality. Chem Phys Lipids 125:27–39

Christ WJ, Asano O, Robidoux AL, Perez M, Wang Y, Dubuc GR, Gavin WE, Hawkins LD, McGuinness PD, Mullarkey MA, Lewis MD, Kishi Y, Kawata T, Bristol JR, Rose JR, Rossignol DP, Kobayashi S, Hishinuma I, Kimura A, Asakawa N, Katayama K, Yamatsu I (1995) E5531, a pure endotoxin antagonist of high potency. Science 268:80–83

Ciucanu I, Kerek F (1984) A simple method for the permethylation of carbohydrates. Carbohydr Res 131:209–217

Corsaro MM, De Castro C, Molinaro A, Parrilli M (2001) Structure of lipopolysaccharides from phytopathogenic bacteria. Recent Research Developments in Phytochemistry 5:119–138

De Castro C, Molinaro A, Lanzetta R, Silipo A, Parrilli M (2008) Lipopolysaccharide structures from *Agrobacterium* and other *Rhizobiaceae* species. Carbohydr Res 343(12):1924–1933

Dow JM, Newman M-A, von Roepenack E (2000) The induction and modulation of plant defence responses by bacterial lipopolysaccharides. Annu Rev Phytopathol 38:241–261

Estabrook MM, Griffiss JM, Jarvis GA (1997) Sialylation of *Neisseria meningitidis* lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect Immun 65:4436–4444

Fitzgerald KA, Golenbock DT (2007) The shape of things to come. Science 316:1574–1576

Galanos C, Luderitz O, Westphal O (1969) A new method for the extraction of R lipopolysaccharides. Eur J Biochem 9:245–249

Holst O (1999) Chemical structure of the core region of lipopolysaccharides. In: Brade H, Morrison DC, Opal S, Vogel S (eds) Endotoxin in health and disease. Marcel Dekker, New York, pp 115–154

Holst O (2000) Methods in molecular biology, bacterial toxins: methods and protocols. Humana Press, Totowa, NJ, pp 345–353

Holst O (2007) The structures of core regions from enterobacterial lipopolysaccharides - an update. FEMS Microbiol Lett 1:3–11

Jansson P-E (1999) The chemistry of O-polysaccharide chains in bacterial lipopolysaccharides. In: Brade H, Morrison DC, Opal S, Vogel S (eds) Endotoxin in health and disease. Marcel Dekker Inc, New York, pp 155–178

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Knirel YA, Bystrova OV, Kocharova NA, Zähringer U, Pier GB (2006) Conserved and variable structural features in the lipopolysaccharide of *Pseudomonas aeruginosa*. J Endotoxin Res 12:324–3036

Leontein K, Lönngren J (1978) Determination of the absolute configuration of sugars by Gas-Liquid Chromatography of their acetylated 2-octyl glycosides. Methods Carbohydr Chem 62:359–362

Mata-Haro V, Cekic C, Chilton MM, PM CCR, Mitchell TC (2007) The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR. Science 316:1628–1632

Medzhitov R (2001) Toll-like receptors and innate immunity. Nature Rev Immunol 1:135–145
Meredith TC, Mamat U, Kaczynski Z, Lindner B, Holst O, Woodard RW (2007) Modification of lipopolysaccharide with colanic acid (M-antigen) repeats in *Escherichia coli*. J Biol Chem 282:7790–7798
Molinaro A, De Castro C, Petersen BO, Duus JO, Parrilli M, Holst O (2000) Acetyl substitution of the O-specific caryan from the lipopolysaccharide of *Pseudomonas* (*Burkholderia*) *caryophylli* leads to a block pattern. Angew Chem Int Ed 39:156–160
Molinaro A, Lindner B, De Castro C, Nolting B, Silipo A, Lanzetta R, Parrilli M, Holst O (2003) The structure of the lipid A of the lipopolysaccharide from *Burkholderia caryophylli* possessing a 4-amino-4-deoxy-L-arabinopyranose-1-phosphate residue exclusively in glycosidic linkage. Chem Eur J 9:1542–1548
Moran AP (2008) Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen *Helicobacter pilori*. Carbohydr Res 343(12):1952–1965
Moran AP, Prendergast MM, Appelmelk BJ (1996) Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. FEMS Immunol Med Microbiol 16:105–115
Munford SR, Varley AW (2006) Shield as signal: lipopolysaccharides and the evolution of immunity to Gram-negative bacteria. PLOS Pathogens 2:467–471
Newman M-A, von Roepenack-Lahaye E, Parr A, Daniels MJ, Dow JM (2002) Prior exposure to lipopolysaccharide potentiates expression of plant defences in response to bacteria. Plant J 29:485–497
Newman M-A, Dow JM, Molinaro A, Parrilli M (2007) Priming, induction and modulation of plant defence responses by bacterial lipopolysaccharides. J Endotox Res 13:69–84
Ohto U, Fukase K, Miyake K, Satow Y (2007) Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science 316:1632–1634
Plötz BM, Lindner B, Stetter KO, Holst O (2000) Characterization of a novel lipid A containing D-galacturonic acid that replaces phosphate residues. The structure of the lipid A of the lipopolysaccharide from the hyperthermophilic bacterium *Aquifex pyrophilus*. J Biol Chem 275:11222–11228
Poltorak A, Ricciardi-Castagnoli P, Citterio S, Beutler B (2000) Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. Proc Natl Acad Sci USA 97:2163–2167
Que NLS, Lin SH, Cotter RJ, Raetz CHR (2000) Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*. J Biol Chem 275:28006–28016
Raetz CRH, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71:635–700
Raetz CRH, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76:295–329
Ribeiro AA, Zhou Z, Raetz CRH (1999) Multi-dimensional NMR structural analyses of purified Lipid X and Lipid A (endotoxin). Magn Reson Chem 37:620–630
Rietschel ETh (1976) Absolute configuration of 3-hydroxy fatty acids present in lipopolysaccharides from various bacterial groups. Eur J Biochem 64:423–428
Rietschel ETh, Westphal O (1999) Endotoxins: historical perspectives. In: Brade H, Morrison DC, Opal S, Vogel S (eds) Endotoxin in health and disease. Marcel Dekker, New York, pp 1–30
Russa R, Urbanik-Sypniewska T, Choma A, Mayer H (1991) Identification of 3-deoxy-*lyxo*-2-heptulosaric acid in the core region of the lipopolysaccharides from *Rhizobiaceae*. FEMS Microbiol Lett 84:337–344
Salimath PV, Weckesser J, Strittmatter W, Mayer H (1983) Structural studies on the non-toxic lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023. Eur J Biochem 136:95–200
Schwudke D, Linscheid M, Strauch E, Appel B, Zahringer U, Moll H, Muller M, Brecker L, Gronow S, Lindner B (2003) The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing alpha-D-Mannoses that replace phosphate residues: similarities and differences between the lipid As and the lipopolysaccharides of the wild type strain *B. bacteriovorus* HD100 and its host-independent derivative HI100. J Biol Chem 278: 27502–27512

Seydel U, Labischinski H, Kastowsky M, Brandenburg K (1993) Phase behaviour, supramolecular structure, and molecular conformation of lipopolysaccharide. Immunobiology 187:191–211

Seydel U, Oikawa M, Fukase K, Kusumoto S, Brandenburg K (2000) Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. Eur J Biochem 267:3032–3039

Sforza S, Silipo A, Molinaro A, Marchelli R, Parrilli M, Lanzetta R (2004) Determination of fatty acid positions in native lipid A by positive and negative electrospray ionization mass spectrometry. J Mass Spectrom 39:378–383

Silipo A, Lanzetta R, Amoresano A, Parrilli M, Molinaro A (2002) Ammonium hydroxide hydrolysis: a valuable support in the MALDI-TOF mass spectrometry analysis of Lipid A fatty acid distribution. J Lipid Res 43:2188–2195

Silipo A, De Castro C, Lanzetta R, Molinaro A, Parrilli M (2004) Full structural characterization of the lipid A components from the *Agrobacterium tumefaciens* strain C58 lipopolysaccharide fraction. Glycobiology 14:805–815

Silipo A, Molinaro A, Cescutti P, Bedini E, Rizzo R, Parrilli M, Lanzetta R (2005a) Complete structural characterization of the lipid A fraction of a clinical strain of *B. cepacia* genomovar I lipopolysaccharide. Glycobiology 15:561–570

Silipo A, Molinaro A, Nazarenko EL, Sturiale L, Garozzo D, Gorshkova RP, Nedashkovskaya OI, Lanzetta R, Parrilli M (2005b) Structural characterization of the carbohydrate backbone of the lipooligosaccharide of the marine bacterium *Arenibacter certesii* strain KMM 3941(T). Carbohydr Res 340:2540–2549

Silipo A, Molinaro A, Sturiale L, Dow JM, Erbs G, Lanzetta R, Newman MA, Parrilli M (2005c) The elicitation of plant innate immunity by lipooligosaccharide of *Xanthomonas campestris*. J Biol Chem 280:33660–33668

Silipo A, Sturiale L, Garozzo D, Dow JM, Erbs G, Lanzetta R, Newman MA, Parrilli M, Molinaro A (2008) The acylation and phosphorylation pattern of lipid A strongly influence its ability to trigger the innate immune response in *Arabidopsis*: *Xanthomonas campestris pv. campestris* mutant strain 8530 synthesizes a chemically different LPS that it is not recognized by innate immunity in plants. Chembiochem 9(6):896–904

Stenutz R, Weintraub A, Widmalm G (2006) The structures of *Escherichia coli* O-polysaccharide antigens. FEMS Microbiol Rev 30:382–403

Strittmatter W, Weckesser J, Salimath PV, Galanos C (1983) Non-toxic lipopolysaccharide from *Rhodopseudomonas sphaeroides* ATCC 17023. J Bacteriol 155:153–158

Sturiale L, Garozzo D, Silipo A, Lanzetta R, Parrilli M, Molinaro A (2005) MALDI mass spectrometry of native bacterial lipooligosaccharides. Rapid Commun Mass Spectrom 19:1829–1834

Trent MS, Ribeiro AA, Lin S, Cotter RJ, RH RC (2001) An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. J Biol Chem 276: 43122–43131

Vedam V, Kannenberg E, Haynes-Gann JG, Sherrier DJ, Datta A, Carlson RW (2003) A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. J Bacteriol 185:1841–1850

Vedam V, Kannenberg E, Datta A, Brown D, Haynes-Gann JG, Sherrier DJ, Carlson RW (2006) The pea nodule environment restores the ability of a *Rhizobium leguminosarum* lipopolysaccharide acpXL mutant to add 27-hydroxyoctacosanoic acid to its lipid a. J Bacteriol 188:2126–2133

Vinogradov E, Bock K (1999) The structure of the core part of *Proteus mirabilis* O27 lipopolysaccharide with a new type of glycosydic linkage. Carbohydr Res 319:92–101

Vinogradov EV, Bock K, Petersen BO, Holst O, Brade H (1997) The structure of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter* strain ATCC 17905. Eur J Biochem 243:122–127

Vinogradov E, Korenevsky A, Beveridge TJ (2003) The structure of the rough-type lipopolysaccharide from *Shewanella oneidensis* MR-1, containing 8-amino-8-deoxy-Kdo and an open-chain form of 2-acetamido-2-deoxy-D-galactose. Carbohydr Res 338:1991–1997

Westphal O, Jahn K (1965) Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. Methods Carbohydr Chem 5:83–91

Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem 75:39–68

Zähringer U, Lindner B, Rietschel ETh (1994) Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. Adv Carbohydr Chem Biochem 50:211–276

Zeidler D, Zähringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J (2004) Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc Natl Acad Sci USA 101:15811–15816

Zipfel C, Felix G (2005) Plants and animals: a different taste for microbes. Curr Opin Plant Pathol 8:353–360

Chapter 5
Structure, Biosynthesis, and Function of Teichoic Acids and Related Cell Wall Glycopolymers in the Gram-positive Cell Envelope

Maren Rautenberg, Thomas Kohler, Guoqing Xia, Emir Kulauzovic, and Andreas Peschel

5.1 Introduction

The cell wall of most Gram-positive bacteria contains two types of structural components (Fig. 5.1). Peptidoglycan (PG), which is a highly conserved component, represents the first and almost omnipresent polymer. The second is represented by additional cell wall glycopolymers (CWGs), which are very variable between species and even between individual strains. Peptidoglycan has the well-studied function of imparting mechanical stability on the bacterial cell, whereas the function of the CWGs is still elusive. Most Gram-positive bacteria contain two types of CWGs: (1) the covalently PG-anchored polymers (P-CWG) and (2) membrane glycolipid-anchored polymers (M-CWG) (Fischer 1988; Neuhaus and Baddiley 2003; Weidenmaier and Peschel 2008). There are bacterial species such as *Bacillus subtilis* that even contain three or four different types of CWGs. Cell wall glycopolymers are composed of repeating units formed by one or more sugar building blocks and, in many cases, additional non-sugar residues such as phosphate, alanine, succinate, pyruvate, choline, or mycolic acides, to name but a few. If the polymer backbone contains diester-linked phosphate groups the CWG is usually named teichoic acid (TA), according to James Baddiley's nomenclature from the 1950s (Armstrong et al. 1959). Most TA exhibit zwitterionic properties because of the negatively charged phosphate groups and additional D-alanine residues on the repeating units, which have free positively charged amino groups. Some Gram-positive bacteria produce CWGs without phosphate groups; hence their polymers are uncharged as in many actinobacteria or certain bacilli, or they are anionic because their repeating units contain uronic acid, pyruvyl, or succinyl groups (Delmas et al. 1997; Greenberg et al. 1996; Powell et al. 1975; Schäffer and

A. Peschel (✉)
Cellular and Molecular Microbiology Section, Medical Microbiology Department, University of Tübingen, 72076 Tübingen, Germany
e-mail: andreas.peschel@uni-tuebingen.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_5,

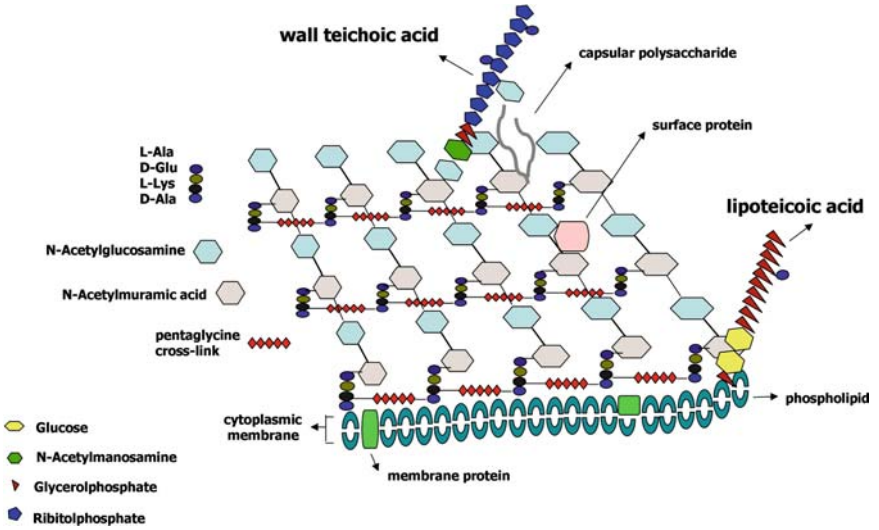

Fig. 5.1 Molecular structure of the cell wall of *S. aureus,* composed of peptidoglycan, teichoic acids, proteins, and capsular polysaccharide. With permission (Kohler et al. 2009b)

Messner 2005; Soldo et al. 1999; Ward 1981). This review will focus on TA-like CWGs with special emphasis on TA structures, biosynthetic pathways, and functions. Other types of CWGs will be mentioned briefly.

5.2 Teichoic Acid Structures

5.2.1 Wall Teichoic Acid and Other Peptidoglycan-Anchored Polymers

Peptidoglycan-anchored polymers are usually attached to PG via more or less conserved linkage units. In most cases these linkage units start with *N*-acetylglucosamine (GlcNAc), which is linked to the *N*-acetylmuramic acid of the PG via a phosphodiester bond. The GlcNAc can be connected to further sugars of the linkage unit or it is connected directly to the CWG (Araki and Ito 1989; Naumova and Shashkov 1997). Up to 50% of the entire cell wall mass is formed by TA of the P-CWG type, which are referred to as wall teichoic acids (WTA) (Fig. 5.2). Since WTA composition is eminently diverse its structure differs profoundly between species or even strains (Endl et al. 1983; Fischer et al. 1993; Naumova and Shashkov 1997; Potekhina et al. 1993). The WTA of *Staphylococcus aureus* is particularly well studied. The linkage unit consists of one GlcNAc residue, one *N*-acetylmannosamine and two glycerolphosphate (Gro-P) residues. The WTA repeating units contain sugars of various sizes ranging from trioses to hexoses.

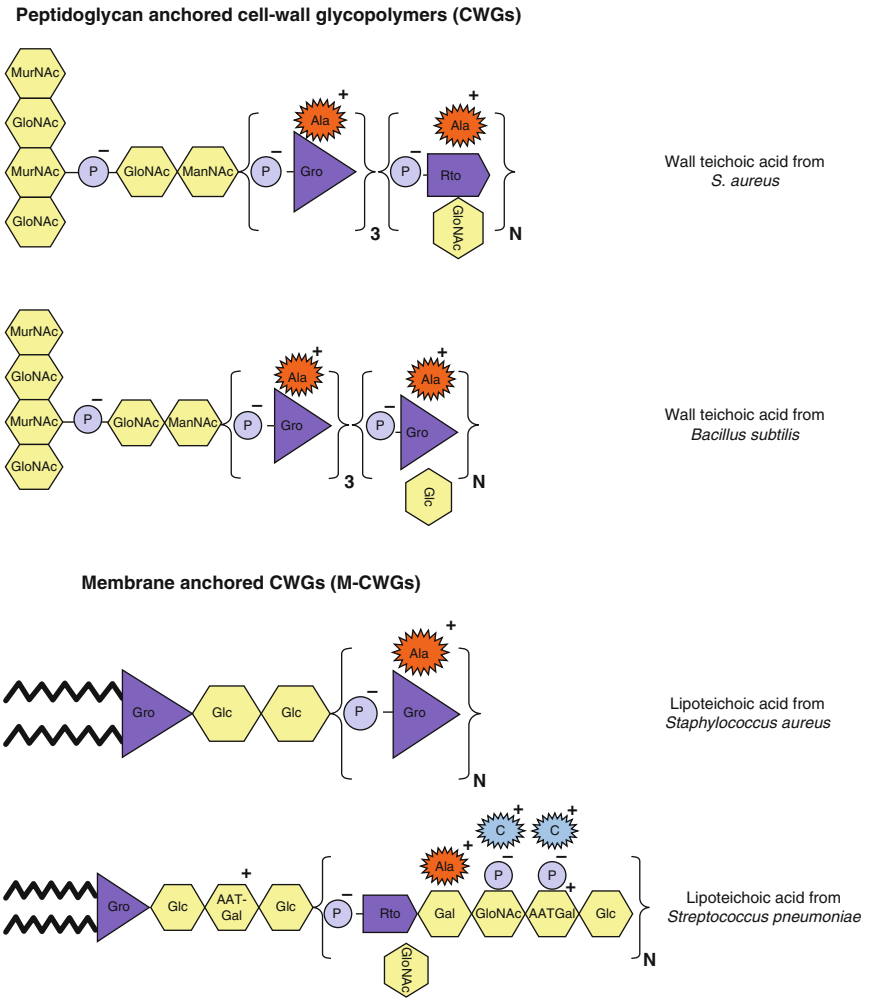

Fig. 5.2 Selected WTA and LTA structures. Trioses, peptoses, and hexoses are shown as triangles, pentagons, and hexagons, respectively. Fatty acids are shown as zigzag lines. Non-glycosyl residues: Ala, D-alanine; C, choline; P, phosphate; Glycosyl residues: AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; Gro, glycerol; ManNAc, *N*-acetylmannosamine; Rto, ribitol. With permission (Kohler et al. 2009b)

Often these sugars are reduced to their corresponding polyols like glycerol or ribitol. Most *S. aureus* strains produce a WTA polymer, which is formed by up to 40 repeating units of ribitolphosphate (Endl et al. 1983). In *Staphylococcus epidermidis* and other staphylococcal species simpler WTA structures can be found. Here Gro-P units form the entire polymer and are not only found in the linkage unit. A more complicated WTA structure with repeating units composed of

glycerolphosphate occurs, e.g., in *Staphylococcus hyicus* (Endl et al. 1983). Among the known polymers, the most complex WTA-like molecule has been found in *Streptococcus agalactiae*. This polymer is extremely complex and branched. It is composed of different types of repeating units, which form the individual branches of the molecule (Sutcliffe et al. 2008). Notably, this polymer represents the species-specific antigen for serological differentiation of streptococci according to Rebecca Lancerfield (Lancefield and Freimer 1966). The repeating units of the WTA molecules are usually further substituted with a diverse set of additional sugars and D-alanine (Neuhaus and Baddiley 2003), other amino acids such as glutamate or lysine (in certain Actinobacteria) (Potekhina et al. 1993; Shashkov et al. 2006), or phosphocholine (*S. pneumoniae*) (Fischer 2000). The negative charge, which derives from the phosphate groups, is neutralized to a large extent by the incorporation of D-alanine (Neuhaus and Baddiley 2003).

A less well studied group of so-called "secondary cell wall polymers" are the anionic P-CWGs, which lack phosphate. This group includes the teichuronic acids (TUA), which are distinguished by the presence of uronic acid residues in their repeating units (e.g., in many bacilli and micrococci) (Soldo et al. 1999; Ward 1981) and pyruvylated polymers (e.g., in *Bacillus anthracis* and *B. cereus*) (Choudhury et al. 2006; Leoff et al. 2007; Schäffer and Messner 2005). When grown under phosphate limitation *B. subtilis* expresses TUA instead of WTA. The linkage unit of *B. subtilis* TUA is similar to that of the WTA. It consists of GlcNAc and glucuronic acid (Soldo et al. 1999). Recently the pyruvylated *B. anthracis* P-CWG has been characterized. It consists of repeating units formed by several hexoses (Choudhury et al. 2006). Many actinobacteria produce uncharged, often branched P-CWG, especially those bacteria with an outer membrane-like mycolic acid layer. For instance, *Mycobacterium tuberculosis* has a branched arabinogalactan polymer, which connects PG and the mycolic acid membrane via the covalently bound mycolic acid (Brennan 2003; Takayama et al. 2005).

5.2.2 *Lipoteichoic Acid and Other Membrane Glycolipid-Anchored Polymers*

Membrane glycolipid-anchored polymers such as lipoteichoic acids (LTA) are attached to the cytoplasmic membrane by linking the polymer to glycolipids. In *S. aureus* the lipid anchor consists of a diglycosylated diacylglycerol (Fischer 1988). In *S. aureus,* as in many other Gram-positive bacteria, the backbone is formed by Gro-P repeating units. As a likely consequence of the unique biosynthetic pathway, the structures of LTA are usually less diverse than those of WTA (see below) (Fischer 1994). In *Streptococcus pneumoniae* a very complex LTA structure can be found. Its repeating units contain phosphocholine and they are identical to those of pneumococcal WTA (Fischer et al. 1993). At the 2-hydroxy group of the glycerol most LTA polymers are substituted with D-alanine and additional sugars as in WTA.

In many actinobacteria, M-CWGs are produced without phosphate in the repeating units. Because of the substitution with succinyl groups such polymers are often anionic (Delmas et al. 1997; Greenberg et al. 1996; Powell et al. 1975). In many mycolic acid-producing bacteria uncharged, branched lipoarabinomannans (LAM) can be found (Briken et al. 2004; Sutcliffe 2005).

5.3 Biosynthesis of Wall Teichoic Acid and Lipoteichoic Acid

Wall teichoic acid and LTA usually rely on profoundly different biosynthetic pathways and precursor molecules (Fig. 5.3), although they exhibit structural similarity. It has been difficult to identify pathways and biosynthetic genes by comparative genomics, probably because of the enormous structural diversity of P-CWGs; this notion implies that most bacterial species require different sets of genes. In *B. subtilis 168* only 13 genes are required for the rather simple Gro-P WTA (Qian et al. 2006) whereas *S. agalactiae* is supposed to depend on more than 120 genes for the synthesis of the extremely complex WTA-like polymer (Sutcliffe et al. 2008). In any case, certain principles are widely conserved among P-CWG-producing bacteria, such as the use of a C_{55} lipid carrier during the assembly process, the enzymes mediating the first steps of linkage unit biosynthesis, and the allocation of biosynthetic genes in clusters. In *B. subtilis* and *S. aureus* most knowledge has been gathered as outlined in the section below (Bhavsar and Brown 2006; Xia and Peschel 2008).

5.3.1 Wall Teichoic Acid Biosythesis in Bacillus subtilis and Staphylococcus aureus

The involvement of nucleotide-activated precursor molecules such as CDP-glycerol, CDP-ribitol, and UDP-GlcNAc has been demonstrated using sophisticated in vitro reconstitution studies of certain WTA-biosynthetic steps (Baddiley 1989; Bracha et al. 1978; Brooks et al. 1971; Nathenson et al. 1966). Biosynthesis is initiated on the undecaprenyl pyrophosphate lipid carrier (C_{55}), which is also used for PG or capsular polysaccharide biosynthesis, at the inside of the cytoplasmatic membrane (Anderson et al. 1972). In *B. subtilis* 168 the first gene cluster involved in biosynthesis of WTA–*tagABCDEFGH*– has been identified by the laboratory of Dimitri Karamata by analysis of temperature-sensitive mutants (Pooley and Karamata 1994). In the last couple of years large-scale sequencing projects and comparative genomics have been used to identify further genes such as *tagO* and the more complex WTA gene clusters of *B. subtilis W23* and *S. aureus*, both of which produce ribitolphosphate (Rbo-P) WTA (Qian et al. 2006). As outlined below, biochemical studies with crude enzymatic preparations derived from temperature-sensitive mutant strains or with recombinant enzymes produced in *Escherichia coli* led to functional predictions for most of

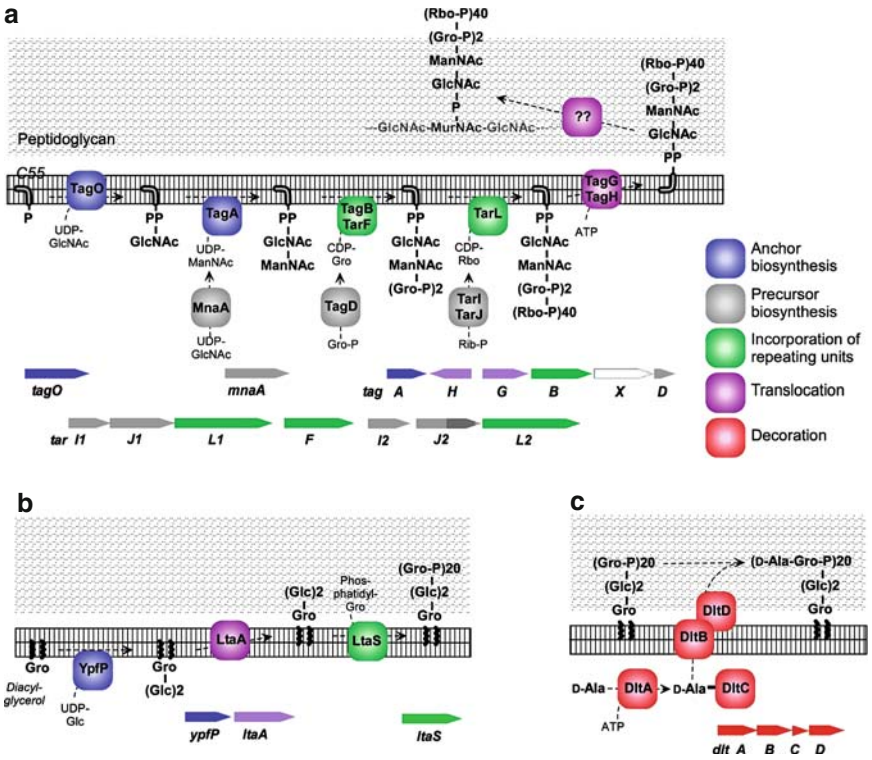

Fig. 5.3 Pathways of *S. aureus* wall teichoic acid (WTA) biosynthesis (**a**), lipoteichoic acid (LTA) biosynthesis (**b**), and D-alanine incorporation into LTA and WTA (**c**). CDP-Gro, cytidyldiphosphate-glycerol; CDP-Rbo, cytidyldiphosphate-ribitol; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gro, glycerol; Gro-P, glycerolphosphate; ManNAc, *N*-acetylmannosamine; MurNAc, *N*-acetyl muramic acid; Rbo-P, ribitol phosphate; Rib-P, ribulose-5-phosphate; UDP-Glc, undecaprenylphosphate-glucose; UDP-GlcNAc, uridine-5′-diphosphate-glucose; UDP-GlcNAc, uridine-5′-*N*-acetylglucosamine; UDP-ManNAc, uridine-5′-*N*-acetylmannosamine. With permission (Kohler et al. 2009b)

the WTA-biosynthetic gene products. Fig. 5.3 shows CWG-biosynthetic steps, which can be divided into four groups:

1. The synthesis of linkage units that usually have conserved structures and connect the polymers with PG or the membrane belong to the first group of WTA-biosynthetic enzymes (Araki and Ito 1989). Hence, an easy identification of CWG gene clusters is feasible since the genes involved, *tagO* and *tagA*, are well conserved among many Gram-positive bacteria (Ginsberg et al. 2006; Weidenmaier et al. 2004). TagO transfers GlcNAc phosphate to C_{55} phosphate (Soldo et al. 2002a) and TagA adds a ManNAc unit using UDP-ManNAc precursors (Ginsberg et al. 2006).
2. The generation of special nucleotide-activated precursor molecules such as UDP-ManNAc (ManA) (Soldo et al. 2002b), CDP-glycerol (TagD) (Park et al.

1993), or CDP-ribitol (TarI, TarJ) (Pereira and Brown 2004) is mediated by the second group of enzymes. Due to the interaction with the nucleotides, many of these genes share conserved domains.

3. Incorporation of the preformed repeating units into CWGs is mediated through the third group of enzymes, which includes both priming and polymerizing enzymes. The Gro-P WTA polymer of *B. subtilis 168* depends on the primase TagB adding the first repeating unit to the C_{55}-bound linkage unit and the polymerase TagF, which adds the additional Gro-P units (Ginsberg et al. 2006). In *S. aureus* Rbo-P WTA biosynthesis the situation is more complicated. Here the TagB reaction is followed by the addition of only one additional Gro-P unit mediated by the TarF enzyme (Brown et al. 2008). Subsequently, the TarL polymerase synthesizes the RboP polymer (Brown et al. 2008; Pereira et al. 2008). No Rbo-P primase seems to be involved in *S. aureus*, whereas in *B. subtilis W23* such an enzyme has been implicated in Rbo-P WTA biosynthesis (TarK) (Bhavsar and Brown 2006). The major challenge in studying enzyme functions is that three genes which are involved in Rbo-P generation and incorporation seem to be duplicated (Qian et al. 2006). Recent studies indicate that the same types of reaction are mediated by the two TarL enzymes, albeit leading to WTA of different chain length and electrophoretic migration. Apparently *S. aureus* can control WTA structure according to bacterial density and environmental changes, since one of the *tarL* genes (also been named *tarK*) is regulated by the *agr* quorum sensing system (Brown et al. 2008).
4. The fourth group consists of proteins which mediate the transfer of the WTA polymers to the outer membrane leaflet (TagG, TagH, forming an ABC transporter) (Lazarevic and Karamata 1995) and the transfer from C_{55} to the acceptor molecules (responsible proteins still unknown).
5. Decoration of WTA/LTA repeating units with sugars, D-alanine, choline, pyruvate or other residues is mediated by further proteins, which are allocated into group v. For WTA with more complex hexose-containing repeating units, such as the minor WTA of *B. subtilis* (Freymond et al. 2006) or the branched WTA-like polymer of *S. agalactiae* (Sutcliffe et al. 2008), different biosynthetic pathways have been proposed which only share *tagO* and (in many cases) *tagA* genes with those described above.

5.3.2 *Lipoteichoic Acid Biosynthesis in Staphylococcus aureus*

The Gro-P repeating units of LTA are derived from a major constituent of bacterial membranes, namely the phospholipid phosphatidylglycerol, but not from a nucleotide-activated precursor (Glaser and Lindsay 1974). Lipoteichoic acid is not polymerized on C_{55} but directly on the glycolipid serving as the membrane anchor for LTA, which is a second major difference between LTA and WTA biosynthesis (Fischer 1988; Koch et al. 1984). Different genes have been implicated in glycolipid biosynthesis of *S. aureus* and *S. agalactiae*, as the glycolipids differ between species

(Doran et al. 2005; Kiriukhin et al. 2001). Lipoteichoic acid is linked to diglycosyldiacylglycerol in *S. aureus*. This lipid is generated by the YpfP enzyme, which adds two glucose residues from UDP-glucose to diacylglycerol (Jorasch et al. 1998, 2000; Kiriukhin et al. 2001). A membrane protein encoded by the *ltaA* gene, which is necessary for efficient LTA biosynthesis, is thought to be a flippase that translocates the glycolipid from the inner to the outer leaflet of the cytoplasmic membrane (Grundling and Schneewind 2007b). Recently, the LTA polymerase LtaS has been discovered by Angelika Gründling and Olaf Schneewind. It utilizes Gro-P units from phosphatidylglycerol to synthesize the LTA polymer at the outer surface of the cytoplasmic membrane (Grundling and Schneewind 2007a). Lipoteichoic acid biosynthesis seems to be a rather conserved process since one or several *ltaS*-related genes are found in most LTA-producing bacteria. Amazingly, deletion of *ypfP* does not block biosynthesis of LTA but leads to synthesis of LTA whose polymer is attached to diacylglycerol (Fedtke et al. 2007; Kiriukhin et al. 2001). For unknown reasons *ypfP* mutants produce unaltered or strongly reduced amounts of the altered LTA compared to the wild-type strains depending on the *S. aureus* strain background (Fedtke et al. 2007). In conclusion, far fewer genes are required for LTA biosynthesis compared to WTA biosynthesis. Nevertheless, highly complex LTA polymers, which are most probably synthesized in a C_{55}-dependent fashion, are produced by bacteria such as *S. pneumoniae* (Draing et al. 2006; Fischer et al. 1993).

5.3.3 *Incorporation of D-Alanine into Wall Teichoic Acid and Lipoteichoic Acid*

A very constant trait of most TA polymers is the modification with D-alanine, whereas most other WTA and LTA components are variable. These substituents seem to be absent in TA molecules without polyol constituents in the repeating units such as the minor WTA of *B. subtilis* (Freymond et al. 2006). The *dltABCD* genes responsible for D-alanine activation and incorporation into WTA and LTA are highly conserved and always seem to form an operon (Neuhaus et al. 1996; Neuhaus and Baddiley 2003), which is in accord with the high prevalence of D-alanylation. Teichoic acid net charge is profoundly affected by D-alanine modification. Accordingly, disruption of the *dltABCD* genes has crucial consequences regarding resistance to antimicrobial peptides, adhesion to host cell receptors and biofilm formation (Weidenmaier and Peschel 2008). The *dlt* operons of *S. aureus* and *S. epidermidis* are controlled via the ApsXRS (also named GraXRS) regulatory system in response to antimicrobial peptide challenge and cell wall stress (Herbert et al. 2007; Kraus et al. 2008; Li et al. 2007). D-Alanine is incorporated after biosynthesis of the TA polymers is completed. D-Alanine can be repeatedly incorporated into a given molecule, since the D-alanine esters are rather labile and are easily lost (Koch et al. 1985). Four proteins (DltA, -B, -C, -D) are required for transfer of D-alanine into TAs. These four proteins form a pathway that includes

activation of D-alanine in the cytoplasm (DltA), linkage to a D-alanine carrier protein (DltC), translocation, and incorporation of D-alanine into TAs (DltB, DltD) (Neuhaus and Baddiley 2003). Hydrolysis of ATP is required for the first DltA-catalyzed step. DltA is homologous to the activating domains of peptide synthetases (Heaton and Neuhaus 1992; Neuhaus et al. 1996). Similarly to the biosynthesis of non-ribosomally synthesized peptides or fatty acids, D-alanine is activated and transferred to a dedicated carrier protein. All these pathways involve intermediates linked to the phosphopantetheine prosthetic groups of the carrier proteins by energy-rich thioester bonds. Transferring the D-alanine into TAs is the last step, which is less well understood. DltB, an integral membrane protein, and DltD, a membrane-tethered hydrophilic protein, seem to be required (Debabov et al. 2000). Thus Dlt proteins represent promising targets for blocking D-alanylation through inhibitory compounds. Recently, it has been reported that bacteria became more susceptible to cationic antimicrobial molecules such as defensins by specifically inhibiting DltA (May et al. 2005). Accordingly, such inhibitors seem to be very efficient in clearing bacterial infections in vivo (Escaich et al. 2007).

5.4 Roles of Wall Teichoic Acid and Lipoteichoic Acid in Bacterial Physiology

Very important roles of WTA and LTA-like polymers for bacterial integrity and fitness are suggested, since bacterial cells commit considerable amounts of energy and genetic information to their biosynthesis and because of the universal presence of CWGs in Gram-positive bacteria. Recently, more detailed characterization of CWG functions has become feasible through the availability of defined mutants lacking CWG (D'Elia et al. 2006; Weidenmaier et al. 2004) or exhibiting altered CWG structures (Doran et al. 2005; Kristian et al. 2005; Peschel et al. 1999). Wall teichoic acid has been shown to be dispensable for viability of *S. aureus* and *B. subtilis* (D'Elia et al. 2006; Weidenmaier et al. 2004). When grown under laboratory conditions these mutants displayed only minor defects. In contrast LTA is indispensable in *S. aureus* at normal temperature (Grundling and Schneewind 2007a) but seems to be dispensable at low temperatures below 30°C (Oku et al. 2008). Amazingly, in vitro growth behavior is not affected by a strongly reduced LTA content in the cell envelope (Fedtke et al. 2007). Accordingly, many of the functions assigned to CWGs appear not to be essential. Many of those functions are supposed to be critical only in certain instances such as exposure to environmental stresses or to host defense factors (Weidenmaier and Peschel 2008). In fact, many reports suggest critical functions of WTA and LTA in protecting the cell envelope from penetration by harmful molecules such as host defense molecules, bacteriocins and antibiotics. This protective function may be either direct by blocking pores and cavities between PG layers or indirect by attachment of outer protection layers such as S-layer proteins (e.g., *B. anthracis*) (Mesnage et al. 2000) or mycolic acids

(e.g., *M. tuberculosis*) (Brennan 2003). Mutants lacking D-alanine in WTA and LTA are more susceptible to cationic antimicrobial peptides (Peschel et al. 1999, 2000; Peschel and Sahl 2006). Moreover, *S. aureus* WTA contributes to lysozyme resistance as well as to resistance against antimicrobial fatty acids (Bera et al. 2007; Kohler et al. 2009a, b). On the other hand, WTA can also increase susceptibility for harmful molecules such as certain phages that use WTA as a receptor (Lopez et al. 1982; Park et al. 1974; Wendlinger et al. 1996) or the human antimicrobial defensin hBD3 and secretory group IIA phospholipase A2 (Koprivnjak et al. 2008).

Certain bacterial proteins are non-covalently anchored by CWG to the cell wall, e.g. in *S. pneumoiae* where many virulence factors are anchored to the phosphocholine residues of the CWG (Bergmann and Hammerschmidt 2006). Furthermore, *B. anthracis* and relatives attach their S-layer proteins to the bacterial surface by the use of pyruvylated CWG (Mesnage et al. 2000). Autolysins of *S. aureus* and other bacteria show a high affinity for WTA and LTA (Bierbaum and Sahl 1987; Giudicelli and Tomasz 1984) but it is proposed that autolysins bind to the cell wall independently of CWG, since *S. aureus* mutants with reduced LTA exhibited no reduced amounts of autolysins (Fedtke et al. 2007). Wall teichoic acid and LTA play a profound role in controlling autolysin activity by interactions that may involve CWG-bound bivalent cations; however, these interactions are only partially understood. It is assumed that LTA interacts with components of the membrane-bound cell division machinery and contributes to its proper placement or regulation, because depletion of LTA leads to bacterial cells with distorted shapes and division sites in *S. aureus* (Grundling and Schneewind 2007a).

Important roles in shaping the ionic milieu in the cell wall have been proposed for the zwitterionic and anionic CWG types that have ion-exchanger-like properties. In particular, TAs have high affinities for magnesium ions and are regarded as magnesium ion storage molecules (Heptinstall et al. 1970). Surface-exposed CWGs are highly hydrophilic, which has a strong influence on the physicochemical properties of bacterial cell surfaces. A strong impact on biofilm formation on biomaterials has been observed in *S. aureus* and *Enterococcus faecalis* mutants with altered CWGs. Furthermore these mutants are attenuated in virulence in animal models (Fabretti et al. 2006; Fedtke et al. 2007; Gross et al. 2001; Kristian et al. 2003). CWGs can play a second role in biofilm formation when they are released by the bacteria to form parts of the biofilm matrix, which protects bacterial cells and glues them together (Sadovskaya et al. 2004; Vinogradov et al. 2006). The precise mechanism by which CWGs are shed from the cell wall or membrane anchors is still unknown.

5.5 Lipoteichoic Acid and Interactions with Host Cell Receptors

Bacteria colonizing or infecting animal hosts (Fig. 5.4) appear to depend on CWGs, which play various crucial roles in microbe–host interactions. A number of recent studies indicate that WTA and LTA can shape the entire infection process of

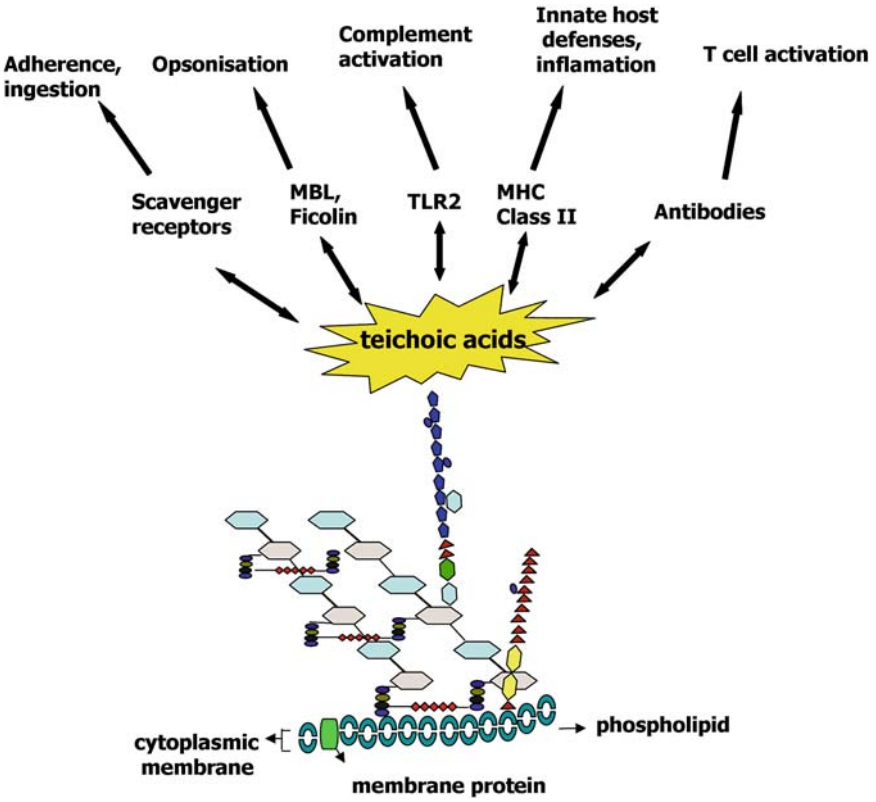

Fig. 5.4 Interaction of LTA and WTA with host molecules. Scavenger receptors, mannose-binding lectin, and ficolins interact with different CWGs, which leads to binding and internalization by host cells or complement activation. Some M-CWGs seem to elicit proinflammatory responses through TLR2. MBL, Mannose-binding lectin; MHC, major histocompatibility complex; TLR2, Toll-like receptor 2. With permission (Kohler et al. 2009b)

S. aureus from initial colonization to activation of innate immunity and to recognition by the adaptive immune system (Weidenmaier and Peschel 2008). Binding to epithelial and endothelial cells has been abrogated in *S. aureus* mutants lacking WTA. Furthermore, these mutants have lost the ability to colonize the nose in animal models (Weidenmaier et al. 2004, 2008) or to leave the bloodstream and infect subendothelial tissues in endovascular infections (Weidenmaier et al. 2005b). Similar impacts on bacterial host cell binding have been observed upon altering TA structure by disrupting the D-alanylation pathway in *S. aureus* (Weidenmaier et al. 2004, 2005a), *Streptococcus pyogenes* (Kristian et al. 2005) and *Listeria monocytogenes* (Abachin et al. 2002) or by altering LTA membrane anchoring in *S. agalactiae* (Abachin et al. 2002). There is evidence for direct binding of WTA to as yet unidentified receptors on epithelial and endothelial cells. Of interest, these interactions seem to contribute to *S. aureus* host cell attachment to a similar extent

as the staphylococcal adhesion proteins (Weidenmaier et al. 2008), which bind, e.g., keratin, fibronectin, or fibrinogen (Foster and Hook 1998; Mongodin et al. 2002; Navarre and Schneewind 1999). Wall teichoic acid-mediated binding can be inhibited by polyinosinic acid, an established inhibitor of scavenger receptors (SR), indicating that SR-like receptors play a major role in the WTA-mediated staphylococcal binding to host cells (Weidenmaier et al. 2008). Accordingly, some members of the SR family that have been identified on mammalian cells have been shown to bind purified CWG and intact *S. aureus* cells. SCARA5 and LOX1, which are expressed by airway epithelial and endothelial cells, respectively, are candidate receptors for WTA-mediated binding (Jiang et al. 2006; Shimaoka et al. 2001).

While SRs appear to mediate bacterial attachment, other host receptors have been shown to stimulate inflammatory processes upon CWG binding. Pathogen-Associated Molecular Pattern (PAMP) molecules such as LTA, mycobacterial LAM and other M-CWG activate the innate immune system via Toll-like receptor 2 (TLR2) (Hermann et al. 2002; Hoebe et al. 2005; Sugawara et al. 2003; Tapping and Tobias 2003). LTA-mediated TLR2 activation seems to require co-receptors such as TLR6, CD14, LBP, and CD36 (Chavakis et al. 2002; Han et al. 2003; Henneke et al. 2005; Hoebe et al. 2005). However, many of the commonly used M-CWG preparations have been shown to be contaminated with lipopeptides, which account for a large percentage of the proinflammatory activity (Hashimoto et al. 2006, 2007). Thus, the proinflammatory potency of M-CWG is still a matter of debate. Nevertheless, synthetic LTA analogs seem to stimulate TLR2 (Deininger et al. 2003). Lipoteichoic acid and LAM also have been shown to bind to soluble C-type lectins such as mannose-binding lectin (MBL), L-ficolin, and the lung surfactant proteins A and D (Ferguson et al. 1999; Lynch et al. 2004; Polotsky et al. 1996, 1997; Sidobre et al. 2000; van de Wetering et al. 2001). The lectin-initiated complement pathway is activated by MBL and L-ficolin upon binding to CWG on the bacterial surface, which leads to bacterial opsonization and release of chemotactic complement split products (Endo et al. 2007; Takahashi et al. 2007).

For many decades CWGs such as *S. aureus* TA have been well-known targets for antibodies (Kumar et al. 2005; Verbrugh et al. 1981; Verhoef et al. 1983). Accordingly, CWGs have been considered as vaccination targets. Indeed it has been shown that vaccination with enterococcal LTA induced the production of protective, opsonic antibodies (Theilacker et al. 2006). Furthermore, promising results were obtained by passive vaccination with a humanized monoclonal antibody targeting staphylococcal LTA (Weisman 2007). Traditionally glycopolymers have been regarded as T cell-independent antigens. There is increasing evidence that zwitterionic glycopolymers can lead to activation of T cells through processing of ingested CWG by antigen-presenting cells and subsequent presentation via MHC class II molecules (Kalka-Moll et al. 2002; Mazmanian and Kasper 2006). Most detailed studies of this new pathway are available for *Bacteroides fragilis* capsular polysaccharides. Recent studies have led to the suggestion that the equally zwitterionic *S. aureus* WTA might serve as a T cell-independent antigen (McLoughlin et al. 2006; Tzianabos et al. 2001). Although these studies are still in their infancy,

CWGs should increasingly be regarded as vaccine candidates with the capacity to elicit immunological memory, if this new concept can be confirmed. Mycobacterial LAM has been shown to stimulate T cells restricted to the MHC-like molecule CD1, which is known to present certain lipid antigens, which reveals another way how CWGs might stimulate specific T cells (Prigozy et al. 1997). It is tempting to speculate that M-CWGs represent more general substrates for CD1 presentation than previously thought.

5.6 Conclusions

After the discovery of TA and other CWGs in the 1960s, these polymers represented a field of active research. However, subsequently the chemical, biochemical, and genetic basis of CWG biosynthesis was studied by only a small group of scientists. Certain CWGs have only recently received increasing attention within the scientific community, since these polymers turned out to be of pivotal importance in microbe–host interaction. Moreover, CWG chemistry and biology has been put on the list of major scientific challenges, since there is a desperate need for new antimicrobial target structures. Recently promising studies have revealed the suitability of CWGs as targets for new antibiotics or vaccines (May et al. 2005; Mikusova et al. 1995; Theilacker et al. 2004). A broader view of the diversity and variability of CWG structures can be achieved in the near future now that improved glycochemical methods are available. The availability of genomic and metagenomic databases represents a valuable basis for predicting CWG biosynthetic pathways by bioinformatics methods. Predicted enzyme functions can be confirmed with the help of in vitro reconstitution of biosynthetic steps, which has recently yielded major scientific progress. Moreover, this progress will assist the use of the reactions in high-throughput screening programs in the search for new antibiotics. In *B. subtilis* the WTA biosynthetic enzymes seem to form a membrane-associated complex, which indicates that CWG biosynthesis is a highly organized process (Formstone et al. 2008). It can be assumed that the CWG and PG biosynthetic machineries are coordinated in sophisticated ways, maybe in cooperation with cytoskeletal elements and with the cell division apparatus.

Structural features of CWGs might be correlated with certain functions in cell wall physiology or host interaction with the help of the increasing availability of defined mutants. The proinflammatory capacity of M-CWGs and the potential of CWGs to activate specific T cells upon processing and presentation in MHC class II or CD1 molecules are the major open questions which need to be approached. Furthermore, many host receptors recognizing and binding CWGs remain to be identified. Because of cell- and species-specific differences in expression of CWG-binding molecules, it is tempting to speculate that the enormous diversity of CWG structures plays a role in bacterial cell and host tropism.

Acknowledgments Our research is supported by grants from from the German Research Foundation (TR34, FOR449, GRK685, SFB685, SFB76, SPP1130), the European Union (LSHM-CT-2004-512093), the German Ministry of Education and Research (NGFN2, SkinStaph) and the IZKF program of the Medical Faculty, University of Tübingen, to A.P.

References

Abachin E, Poyart C, Pellegrini E, Milohanic E, Fiedler F, Berche P, Trieu-Cuot P (2002) Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Mol Microbiol 43:1–14

Anderson RG, Hussey H, Baddiley J (1972) The mechanism of wall synthesis in bacteria. The organization of enzymes and isoprenoid phosphates in the membrane. Biochem J 127:11–25

Araki Y, Ito E (1989) Linkage units in cell walls of Gram-positive bacteria. Crit Rev Microbiol 17:121–135

Armstrong JJ, Baddiley J, Buchanan JG, Davision AL, Kelemen MV, Neuhaus FC (1959) Composition of teichoic acids from a number of bacterial walls. Nature 184:247–248

Baddiley J (1989) Bacterial cell walls and membranes. Discovery of the teichoic acids. Bioessays 10:207–210

Bera A, Biswas R, Herbert S, Kulauzovic E, Weidenmaier C, Peschel A, Gotz F (2007) Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. J Bacteriol 189:280–283

Bergmann S, Hammerschmidt S (2006) Versatility of pneumococcal surface proteins. Microbiology 152:295–303

Bhavsar AP, Brown ED (2006) Cell wall assembly in *Bacillus subtilis*: how spirals and spaces challenge paradigms. Mol Microbiol 60:1077–1090

Bierbaum G, Sahl HG (1987) Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of *N*-acetylmuramoyl-L-alanine amidase. J Bacteriol 169:5452–5458

Bracha R, Chang M, Fiedler F, Glaser L (1978) Biosynthesis of teichoic acids. Methods Enzymol 50:387–40

Brennan PJ (2003) Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis (Edinburgh) 83:91–97

Briken V, Porcelli SA, Besra GS, Kremer L (2004) Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. Mol Microbiol 53:391–403

Brooks D, Mays LL, Hatefi Y, Young FE (1971) Glucosylation of teichoic acid: solubilization and partial characterization of the uridine diphosphoglucose:polyglycerolteichoic acid glucosyl transferase from membranes of *Bacillus subtilis*. J Bacteriol 107:223–229

Brown S, Zhang YH, Walker S (2008) A revised pathway proposed for *Staphylococcus aureus* wall teichoic acid biosynthesis based on *in vitro* reconstitution of the intracellular steps. Chem Biol 15:12–21

Chavakis T, Hussain M, Kanse SM, Peters G, Bretzel RG, Flock JI, Herrmann M, Preissner KT (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti inflammatory factor by inhibiting the recruitment of host leukocytes. Nat Med 8:687–693

Choudhury B, Leoff C, Saile E, Wilkins P, Quinn CP, Kannenberg EL, Carlson RW (2006) The structure of the major cell wall polysaccharide of *Bacillus anthracis* is species specific. J Biol Chem 281:27932–27941

D'Elia MA, Millar KE, Beveridge TJ, Brown ED (2006) Wall teichoic acid polymers are dispensable for cell viability in *Bacillus subtilis*. J Bacteriol 188:8313–8316

Debabov DV, Kiriukhin MY, Neuhaus FC (2000) Biosynthesis of lipoteichoic acid in *Lactobacillus rhamnosus*: role of DltD in D-alanylation. J Bacteriol 182:2855–2864

Deininger S, Stadelmaier A, von Aulock S, Morath S, Schmidt RR, Hartung T (2003) Definition of structural prerequisites for lipoteichoic acid-inducible cytokine induction by synthetic derivatives. J Immunol 170:4134–4138

Delmas C, Gilleron M, Brando T, Vercellone A, Gheorghui M, Riviere M, Puzo G (1997) Comparative structural study of the mannosylated-lipoarabinomannans from *Mycobacterium bovis* BCG vaccine strains: characterization and localization of succinates. Glycobiology 7:811–817

Doran KS, Engelson EJ, Khosravi AMH, Fedtke I, Equils O, Michelsen KS, Arditi M, Peschel A, Nizet V (2005) Group B *Streptococcus* blood-brain barrier invasion depends upon proper cell surface anchoring of lipoteichoic acid. J Clin Invest 115:2499–2507

Draing C, Pfitzenmaier M, Zummo S, Mancuso G, Geyer A, Hartung T, von Aulock S (2006) Comparison of lipoteichoic acid from different serotypes of *Streptococcus pneumoniae*. J Biol Chem 281:33849–33859

Endl J, Seidl HP, Fiedler F, Schleifer KH (1983) Chemical composition and structure of the cell wall teichoic acids of staphylococci. Arch Microbiol 135:215–223

Endo Y, Matsushita M, Fujita T (2007) Role of ficolin in innate immunity and its molecular basis. Immunobiology 212:371–379

Escaich S, Moreau F, Vongsouthi VSC, Malacain E, Prouvensier L, Gerusz V, Denis M, Saccomani M, Oxoby M (2007) Discovery of new Gram-positive antivirulence drugs: the first antivirulence molecule active in vivo. ICAAC Conference 2007. F2-958

Fabretti F, Theilacker C, Baldassarri L, Kaczynski Z, Kropec A, Holst O, Huebner J (2006) Alanine esters of enterococcal lipoteichoic acid play a role in biofilm formation and resistance to antimicrobial peptides. Infect Immun 74:4164–4171

Fedtke I, Mader D, Kohler T, Moll H, Nicholson G, Biswas R, Henseler K, Gotz F, Zahringer U, Peschel A (2007) A *Staphylococcus aureus ypfP* mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity. Mol Microbiol 65:1078–1091

Ferguson JS, Voelker DR, McCormack FX, Schlesinger LS (1999) Surfactant protein D binds to *Mycobacterium tuberculosis* bacilli and lipoarabinomannan via carbohydrate-lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages. J Immunol 163:312–321

Fischer W (1988) Physiology of lipoteichoic acids in bacteria. Adv Microbiol Physiol 29:233–302

Fischer W (1994) Lipoteichoic acids and lipoglycans. In: Ghuysen J-M, Hakenbeck R (eds) Bacterial cell wall. Elsevier Science B.V, Amsterdam, The Netherlands, pp 199–215

Fischer W (2000) Phosphocholine of pneumococcal teichoic acids: role in bacterial physiology and pneumococcal infection. Res Microbiol 151:421–427

Fischer W, Behr T, Hartmann R, Peter-Katalinic J, Egge H (1993) Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures. A reinvestigation of teichoid acid (C polysaccharide). Eur J Biochem 215:851–857

Formstone A, Carballido-Lopez R, Noirot P, Errington J, Scheffers DJ (2008) Localization and interactions of teichoic acid synthetic enzymes in *Bacillus subtilis*. J Bacteriol 190:1812–1821

Foster TJ, Hook M (1998) Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol 6:484–488

Freymond PP, Lazarevic V, Soldo B, Karamata D (2006) Poly(glucosyl-*N*-acetylgalactosamine 1-phosphate), a wall teichoic acid of *Bacillus subtilis* 168: its biosynthetic pathway and mode of attachment to peptidoglycan. Microbiology 152:1709–1718

Ginsberg C, Zhang YH, Yuan Y, Walker S (2006) *In vitro* reconstitution of two essential steps in wall teichoic acid biosynthesis. ACS Chem Biol 1:25–28

Giudicelli S, Tomasz A (1984) Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. J Bacteriol 158:1188–1190

Glaser L, Lindsay B (1974) The synthesis of lipoteichoic acid carrier. Biochem Biophys Res Commun 59:1131–1136

Greenberg JW, Fischer W, Joiner KA (1996) Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. Infect Immun 64:3318–3325

Gross M, Cramton S, Götz F, Peschel A (2001) Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun 69:3423–3426

Grundling A, Schneewind O (2007a) Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. Proc Natl Acad Sci USA 104:8478–8483

Grundling A, Schneewind O (2007b) Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. J Bacteriol 189(6):2521–30

Han SH, Kim JH, Martin M, Michalek SM, Nahm MH (2003) Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. Infect Immun 71:5541–5548

Hashimoto M, Tawaratsumida K, Kariya H, Kiyohara A, Suda Y, Krikae F, Kirikae T, Gotz F (2006) Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in *Staphylococcus aureus*. J Immunol 177:3162–3169

Hashimoto M, Furuyashiki M, Kaseya R, Fukada Y, Akimaru M, Aoyama K, Okuno T, Tamura T, Kirikae T, Kirikae F, Eiraku N, Morioka H, Fujimoto Y, Fukase K, Takashige K, Moriya Y, Kusumoto S, Suda Y (2007) Evidence of immunostimulating lipoprotein existing in the natural lipoteichoic acid fraction. Infect Immun 75:1926–1932

Heaton MP, Neuhaus FC (1992) Biosynthesis of D-alanyl-lipoteichoc acid: cloning, nucleotide sequence, and expression of the *Lactobacillus casei* gene for the D-alanine-activating enzyme. J Bacteriol 174:4707–4717

Henneke P, Morath S, Uematsu S, Weichert S, Pfitzenmaier M, Takeuchi O, Muller A, Poyart C, Akira S, Berner R, Teti G, Geyer A, Hartung T, Trieu-Cuot P, Kasper DL, Golenbock DT (2005) Role of lipoteichoic acid in the phagocyte response to group B *streptococcus*. J Immunol 174:6449–6455

Heptinstall S, Archibald AR, Baddiley J (1970) Teichoic acids and membrane function in bacteria. Nature 225:519–521

Herbert S, Bera A, Nerz C, Kraus D, Peschel A, Goerke C, Meehl M, Cheung A, Gotz F (2007) Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog 3:981–994

Hermann C, Spreitzer I, Schroder NW, Morath S, Lehner MD, Fischer W, Schutt C, Schumann RR, Hartung T (2002) Cytokine induction by purified lipoteichoic acids from various bacterial species – role of LBP, sCD14, CD14 and failure to induce IL-12 and subsequent IFN-gamma release. Eur J Immunol 32:541–551

Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K, Sovath S, Shamel L, Hartung T, Zahringer U, Beutler B (2005) CD36 is a sensor of diacylglycerides. Nature 433:523–527

Jiang Y, Oliver P, Davies KE, Platt N (2006) Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells. J Biol Chem 281:11834–11845

Jorasch P, Wolter FP, Zähringer U, Heinz E (1998) A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1, 2-diacylglycerol: expression of *ypfP* in *Escherichia coli* and structural analysis of its reaction products. Mol Microbiol 29:419–30

Jorasch P, Warnecke DC, Lindner B, Zähringer U, Heinz E (2000) Novel processive and nonprocessive glycosyltransferases from *Staphylococcus aureus* and *Arabidopsis thaliana* synthesize glycoglycerolipids, glycophospholipids, glycosphingolipids and glycosylsterols. Eur J Biochem 267:3770–3783

Kalka-Moll WM, Tzianabos AO, Bryant PW, Niemeyer M, Ploegh HL, Kasper DL (2002) Zwitterionic polysaccharides stimulate T cells by MHC class II-dependent interactions. J Immunol 169:6149–6153

Kiriukhin MY, Debabov DV, Shinabarger DL, Neuhaus FC (2001) Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus* RN4220: role of YpfP, the diglucosyldiacylglycerol synthase. J Bacteriol 183:3506–3514

Koch HU, Haas R, Fischer W (1984) The role of lipoteichoic acid biosynthesis in membrane lipid metabolism of growing *Staphylococcus aureus*. Eur J Biochem 138:357–363

Koch HU, Doker R, Fischer W (1985) Maintenance of D-alanine ester substitution of lipoteichoic acid by reesterification in *Staphylococcus aureus*. J Bacteriol 164:1211–1217

Kohler T, Weidenmaier C, Peschel A (2009a) Wall teichoic acids protects *Staphylococcus aureus* against antimicrobial fatty acids from human skin. J Bacteriol 191(13):4482–4484

Kohler T, Xia G, Kulausovic E, Peschel A (2009b) Teichoic acids, lipoteichoic acids, and related cell wall glycopolymers of Gram-positive bacteria. In: Moran A (ed) Microbial Glycobiology: structures, relevance and applications. Elsevier, Amsterdam, pp 75–91

Koprivnjak T, Weidenmaier C, Peschel A, Weiss JP (2008) Wall teichoic acid deficiency in *Staphylococcus aureus* confers selective resistance to mammalian group IIA phospholipase A (2) and human beta-defensin 3. Infect Immun 76:2169–2176

Kraus D, Herbert S, Kristian SA, Khosravi A, Nizet V, Gotz F, Peschel A (2008) The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defenses. BMC Microbiol 8:85

Kristian SA, Lauth X, Nizet V, Götz F, Neumeister B, Peschel A, Landmann R (2003) Alanylation of teichoic acids protects *Staphylococcus aureus* against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model. J Infect Dis 188:414–423

Kristian SA, Datta V, Weidenmaier C, Kansal R, Fedtke I, Peschel A, Gallo RL, Nizet V (2005) D-alanylation of teichoic acid promotes group A *Streptococcus* antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. J Bacteriol 187:6719–6725

Kumar A, Ray P, Kanwar M, Sharma M, Varma S (2005) A comparative analysis of antibody repertoire against *Staphylococcus aureus* antigens in patients with deep-seated versus superficial staphylococcal infections. Int J Med Sci 2:129–136

Lancefield RC, Freimer EH (1966) Type-specific polysaccharide antigens of group B streptococci. J Hyg (London) 64:191–203

Lazarevic V, Karamata D (1995) The *tagGH* operon of *Bacillus subtilis* 168 encodes a two-component ABC transporter involved in the metabolism of two wall teichoic acids. Mol Microbiol 16:345–355

Leoff C, Saile E, Sue D, Wilkins P, Quinn CP, Carlson RW, Kannenberg EL (2007) Cell wall carbohydrate compositions of strains from the *B. cereus* group of species correlate with phylogenetic relatedness. J Bacteriol 190(1):112–121

Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M (2007) Gram-positive three-component antimicrobial peptide-sensing system. Proc Natl Acad Sci USA 104:9469–9474

Lopez R, Garcia E, Garcia P, Ronda C, Tomasz A (1982) Choline-containing bacteriophage receptors in *Streptococcus pneumoniae*. J Bacteriol 151:1581–1590

Lynch NJ, Roscher S, Hartung T, Morath S, Matsushita M, Maennel DN, Kuraya M, Fujita T, Schwaeble WJ (2004) L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement. J Immunol 172:1198–1202

May JJ, Finking R, Wiegeshoff F, Weber TT, Bandur N, Koert U, Marahiel MA (2005) Inhibition of the D-alanine: D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall. FEBS J 272:993–3003

Mazmanian SK, Kasper DL (2006) The love-hate relationship between bacterial polysaccharides and the host immune system. Nat Rev Immunol 6:849–858

McLoughlin RM, Solinga RM, Rich J, Zaleski KJ, Cocchiaro JL, Risley A, Tzianabos AO, Lee JC (2006) CD4+ T cells and CXC chemokines modulate the pathogenesis of *Staphylococcus aureus* wound infections. Proc Natl Acad Sci USA 103:10408–10413

Mesnage S, Fontaine T, Mignot T, Delepierre M, Mock M, Fouet A (2000) Bacterial SLH domain proteins are non-covalently anchored to the cell surface via a conserved mechanism involving wall polysaccharide pyruvylation. EMBO J 19:4473–4484

Mikusova K, Slayden RA, Besra GS, Brennan PJ (1995) Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob Agents Chemother 39:2484–2489

Mongodin E, Bajolet O, Cutrona J, Bonnet N, Dupuit F, Puchelle E, de Bentzmann S (2002) Fibronectin-binding proteins of *Staphylococcus aureus* are involved in adherence to human airway epithelium. Infect Immun 70:620–630

Nathenson SG, Ishimoto N, Anderson JS, Strominger JL (1966) Enzymatic synthesis and immunochemistry of alpha- and beta-*N*-acetylglucosaminylribitol linkages in teichoic acids from several strains of *Staphylococcus aureus*. J Biol Chem 241:651–658

Naumova IB, Shashkov AS (1997) Anionic polymers in cell walls of Gram-positive bacteria. Biochemistry (Moscow) 62:809–840

Navarre WW, Schneewind O (1999) Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 63:174–229

Neuhaus FC, Baddiley J (2003) A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in Gram-positive bacteria. Microbiol Mol Biol Rev 67:686–723

Neuhaus FC, Heaton MP, Debabov DV, Zhang Q (1996) The *dlt* operon in the biosynthesis of D-alanyl-lipoteichoic acid in *Lactobacillus casei*. Microb Drug Resist 2:77–84

Oku Y, Kurokawa K, Matuso M, Yamada S, Lee BL, Sekimizu K (2008) Pleiotropic roles of polyglycerolphosphate synthase of lipoteichoic acid in the growth of *Staphylococcus aureus* cells. J Bacteriol 191:141–151

Park JT, Shaw DR, Chatterjee AN, Mirelman D, Wu T (1974) Mutants of staphylococci with altered cell walls. Ann NY Acad Sci 236:54–62

Park YS, Sweitzer TD, Dixon JE, Kent C (1993) Expression, purification, and characterization of CTP: glycerol-3-phosphate cytidylyltransferase from *Bacillus subtilis*. J Biol Chem 268:16648–16654

Pereira MP, Brown ED (2004) Bifunctional catalysis by CDP-ribitol synthase: convergent recruitment of reductase and cytidylyltransferase activities in *Haemophilus influenzae* and *Staphylococcus aureus*. Biochemistry 43:11802–11812

Pereira MP, Delia MA, Troczynska J, Brown ED (2008) Duplication of teichoic acid biosynthetic genes in *Staphylococcus aureus* leads to functionally redundant poly(ribitol phosphate) polymerases. J Bacteriol 190(16):5642–9

Peschel A, Sahl HG (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol 4:529–536

Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other antimicrobial peptides. J Biol Chem 274:8405–8410

Peschel A, Vuong C, Otto M, Götz F (2000) The D-alanine residues of *Staphylococcusaureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolysins. Antimicrob Agents Chemother 44:2845–2847

Polotsky VY, Fischer W, Ezekowitz AB, Joiner KA (1996) Interactions of human mannose-binding protein with lipoteichoic acids. Infect Immun 64:380–383

Polotsky VY, Belisle JT, Mikusova K, Ezekowitz RA, Joiner KA (1997) Interaction of human mannose-binding protein with *Mycobacterium avium*. J Infect Dis 175:1159–1168

Pooley HM, Karamata D (1994) Teichoic acid synthesis in *Bacillus subtilis*: genetic organization and biological roles. In: Ghuysen J-M, Hakenbeck R (eds) Bacterial Cell Wall. Elsevier Science B.V, Amsterdam, The Netherlands, pp 187–197

Potekhina NV, Tul'skaya EM, Naumova IB, Shashkov AS, Evtushenko LI (1993) Erythritolteichoic acid in the cell wall of *Glycomyces tenuis* VKM Ac-1250. Eur J Biochem 218:371–375

Powell DA, Duckworth M, Baddiley J (1975) A membrane-associated lipomannan in micrococci. Biochem J 151:387–397

Prigozy TI, Sieling PA, Clemens D, Stewart PL, Behar SM, Porcelli SA, Brenner MB, Modlin RL, Kronenberg M (1997) The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. Immunity 6:187–197

Qian Z, Yin Y, Zhang Y, Lu L, Li Y, Jiang Y (2006) Genomic characterization of ribitol teichoic acid synthesis in *Staphylococcus aureus*: genes, genomic organization and gene duplication. BMC Genomics 7:74

Sadovskaya I, Vinogradov E, Li J, Jabbouri S (2004) Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus epidermidis* RP62A, a reference biofilm-positive strain. Carbohydr Res 339:1467–1473

Schäffer C, Messner P (2005) The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. Microbiology 151:643–651

Shashkov AS, Streshinskaya GM, Senchenkova SN, Kozlova YI, Alferova IV, Terekhova LP, Evtushenko LI (2006) Cell wall teichoic acids of streptomycetes of the phenetic cluster '*Streptomyces fulvissimus*'. Carbohydr Res 341:796–802

Shimaoka T, Kume N, Minami M, Hayashida K, Sawamura T, Kita T, Yonehara S (2001) LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. J Immunol 166:5108–5114

Sidobre S, Nigou J, Puzo G, Riviere M (2000) Lipoglycans are putative ligands for the human pulmonary surfactant protein A attachment to mycobacteria. Critical role of the lipids for lectin-carbohydrate recognition. J Biol Chem 275:2415–2422

Soldo B, Lazarevic V, Pagni M, Karamata D (1999) Teichuronic acid operon of *Bacillus subtilis* 168. Mol Microbiol 31:795–805

Soldo B, Lazarevic V, Karamata D (2002a) *tagO* is involved in the synthesis of all anionic cell-wall polymers in *Bacillus subtilis* 168. Microbiology 148:2079–2087

Soldo B, Lazarevic V, Pooley HM, Karamata D (2002b) Characterization of a *Bacillus subtilis* thermosensitive teichoic acid-deficient mutant: gene mnaA (yvyH) encodes the UDP-*N*-acetylglucosamine 2-epimerase. J Bacteriol 184:4316–4320

Sugawara I, Yamada H, Li C, Mizuno S, Takeuchi O, Akira S (2003) Mycobacterial infection in TLR2 and TLR6 knockout mice. Microbiol Immunol 47:327–336

Sutcliffe I (2005) Lipoarabinomannans – structurally diverse and functionally enigmatic macro-amphiphiles of mycobacteria and related actinomycetes. Tuberculosis (Edinburgh) 85:205–206

Sutcliffe IC, Black GW, Harrington DJ (2008) Bioinformatic insights into the biosynthesis of the Group B carbohydrate in *Streptococcus agalactiae*. Microbiology 154:1354–1363

Takahashi M, Mori S, Shigeta S, Fujita T (2007) Role of MBL-associated serine protease (MASP) on activation of the lectin complement pathway. Adv Exp Med Biol 598:93–104

Takayama K, Wang C, Besra GS (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. Clin Microbiol Rev 18:81–101

Tapping RI, Tobias PS (2003) Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. J Endotoxin Res 9:264–268

Theilacker C, Krueger WA, Kropec A, Huebner J (2004) Rationale for the development of immunotherapy regimens against enterococcal infections. Vaccine 22(Suppl 1):S31–S38

Theilacker C, Kaczynski Z, Kropec A, Fabretti F, Sange T, Holst O, Huebner J (2006) Opsonic antibodies to *Enterococcus faecalis* strain 12030 are directed against lipoteichoic acid. Infect Immun 74:5703–5712

Tzianabos AO, Wang JY, Lee JC (2001) Structural rationale for the modulation of abscess formation by *Staphylococcus aureus* capsular polysaccharides. Proc Natl Acad Sci USA 98:9365–9370

van de Wetering JK, van Eijk M, van Golde LM, Hartung T, van Strijp JA, Batenburg JJ (2001) Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, 2 major cell wall components of Gram-positive bacteria. J Infect Dis 184:1143–1151

Verbrugh HA, Peters R, Rozenberg-Arska M, Peterson PK, Verhoef J (1981) Antibodies to cell wall peptidoglycan of *Staphylococcus aureus i*n patients with serious staphylococcal infections. J Infect Dis 144:1–9

Verhoef J, Musher DM, Spika JS, Verbrugh HA, Jasper FC (1983) The effect of staphylococcal peptidoglycan on polymorphonuclear leukocytes *in vitro* and *in vivo*. Scand J Infect Dis Suppl 41:79–86

Vinogradov E, Sadovskaya I, Li J, Jabbouri S (2006) Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus aureus* MN8m, a biofilm forming strain. Carbohydr Res 341:738–743

Ward JB (1981) Teichoic and teichuronic acids: biosynthesis, assembly and location. Microbiol Rev 45:211–243

Weidenmaier C, Peschel A (2008) Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat Rev Microbiol 6:276–287

Weidenmaier C, Kokai-Kun JF, Kristian SA, Chanturyia T, Kalbacher H, Gross M, Nicholson G, Neumeister B, Mond JJ, Peschel A (2004) Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. Nat Med 10:243–245

Weidenmaier C, Peschel A, Xiong YQ, Kristian SA, Dietz K, Yeaman MR, Bayer AS (2005a) Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. J Infect Dis 191:1771–1777

Weidenmaier C, Peschel A, Kempf VA, Lucindo N, Yeaman MR, Bayer AS (2005b) DltABCD- and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. Infect Immun 73:8033–8038

Weidenmaier C, Kokai-Kun JF, Kulauzovic E, Kohler T, Thumm G, Stoll H, Gotz F, Peschel A (2008) Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. Int J Med Microbiol 298(5–6):505–13

Weisman LE (2007) Antibody for the prevention of neonatal nosocomial staphylococcal infection: a review of the literature. Arch Pediatr 14(Suppl 1):S31–S34

Wendlinger G, Loessner MJ, Scherer S (1996) Bacteriophage receptors on *Listeria monocytogenes* cells are the *N*-acetylglucosamine and rhamnose substituents of teichoic acids or the peptidoglycan itself. Microbiology 142:985–992

Xia G, Peschel A (2008) Toward the pathway of *S. aureus* WTA biosynthesis. Chem Biol 15:95–96

Chapter 6
Outer Membrane Proteins

Oliver Mirus, Alexander Hahn, and Enrico Schleiff

6.1 Introduction

Cellular systems are generally surrounded by membranes. The fundamental concept of lipid membranes surrounding cells dates back to the nineteenth century and its formulation marked a new area of "cellular" biology (Edidin 2003). Interestingly, Gram-negative bacteria are even protected by two individual membranes designated plasma, cytoplasmic or inner membrane and outer membrane. The outer membrane was initially described as a lipoprotein layer, but this model was subsequently refined into a mosaic ensemble of lipopolysaccharides (LPS), lipoproteins and phospholipids (e.g., Remsen and Lundgren 1966; dePetris 1967).

Hence, a milestone for the understanding of the membrane systems after their discovery was set by the consideration of proteins as components of the membrane system, even though the initial concept merely considered associated proteins (Danielli and Davson 1935). In subsequent years the model of the membranes was refined by (1) the consideration of membrane-spanning proteins (e.g., Lenard and Singer 1966) and (2) the observation of a highly dynamic ensemble of this protein–lipid mixture leading to the fluid mosaic model (Singer and Nicolson 1972). Later, a trend began in which membrane function was almost exclusively attributed to the protein content, leaving lipids a function as "passive protein carriers" before the lipids re-entered the stage in the late 1990s (e.g., Dowhan 1997). Today it is widely accepted that the coordinated interaction and coaction of all components are required for the function of each membrane.

The membrane proteins can be divided into three classes: helical anchored proteins, lipoproteins and the outer membrane proteins (OMPs) with a so-called

E. Schleiff (✉)
Department of Biosciences, JWGU Frankfurt am Main, Cluster of Excellence Macromolecular Complexes, Centre of Membrane Proteomics, Molecular Cell Biology of Plants, Max-von-Laue Str. 9, 60438 Frankfurt, Germany
e-mail: schleiff@bio.uni-frankfurt.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_6,

β-barrel fold. The helical proteins are the classical constituents of the plasma membrane. The helical conformation of proteins was first demonstrated in non-aqueous solvents (e.g., Singer 1962) and was subsequently confirmed by electron microscopy (Henderson and Unwin 1975). During the last thirty years this picture of membrane proteins has been generalized. The second class of proteins contains the so-called lipoproteins, which are attached to the membrane via an N-terminal *N*-acyl-diacylglycerylcysteine and which are found at both the periplasmic and the extracellular face of the cellular membranes (Bos and Robert 2007). This review, however, will focus on the third class of proteins as they are the major constituents of the outer membrane.

A discrepancy of the secondary structure of integral OMPs when compared to plasma membrane-inserted proteins was first noticed in 1974. Using circular dichroism (CD) spectroscopy and absorption spectroscopy, a high β-sheet rather than an α-helical content was observed in an analysis of the major OMP of *Escherichia coli* (Rosenbusch 1974). Based on topology modeling, Vogel and Jähnig (1986) for the first time suggested an amphiphilic barrel conformation of these proteins, inspired by the observed structural folds of soluble proteins (Richardson 1977). In the following sections. firstly the global properties of such proteins will be described and subsequently the different functional classes of the OMPs and their complexes will be discussed.

6.2 The Outer Membrane Proteins

The outer membrane is the first line of external attack and has a protective function, but at the same time this membrane has to permit the exchange of solutes between the cell and the environment in particular. On top of that the outer membrane is last to receive "support" from cellular utilities, as the nature of signal transduction cascades enforces a delay. Hence, the existence of components in the outer membrane themselves responding to environmental changes is essential for the functionality of this membrane and thereby for cell survival. Such components are the proteins either attached or embedded in this membrane. In the following section the global properties of the proteins embedded in the outer membrane are described.

6.2.1 The Properties of β-Barrel Proteins

Almost all outer membrane-embedded proteins share the same structural concept; they have a β-barrel as the membrane-inserted domain. The β-barrel structure is formed in general by an even number of β-strands which are antiparallel, locally connected to their next neighbors, and the N and C termini are usually facing the periplasmic space (Fig. 6.1a; Schulz 2000, 2002; Wimley 2003). Interestingly, the recently solved structure of the voltage-dependent anion channel – a β-barrel

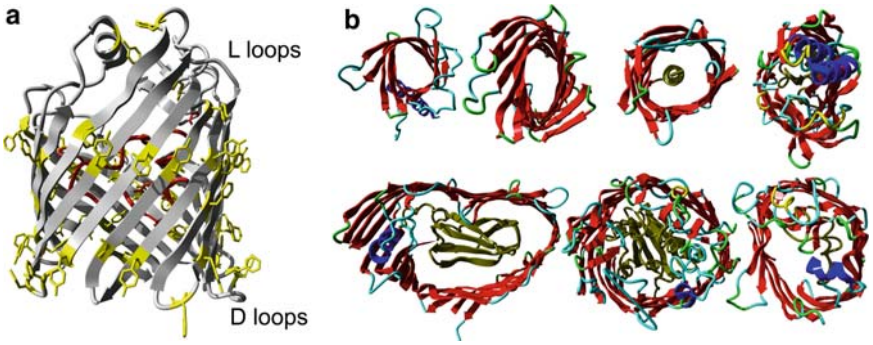

Fig. 6.1 Properties of β-barrel outer membrane proteins. (**a**) The structure of maltoporin–maltotriose complex from *Escherichia coli* (pdb: 1MPN, Dutzler et al. 1996); all aromatic amino acids are *highlighted in yellow* to visualize the aromatic belt on the outer rim of the barrel. The plug domain in the interior of the channel is shown in *red* and the β-strands in *grey*. (**b**) Top view (from the extracellular space) of β-barrels (*from left to right and from top to bottom*) of outer membrane PagP from *E. coli* (pdb: 1THQ, Ahn et al. 2004), outer membrane protease OmpT from *E. coli* (pdb: 1I78, Vandeputte-Rutten et al. 2001), autotransporter NalP from *Neisseria meningitidis* (pdb: 1UYN, Oomen et al. 2004), bacterial fatty acid transporter FadL from *E. coli* (pdb: 1T16, van den Berg et al. 2004), P pilus usher (PapC) translocation pore from *E. coli* (pdb: 2VQI, Remaut et al. 2008), ferric enterobactin receptor FepA from *E. coli* (pdb: 1FEP, Buchanan et al. 1999), and osmoporin from *Klebsiella pneumoniae* (pdb: 1OSM, Dutzler et al. 1999) with 8, 10, 12, 14, 24, 22, 16 β-strands, respectively, to visualize the β-barrel structure and the pore diameter as well as the sealing (plug) domains (*yellow*)

protein of the mitochondrial outer membrane – revealed the first exception of the "even strand number" rule (Bayrhuber et al. 2008; Hiller et al. 2008; Ujwal et al. 2008) and it remains to be explored whether this is an eukaryotic invention or whether barrel proteins with uneven strand numbers may also be discovered in bacteria. The membrane-spanning segment of the β-strands needs to be only seven to nine amino acids long. However, due to the inclination of the strand with respect to the bilayer they can be up to 24 residues long. The loops facing the periplasmic space are typically short (Fig. 6.1a; termed D loops), whereas the external loops are classically rather long (termed L loops). The β-barrel exhibits two surfaces, the hydrophilic channel interior, which can also be charged, and the nonpolar exterior, which consists of aliphatic side-chains. In many cases the aliphatic exterior of the β-strands is bordered by aromatic side chains with intermediate polarity. These aromatic amino acids are thought to contact the two nonpolar–polar interface layers of the membrane and are suggested to stabilize the vertical position of the β-barrel in the membrane. This idea is supported by the observed preference of aromatic amino acids in the nonpolar–polar regions of the membrane (e.g., Yau et al. 1998; Wimley and White 1996).

In recent decades, several structures of membrane-embedded β-barrel proteins have been solved, leading to the proposal of certain rules for this protein class with respect to global structural features and amino acid composition. With respect to the global fold, the barrel is defined by its strand number (n), which typically ranges

Table 6.1 Physical properties of some examples of prokaryotic β-barrel proteins, showing the name, the strand number *n* (in brackets of the monomer), the shear number *S*, the tilt angle α of the strands and a reference of the structure. np, not published

	n	*S*	α (degrees)	Reference
OmpX	8	8	37	Vogt and Schulz (1999)
OmpA	8	10	43	Pautsch and Schulz (1998)
OmpT	10	12	42	Vandeputte-Rutten et al. (2001)
OmpLA	12	12	37	Snijder et al. (1999)
TolC	12 (4)	20	51	Koronakis et al. (2000)
α-Hemolysin	14	14	37	Song et al. (1996)
OmpF	16	20	43	Cowan et al. (1995)
FhaC	16	20	~42	Clantin et al. (2007)
Maltoporin	18	20	40	Dutzler et al. (1996)
FhuA	22	24	39	Eisenhauer et al. (2005)
PapC	24	np	~45	Remaut et al. (2008)

from 8 to 24 and can be formed either by a single molecule or by oligomeric complexes (Fig. 6.1b). The second important parameter is the shear number (Liu 1998). This (integer) number is the minimum amino acid distance within one strand and is thereby a measure for the inclination angle of the strands against the barrel axis. Hence, the shear number is related to the tilt angle of the strands as depicted in Fig. 6.1, and the values for some representative porins are listed in Table 6.1. Here, two parameters are introduced, which represent the distance between two Cα atoms in one strand (a = 3.3 Å) and the distance between two Cα atoms of two amino acids in two neighboring strands forming a hydrogen bond (b = 4.4 Å; Schulz 2002).

$$\tan\alpha = S * a/n/b \tag{6.1}$$

Summarizing, the structure of the β-barrel membrane anchor is quite conserved and the physical properties can be evaluated. However, particularly for the larger barrels, the structural features become more complex as they contain plug domains which regulate the functionality and specificity of the protein. In addition, the N-terminus or the L3 loop of some of the proteins has a folded and functional conformation.

Beside the structural description, several approaches targeted the amino acid composition of the barrel region of the OMPs. At first one has to consider that each transmembrane β-strand faces two different environments, namely the hydrophobic exterior of the β-barrel and the aqueous hydrophilic pore interior. This results in an alternating hydrophobic–hydrophilic residue profile with the exception of β-strands, for example, facing an oligomerization interface. Hence, the alternation of hydrophobic and polar residues in the membrane spanning β-strands was used quite early on to assist prediction of β-barrel membrane proteins (Vogel and Jähnig 1986). Subsequently, the first property used for prediction was the so-called alternating hydrophobicity of a protein segment – as represented in Eqn. 6.2 – where h is the hydrophobicity (usually to the scale of Eisenberg et al. 1982, 1984 or Wimley

and White 1996) assigned to the indicated amino acid at position i of the sequence by screening the entire sequence with a sliding window.

$$H_a(i) = [h(i-2) + h(i) + h(i+2) + h(i+4)]/4. \quad (6.2)$$

An extension of this equation is the β-strand moment according to the definition of the helical moment (Eisenberg et al. 1982, 1984). However, it became clear that the application of such simple rules is prone to false-positive predictions and therefore do not represent an ultimate description of membrane-inserted β-barrels. Subsequent to the structure determination, the focus was on the statistical analysis of the amino acid composition of the membrane-inserted β-strands. A "membrane β-strand scale" was developed by aligning the structures with respect to the hypothesized lipid bilayer midplane and determining the statistical frequencies of the residues within the β-strands. This approach was taken even further to discriminate between the frequency within the fatty acid chain (core) region and the head group (interface) region (Table 6.2; Wimley 2002). From this analysis it became evident that tyrosines, phenylalanines and tryptophans are abundant external amino acids in the interface, which is consistent with the structurally determined aromatic belt. In the core region (facing the membrane), besides tyrosine, hydrophobic amino acids like valine and leucine are enriched. In contrast, no preference for certain amino acids was detected for the channel interior.

These two global features were subsequently applied for the identification of new OMPs in prokaryotes (Wimley 2002) and eukaryotes (e.g., Schleiff et al. 2003; Mirus and Schleiff 2005). However, the hydrophobicity pattern and the defined

Table 6.2 The membrane β-strand scale according to Wimley (2002). The values given are the abundance of amino acids in transmembrane β-strands outside (external) or within (internal) the channel in the interface or core region of the membrane normalized to the genomic occurrence of the amino acid

	External face		Internal face	
	Interface	Core	Interface	Core
Ala	0.55	1.19	0.86	0.97
Arg	0.042	0.04	0.85	0.95
Asu	1.0	0.38	1.42	1.83
Asp	0.13	0.13	1.75	0.67
Cys	0.02	0.02	0.02	0.02
Gln	0.34	0.29	1.54	1.35
Glug	0.041	0.02	0.98	0.75
Gly	0.54	0.88	2.06	2.27
His	0.33	0.33	0.61	0.35
Ile	1.4	0.95	0.23	0.22
Leu	1.1	2.37	0.29	0.27
Lys	0.22	0.02	0.98	1.23
Met	0.62	0.80	1.10	0.48
Phe	4.0	2.15	0.36	0.34
Pro	0.38	0.71	0.49	0.06
Ser	0.41	0.29	1.82	2.13
Thr	0.48	1.18	1.87	2.24
Trp	5.3	0.69	0.19	0.37
Tyr	6.7	3.17	1.45	2.02
Val	1.8	2.20	0.30	0.43

amino acid composition have their limits in identification of new membrane-inserted β-barrel proteins (Mirus and Schleiff 2005). On one hand the sequence variability of β-barrel membrane proteins is high compared to soluble proteins, leading to different amino acid patterns not yet reflected by solved crystal structures. In the same light the alternating hydrophobicity profile is not necessarily valid for the oligomerization surface of the β-barrel. On the other hand, the species-specific codon usage is not reflected in the statistics and might impede the detection of β-barrel proteins in various species. Nevertheless, all three features – structural properties, the hydrophobic nature of the outer rim of the barrel and the amino acid distribution – to a large extent globally characterize membrane-inserted β-barrel proteins.

6.2.2 *Why Is the Membrane Anchor a β-Barrel and Not an α-Helix?*

This question cannot yet be answered to full satisfaction. In the past several hypotheses have been made. For instance it was discussed whether the stability of β-barrel structures against denaturation might have been an evolutionary advantage as this would provide a protection against bile salts. However, this notion is questionable in the light of the general stability of membrane-embedded proteins. An alternative idea was that β-barrels have evolved as a consequence of intimate interactions with LPS in the outer leaflet of the outer membrane (Klebba 2002). Such interaction was envisioned as a protective property of the outer membrane towards detergents and bile salts. However, even though interaction between LPS and β-barrel proteins has been reported, the interaction is mostly mediated by the hydrophobic acyl-chains, as found for the interaction between inner membrane proteins and phospholipids (e.g., Marsh 2003)

The most plausible explanation at present is related to the transport of proteins across the outer membrane (see Sect. 6.6.2). A helical transmembrane region would consist of at least 15–18 amino acids with a high hydrophobicity, which generally serves as a signal for the integration of such region into the plasma membrane. Hence, OMPs would not be able to pass the plasma membrane. A β-strand only requires 5–9 amino acids to span the outer membrane and because of its amphipathic nature a transmembrane β-strand is less hydrophobic (Tommassen 1988). Therefore, the signature of the transmembrane β-strand is distinct from that of a transmembrane helix and can cross the inner membrane. Hence, nature might have selected for proteins which could be secreted across the plasma membrane and could still be integrated into the outer membrane.

6.2.3 *The Functional Concepts of Outer Membrane Proteins*

Outer membrane β-barrel transporters can be divided into four groups (Buchanan 2001; Tamm et al. 2004). The first group is composed of the so-called classical

porins (Sect. 6.3), which are 16-stranded β-barrels (Fig. 6.1b; Table 6.1) and function as homotrimers composed of three individual pores (Weiss et al. 1991; Cowan et al. 1992). It is proposed that these are the doors for hydrophilic solutes up to approximately 600 Da. The second group comprises substrate-specific porins (Sect. 6.4). They, however, vary with respect to their strand number and different oligomerization behavior. The best studied examples are transporters for sugar or iron-bound siderophores. Their substrates are proposed to be recognized after translocation across the outer membrane by substrate-specific binding proteins and shuttled further to the inner membrane ATP-binding cassette (ABC) transporters. ABC transporters utilize energy generated by ATP hydrolysis to perform an active transport across the inner membrane into the cytoplasm (Buchanan 2001). The third group of OMPs is not involved in the uptake or export of substrates but in the biogenesis of the outer membrane (Sect. 6.6). Here three prominent proteins are Omp85 involved in the biogenesis of outer membrane porins (e.g., Schleiff and Soll 2005), Imp/ OstA involved in the export of lipid A (e.g., Ruiz et al. 2006) and Wza required for polysaccharide export (Whitfield 2006). These proteins are essential as their function is required for the functional integrity of the outer membrane in general. The last group of proteins, or rather of complexes with components in the outer membrane, concerns the secretion and export of solutes and proteins. Here a large diversity exists as different environmental situations have enforced the evolution of different complexes (Sect. 6.7). In the following, the different outer membrane-embedded proteins will be discussed.

6.3 The Porins

6.3.1 The "Classical" Porins

Nonspecific diffusion of hydrophilic solutes across the outer membrane is essential for the viability of bacteria and this diffusion usually occurs through porins. The flux generated is proportional to the concentration gradient, in this case between exterior and periplasmic space. The three proteins first described as porins are PhoE, OmpF and OmpC from *Escherichia coli* (*E. coli*). These β-barrel proteins with 16 strands and a pore diameter of about 1.2 nm allow the passive diffusion of small hydrophilic solutes with a molecular weight of up to 600 Da (e.g., Nakae 1975). Based on this observation it was proposed that the outer membrane functions as a "molecular sieve". In the outer membrane these proteins form a trimeric complex. In general OmpF and OmpC are slightly cation-selective, whereas PhoE is slightly anion-selective, even though PhoE shares about 72% sequence similarity with OmpF. The difference in charge preference of these two proteins is governed by a single amino acid substitution, namely the replacement of Gly131 in OmpF with the positively charged Lys125 in PhoE (Bauer et al. 1989; Cowan et al. 1992). Furthermore, the active OmpC pore appears to be smaller than the pore formed by

OmpF based on the diffusion of model substrates (Nikaido and Rosenberg 1983; Nikaido et al. 1983). This observation together with the fact that *E. coli* lives in the intestinal tract explains the regulation of their expression in response to osmotic stress. Under high osmotic strength or high temperature, the expression of the protein with the smaller channel (OmpC) is favored, whereas the production of OmpF is repressed. In turn, under low-osmolarity conditions or low temperatures the production of OmpF is enhanced. The regulation occurs by the EnvZ–OmpR two-component system (Sect. 6.4). In contrast, PhoE is a component of the phosphate regulon, which is induced by phosphate starvation (Tommassen and Lugtenberg 1982). Nevertheless, the three porins are very similar in sequence and structure and even form heterotrimeric complexes in native membranes (Gehring and Nikaido 1989).

By computational modeling it was discovered that anions and cations take different routes within the channel; cations flow close to the negative charges of the L3 loop, and anions near the positively charges at the opposite barrel wall (Im and Roux 2002). These observations suggested an interaction of the ions with the channel interior and was thereby consistent with results obtained by electrophysiological experiments while varying ionic strength or pH (e.g., Saint et al. 1996; Alcaraz et al. 2004). For example, the selectivity of OmpF for cations relative to anions increases at low ionic strength and it reverses the ion specificity at $pH < 4$ (Alcaraz et al. 2004). Furthermore, the selectivity was shifted away from the cation selectivity by mutation of the acidic amino acid aspartate within the channel (Saint et al. 1996) and was enhanced by mutation of positively charged arginines of the constriction zone (Alcaraz et al. 2004). In contrast, a correlation between conductance and the presence or absence of large amino acids within the channel is not as evident, strongly supporting the notion that ions do indeed interact with the channel interior.

However, porins are not always in an open conformation in the outer membrane. Analysis of physical properties of classical porins revealed the existence of a so-called threshold potential of about 150–200 mV (e.g., Lakey et al. 1991; Saint et al. 1996). This means that porins can exist in a closed conformation, which can be induced by changes in environmental conditions such as pH or presence of polyamines (delaVega and Delcour 1995; delaVega and Delcour 1996). For example, cadaverine, a polyamine endogenously produced by *E. coli* during anaerobic growth in the presence of its precursor amino acid lysine and associated with the outer membrane after its export from the cytosol (Koski and Vaara 1991), induces the closure of porins, which explains the reduced permeability of the outer membrane induced by cadaverine (e.g., Samartzidou and Delcour 1999). Based on such observations, it is proposed that rapid modulation of porin function by cadaverine secretion could provide an emergency system for short-term and rapid reduction of the outer membrane permeability. However, these observations have an additional impact. Porins are targets for antibiotics and the observed alteration of selectivity depending on the environmental conditions might subsequently lead to a reduced uptake of antibiotics (delaVega and Delcour 1995). Hence, in recent years the importance of the classical porins has moved from simple sieves to well-defined

drug targets and their molecular properties with respect to this function are currently under investigation (Delcour 2009).

6.3.2 Larger, Not as Fast, but Still Classical?

The main porin, for example of *Pseudomonas aeruginosa*, OprF (Hancock et al. 1979), is somewhat distinct in its properties from the three porins described above. The same holds true for its *E. coli* homolog OmpA. It appears that these proteins do not form trimeric complexes in the membrane and they show a distinct size selectivity compared to the porins mentioned above. The diffusion of small solutes such as monosaccharides is significantly slower through OprF than through OmpF (Yoshimura et al. 1983), but at the same time OprF can transport larger solutes than OmpF (Hancock et al. 1979). For example it was demonstrated that production of raffinose (505 Da) – which cannot diffuse through the major *E. coli* porins – leads to the survival of *oprF*$^+$ cells, but not of *oprF* mutants (Bellido et al. 1992).

But how can this obvious discrepancy between reduced transport rate and larger solute acceptance be explained? For the major OMP OmpA (Chai and Foulds 1977) a two-domain structure was proposed based on genetic and biophysical evidence (Bremer et al. 1982; Vogel and Jähnig 1986). It was suggested that the N-terminal portion forms a β-barrel domain composed of eight membrane-spanning β-strands, whereas the C-terminal portion of the protein was proposed to form a periplasmic domain interacting with the peptidoglycan layer (e.g., Koebnik and Kramer 1995). The structure predicted for the N-terminus was subsequently confirmed by determination of crystal structure (Pautsch and Schulz 1998). Interestingly, the structure revealed the absence of a passage for water or solutes within the barrel as the channel is blocked by amino acids which form salt bridges (Fig. 6.2a). This apparently would contradict the ion permeability observed by electrophysiology and the estimated pore diameter of about 1 nm (Sugawara and Nikaido 1992). However, a high flexibility of the salt bridges in the channel interior was observed by NMR experiments and molecular dynamic simulations (Arora et al. 2001; Bond et al. 2002). These observations could be confirmed by the analysis of the gating behavior of mutants of the OmpA targeting these salt bridges (Hong et al. 2006). Nevertheless, the observed salt bridge network and its flexibility explain why the passage of small substances occurs at a slower rate than that found for the other porins. However, it does not yet explain why larger molecules can pass the membrane barrier in an OmpA-dependent manner. Here, the current model was proposed by Stathopoulos (1996). It was proposed that OmpA could form a 16-β-stranded barrel involving the usually periplasmic localized C-terminus (Fig. 6.2b). Indeed such a model is consistent with some experimental evidence. For instance it was observed that only the full-length protein, but not the region forming the N-terminal 8-stranded barrel, can form large pores (Arora et al. 2000). In linewith this, it was observed that the larger channels are thermally inducible and that the formation of larger channels is subsequently irreversible (Zakharian and Reusch 2005). However, the crystal structure of the C-terminus of RmpM, which is

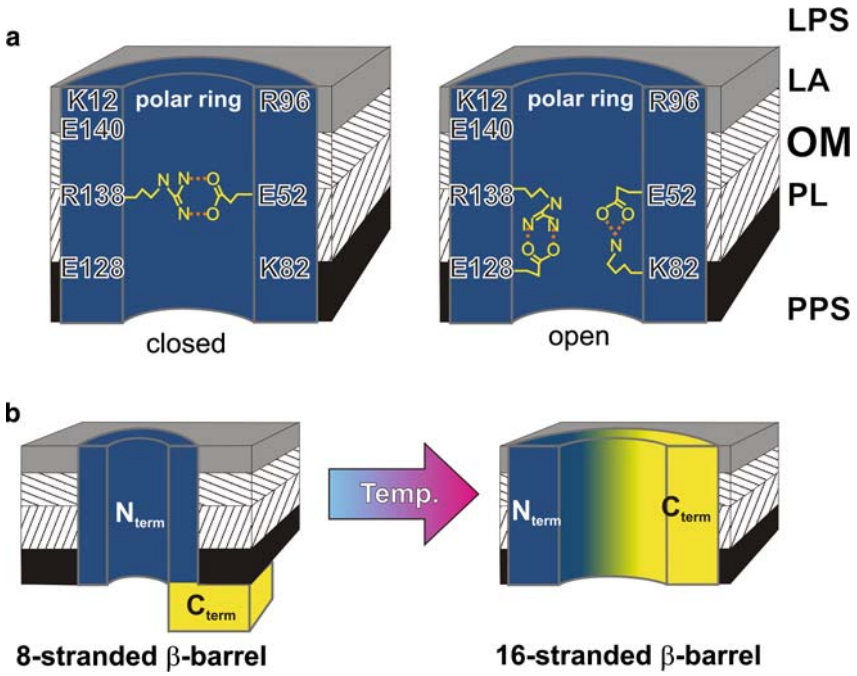

Fig. 6.2 The regulation of OmpA. (**a**) The essential residues involved in the gating of the small channel of OmpA are highlighted. There are charges on the outer surface termed the polar ring. The essential amino acids regulating the opening and closing of the channel are R138, E128, E52 and K82. R138 forms a strong electrostatic interaction with E52 and the resulting salt bridge blocks ion transport through the channel (*closed*). K82 and E128 may serve as counterpart to this interaction and the formation of the two alternative salt bridges would open the channel for ion fluxes (*open*). (**b**) According to the model proposed by Stathopoulos (1996), the channel of OmpA can extend to a 16-stranded β-barrel in a temperature-dependent manner resulting in a channel able to transport large molecules. The figures are according to the models presented in Smith et al. (2007) and Bond et al. (2002). Abbreviations: LA, lipid A layer; LPS, lipopolysaccharide layer of the outer membrane; OM, outer membrane; PL, phospholipid layer of the outer membrane, PPS periplasmic space

homologous to the C-terminus of OmpA, revealed a β-sheet system covered by two helices (Grizot and Buchanan 2004), which explains the hydrophobicity profile leading to the false-positive prediction of membrane-inserted sheets. Furthermore, to the best of our knowledge the final proof that the C-terminus indeed forms membrane-inserted strands has not yet been provided. Hence, the question remains whether the current model for the transport of larger molecules holds true.

6.3.3 The Large Diversity of Porins and Porins Without Pore

After the discovery of the major porins PhoE, OmpF and OmpC in *E. coli*, many additional porins have been described in various species such as *E. coli*. In this

section only a few can be covered, particularly with respect to the porins of species other than *E. coli*. Nevertheless, the principle behind translocation of solutes across the outer membrane is similar to that of the described porins, and only variations of the general theme occur. In this section we focus on the current understanding of some distinct proteins with unique regulatory or functional properties.

OmpD and OmpG are members of the 16-stranded channel proteins. OmpD is a member of the porin family with similarity to OmpF and OmpC. The protein was first isolated from *S. enterica* serovar Typhimurium (Benz et al. 1980) and subsequently in *S. enterica* serovar Enteritidis. The gene is positioned downstream of a gene *yddG* encoding a putative transporter, and a hetero-oligomeric complex was proposed (Chart et al. 1993). Strikingly, the synthesis of OmpD is dependent on the cyclic AMP receptor protein Crp, for which a binding site could be identified (see also Sect. 6.5; Santiviago et al. 2003). Based on this observation Santiviago et al. (2003) postulated that OmpD may be involved in the efflux of toxic compounds made during the catabolism of alternative, poorer carbon sources. OmpG from *Salmonella typhimurium* is a major target of antibodies of infected patients and hence might represent an optimal target for vaccine development (Gil-Cruz et al. 2009). OmpG is a monomeric porin with unusually large channels (Misra and Benson 1989; Fajardo et al. 1998), as confirmed by single-channel conductance and folding studies (Conlan and Bayley 2003; Conlan et al. 2000). The *ompG* gene appears to be a gene in an operon which contains genes involved in uptake of oligosaccharides (Fajardo et al. 1998) and it was proposed to be involved in the transport of larger oligosaccharides across the outer membrane.

Beside 16-stranded barrels, several barrel proteins with 8, 10 or 12 strands are present in the outer membrane. The important functional units within these proteins are the external loops which interact with substrates and targets such as the surface of host cell membranes, and the barrel only serves as a membrane anchor. Members of the 8-stranded barrel protein family are, for example, PagP (Fig. 6.3a; Hwang et al. 2002), OmpX of *E. coli* (Vogt and Schulz 1999) or NspA (Vandeputte-Rutten et al. 2003). PagP, for example, is the only known outer membrane-localized enzyme involved in lipopolysaccharide biosynthesis in *E. coli* and it transfers a palmitoyl residue from phospholipid to the R-3-hydroxymyristate chain at position 2 of lipid A (Fig. 6.3c). Its palmitate recognition pocket, known as the hydrocarbon ruler, resides within the interior of the β-barrel and is only accessible from the outer membrane external leaflet (Ahn et al. 2004). Hydrocarbon rulers are generally "molecular measuring devices" to distinguish between fatty-acyl groups with varying numbers of carbon atoms and are found in several molecules (Wyckoff et al. 1998, Tarshis et al. 1996). In the case of PagP, the ruler has specificity for 16-carbon acyl-chains (Guo et al. 1998) and this specificity is defined by a single glycine residue at position 88 (Ahn et al. 2004). Routes for the lateral access of lipids into the hydrocarbon ruler are provided by two discontinuities in β-strand hydrogen bonding enforced by a conserved proline within the LPS-exposed region (Ahn et al. 2004). It is envisioned that catalysis proceeds when lipid A and the phospholipid form a ternary complex with the enzyme (Bishop 2005).

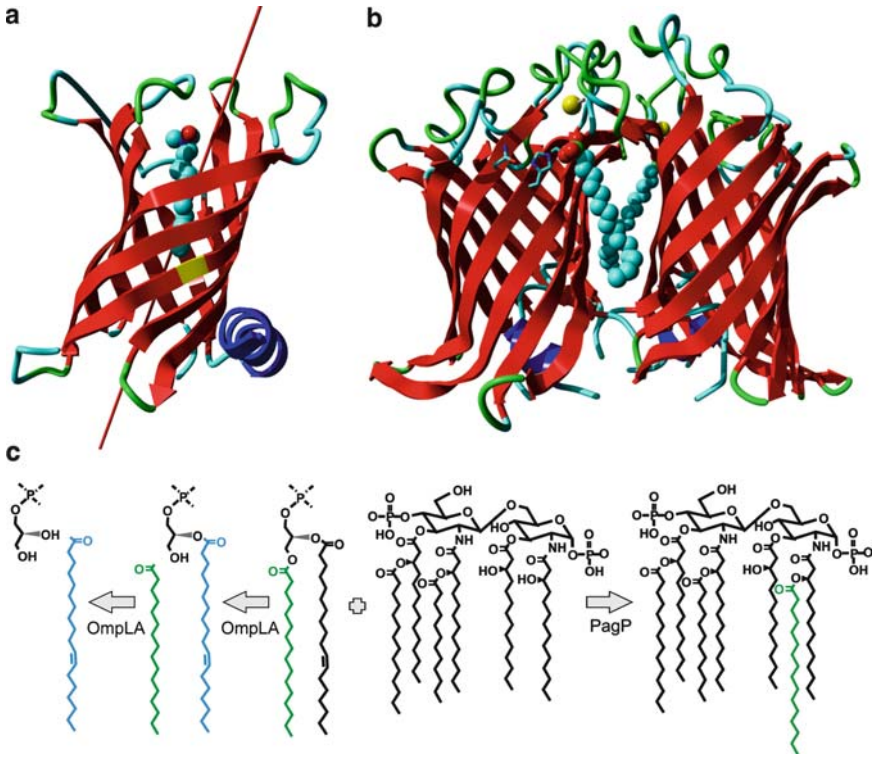

Fig. 6.3 PagP and OmpLA. (**a**) The structure of PagP from *E. coli* is shown (pdb: 1THQ; Ahn et al. 2004). The protein is complexed with a bound lauroyldimethylamine-*N*-oxide molecule (ball representation), which defines the hydrocarbon ruler. The barrel is tilted by 25° as indicated by the orientation of the barrel axis. (**b**) The structure of the dimeric conformation of the outer membrane phospholipase OMPLA from *E. coli* (pdb: 1QD6; Snijder et al. 1999) complexed with bound Ca^{2+} (*yellow*) and hexadecanesulfonyl group (ball representation). (**c**) PagP catalyzes transfer of a palmitate chain (C16:0) from the sn-1 position of a phospholipid to lipid A (*right section*). In the presence of calcium ions OMPLA catalyzes the production of lysophospholipid and the enzyme also displays lysophospholipase activity, at least in vitro (*left section*). The figures were generated according to Bishop (2005, 2008)

The outer membrane-associated protease OmpT or the opacity proteins of *Neisseria meningitidis* are examples of 10-stranded barrels, as confirmed by structure determination (Prince et al. 2002). An example of a 12-stranded barrel is the outer membrane-associated calcium-dependent phospholipase A (OmpLA; Fig. 6.3b; Scandella and Kornberg 1971) exhibiting a function which can be placed in relation to PagP. In an outer membrane with native asymmetry, the protein is monomeric and formation of homodimeric complexes within the membrane environment is triggered by the lipid substrate and calcium (Dekker et al. 1997), and the effect of both molecules is additive (Stanley et al. 2006). The relation to calcium is further manifested by the influence of divalent ions on the outer membrane, as their

presence perturbs the asymmetry and enforces transport of glycerophospholipids into the outer layer of the outer membrane. The recycling of these molecules would be initialized by the function of OmpLA cleaving the glycerophospholipids and producing a fatty acid and lysophospholipid, which subsequently will be degraded by OmpLA as well (Fig. 6.3c). The protein selects a fatty acyl-chain at either the sn-1 or sn-2 position of the glycerophospholipid (Horrevoets et al. 1989). OmpLA is unspecific for the head groups and binds fatty acyl-chains of 14 carbons or more (Stanley et al. 2007). The fatty acids have to be removed from the outer membrane as they would have bilayer destabilizing properties as well. This might be utilized by the FadL- dependent system as described later (Sect. 6.4.2). Based on the structures determined in monomeric and dimeric conformation it was suggested that the enzymatic unit is only formed upon dimerization (Snijder et al. 1999). The interior of the barrel formed by OmpLA appears to be closed by a hydrogen-bonding network such that the protein does not function as a channel. In line with this, the three central residues involved in substrate recognition and processing, namely Asn156, His142, and Ser144, are localized on the exterior of the channel, forming the catalytic center.

Summarizing, proteins classified as "porins" can exhibit different properties. They might form pores for solute transport (see also Sects. 6.3.1 and 6.3.2), pores for substrate processing without transport function such as PagP, or simply β-barrel structures for anchoring enzymatic activities in the outer membrane (OmpLA). The function of the membrane-inserted domain, however, cannot simply be extracted from the barrel size, as OmpA (Sect. 6.3.2) with its eight sheets forms a translocation pore, whereas OmpLA with its 12 sheets is only the membrane anchor for an enzyme. However, as outlined throughout the previous sections, all of them are related to the functionality of the cell and have to be tightly regulated, for instance at transcript level as described in the following section. For further reading on the diversity of porins we would like to draw the attention of the interested reader to the review article by Nikaido (2003) in which the porins of other species are discussed.

6.4 Substrate-Specific Porins

6.4.1 Porins for Sweets

The solute flux across the membrane is dependent on the chemical gradient because of the passive nature of the transport. At low concentrations of required solutes, one option to enhance diffusion would be to enlarge the number of channels (e.g., Forst et al. 1998). However, this poses the risk of disproportionate uptake of salts as well. To overcome this problem substrate-specific porins – so-called specific diffusion channels – have been evolved containing a binding site for the solute to be transported. Prominent examples are transporters for maltodextrins, sucrose, nucleotides (Sect. 6.4.1), fatty acids (Sect. 6.4.2) or siderophores (see Sect. 6.4.3).

Maltoporin (LamB) is an outer membrane transporter specific for maltosaccharides (maltodextrins; Szmelcman and Hofnung 1975), which even can bind saccharides such as starch (e.g., Ferenci et al. 1980). Furthermore, LamB is able to transport melibiose and trehalose and thus can perceive several different saccharides. The dissociation constant (K_D) for different sugars ranges from 10 mmol/L (for maltose) to 60 μmol/L for, e.g., maltopentaose (Andersen et al. 1995). Detailed studies concerning the substrate recognition revealed that the alteration of the dissociation constant is dominated by changes of the off rate rather than the association rate of the different solutes. The protein is composed of an 18-stranded β-barrel (Table 6.1) and forms a trimeric complex in vivo. The outstanding feature observed in the structure determined (Schirmer et al. 1995) is a stretch of aromatic residues inside the channel arranged along a left-handed path named "greasy slide" (Fig. 6.4a). In addition, polar residues are also situated at the constriction site (and are termed polar tracks; Fig. 6.4b). They are proposed to play an important role in maltose and maltodextrin translocation.

Remarkably, the sucrose-specific porin ScrY shows similar structural features to LamB (Forst et al. 1998). However, in contrast to LamB, ScrY allows the diffusion of multiple solutes such as glucose, fructose, arabinose, maltose, lactose, raffinose and maltodextrins in addition to sucrose (Hardesty et al. 1991; Schmid et al. 1991; Schuelein 1991; Andersen et al. 1998). The major difference between LamB and ScrY, which explains the difference in selectivity, can be assigned to three amino acids (LamB: R109, Y118, D121; Fig. 6.4b; ScrY: N192, D201, F204). These residues are at the channel constriction site and hinder transport of the molecule by sterically blocking the fructose ring (in case of LamB). Indeed, replacing these three amino acids in ScrY by those of LamB results in a LamB-like specificity of the ScrY mutant (Ulmke et al. 1999) and the reverse experiment enabled the LamB mutant to transport sucrose (van Gelder et al. 2001). Therefore, cargo discrimination by LamB appears to be the result of steric hindrance rather than specific interaction inside the channel. Based on the crystal structures and many experimental approaches, a model of the molecule transport by maltoporin was recently proposed, and we would like to draw the attention of the interested reader to this article (Ranquin and van Gelder 2004).

Another described substrate-specific diffusion channel with homology to LamB and ScrY is the trimeric BglH (Wang et al. 1997). The protein appears to be specific for aryl-β-glucosides such as arbutin (4-hydroxyphenyl-β-glucoside) or salicin (2-hydroxymethylphenyl-β-glucoside). An apparent dissociation constant for the substrate of about 1–3 mmol/L was determined by transport inhibition experiments (Andersen et al. 1999). However, the reason for the functional distinction remains to be explored.

The nucleoside transporter Tsx is somewhat distinct from the substrate-specific channels described above. The protein is rather small at only 31.4 kDa and forms a 12-stranded β-barrel (Ye and van den Berg 2004). At present there is no convincing evidence for an oligomeric state in the outer membrane. It is suggested that Tsx functions as a substrate-specific channel for nucleosides and deoxynucleosides (Bremer et al. 1990). The presence of the protein further results in uptake of the

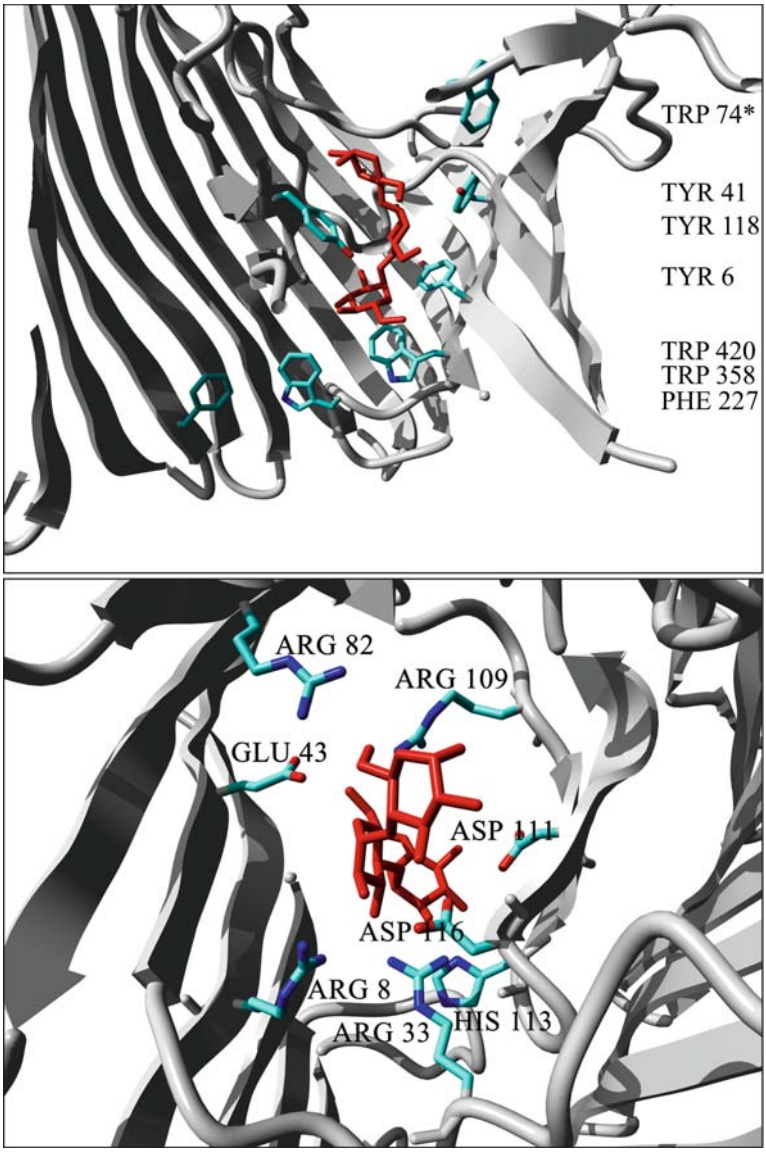

Fig. 6.4 The "greasy slide" and the "polar tracks" of maltoporin. Shown is the interior of the LamB maltoporin (pdb: 1MPN, Dutzler et al. 1996; barrel shown in *grey*) complexed with maltotriose (*red*). *On the top* the amino acids forming the "greasy slide" and *on the bottom* the amino acids of the "polar track" are indicated

antibiotic albicidin, and albicidin-resistant mutants generally contain alterations in this protein (Birch et al. 1990). In the same category of proteins are CymA and KdgM. CymA identified from *Klebsiella oxytoca* is a protein of only 322 residues

(Fiedler et al. 1996), which exists in the outer membrane as a monomer (Pajatsch et al. 1999). The protein binds cyclodextrins at high affinity, as judged from the blocking of the ion conductance produced by this protein, and many features of this protein are reminiscent of those of Tsx. KdgM (of *Erwinia chrysanthemi*; Blot et al. 2002) acts as a monomer and forms an oligogalacturonate channel, and a 14-stranded β-barrel is suggested (Pellinen et al. 2003). Hence, it appears that a Tsx-like channel can be found in several bacteria.

6.4.2 FadL: The Porin for Hydrophobic Molecules

Another challenging task for bacteria is the uptake of long chain fatty acids (LCFA). It is proposed that the need for this uptake is caused by the high energetic costs of fatty acid synthesis and by the requirement of LCFAs as carbon sources (e.g., Black 1991). However, hydrophobic molecules are generally not recognized by the classical porins described above. Hence, a specific transporter for these molecules exists in the outer membrane of many Gram-negative bacteria, namely FadL (in *E. coli* (Black et al. 1987); also named TbuX in *Ralstonia picketii* (Kahng et al. 2000), XylN and CymD in *Pseudomonas putida* (Kasai et al. 2001; Eaton 1997), SalD in *Acinetobacter* (Jones et al. 2000), and CumH in *Pseudomonas fluorescens* (Habe et al. 1996)). This OMP acts in a consortium together with the inner membrane-associated long-chain acyl-CoA synthetase FadD and the long-chain acyl-CoA-responsive transcription factor FadR (Fig. 6.5a). The coaction of these three components leads to the regulation of gene expression in response to LCFA uptake (DiRusso and Black 2004).

FadL is a protein of 46 kDa which apparently exists as a monomer in the outer membrane (Black and DiRusso 1994) forming a 14-stranded β-barrel (van den Berg et al. 2004). The barrel is closed by an N-terminal compact domain of 42 amino acids, which form three short helices. In comparison to the other substrate-specific channels of the outer membrane, FadL exhibits a very high affinity for substrates; the K_D for oleate, for example, is 2×10^{-7} mol/L and mutagenesis experiments revealed the binding side close to the C-terminus (Kumar and Black 1993). Interestingly, oleoyl alcohol and methyl oleate are unable to compete for the binding of FadL to oleate (Black 1990). This suggests that the carboxylate of the fatty acid is required for this interaction and supports the idea that hydrophobic interactions occur between the protein and the acyl chain of the fatty acid chain.

Based on the structure(s) determined, a model for the transport of LCFAs across FadL has been proposed (Fig. 6.5b; van den Berg et al. 2004) and was recently challenged and confirmed by molecular dynamics simulations (Zou et al. 2008). At first the substrate is recognized by a hydrophobic groove (Fig. 6.5b, step 1) between the extracellular loops L3 and L4. This in turn results in a high "local" concentration of the substrate. Second, the substrate diffuses into the binding pocket (Fig. 6.5b, step 2). This is proposed to induce a structural change in the N-terminus, which in turn is thought to lower the affinity for substrate and to create the first part

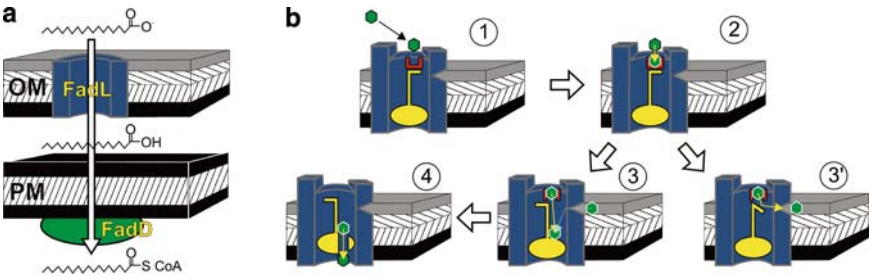

Fig. 6.5 Functional model of FadL action. (**a**) FadL is located in the outer membrane and recognizes long-chain fatty acids. The transfer of the long-chain fatty acid to the cytoplasmic side of the plasma membrane remains elusive. In the cytosol the FadD attached to the inner membrane removes the long-chain fatty acids from the inner membrane and converts them to long-chain acyl-CoA. (**b**) Model of long-chain fatty acid transport across the FadL protein. At first the substrate (*green*) binds into the low-affinity binding pocket (*1*) and then diffuses into the high-affinity binding pocket (*red, 2*). For the subsequent transport two alternative routes were proposed. On the one hand, a conformational change of the N-terminus of the "hatch" results in opening of a passageway (*3*) and further conformational changes in the hatch lead to a channel formation (*4*) for the transport into the periplasm. Alternatively (*3′*) a barrel wall opening formed by the kink in strand S3 results in substrate release to the outer membrane after conformational change of the N-terminus of the "hatch." The models presented were generated according to van den Berg (2005) and Hearn et al. (2009). Abbreviations: PM, plasma membrane; OM, outer membrane

of the substrate channel (Fig. 6.5b, step 3). The initiated substrate movement is proposed to induce additional conformational changes that create the final part of the channel for substrate movement into the periplasm (Fig. 6.5b, step 4). Hence, in contrast to the TonB-dependent transporters (see Sect. 6.3.3), the movement of the hatch of FadL does not require an additional energy supply and might take the energy for the structural rearrangements from the high affinity of the substrate receptor interaction mentioned above. Thermal fluctuations in the structure are probably sufficient not only to release the substrate from its binding site but also to generate a passageway through the hatch domain (van den Berg et al. 2004). This transport model is supported by molecular dynamics simulations, which revealed multiple pathways for fatty acids through the pore (Zou et al. 2008). The model was recently extended, as Hearn et al. (2009) propose a "lateral diffusion" transport model. A kink in the transmembrane β-strand S3 creates a lateral opening in the β-barrel, which facilitates the diffusion of hydrophobic substances into the outer membrane (Fig. 6.5b, step 3). Alternatively, this pathway could also be envisioned as an additional entrance for the fatty acids produced by OmpLA (see Sect. 6.2.3). Hence, it might be possible that either the postulated pathways function in parallel or that one of them has to be rejected by future analysis.

6.4.3 The TonB-Dependent Transporters in the Outer Membrane

Bacteria have to incorporate iron to avoid iron starvation. The outer membrane hosts proteins specialized for this duty. Nevertheless, the iron concentration

within the cell is higher than in the surroundings and hence these transporters actively pump siderophores against a concentration gradient into the periplasm. They are supplied with energy from a cytoplasmic protein complex via TonB (Faraldo-Gómez and Sansom 2003), which is important for the functionality of the TonB-dependent outer membrane transporters (TBDTs) as *ton*B mutants are unable to transport siderophores (Hantke and Braun 1975). Three large classes of TBDTs are defined, namely transferrin/lactoferrin, porphyrin and siderophore transporters (Braun and Killmann 1999). The transferrin or lactoferrin uptake is mediated by the TBDTs TbpA and LbpA, respectively, which is assisted by the extracellular outer membrane-localized TbpB and LbpB (Faraldo-Gómez and Sansom 2003). Porphyrin-transporting TBDTs are, e.g., HasR, HgbA, HmbR, which transport heme (Perkins-Balding et al. 2004), and BtuB, the transporter for vitamin B_{12} (e.g., Ferguson and Deisenhofer 2002). The uptake of heme-type solutes is especially important in bacterial pathogens, where various heme-containing compounds are utilized (Lee 1995).

The most common mechanism of bacterial iron acquisition is iron-chelator (siderophore)-mediated. To avoid iron starvation, bacteria secrete low-molecular-weight iron chelators (siderophores) under iron-limiting conditions to complex environmental iron (e.g., Andrews et al. 2003). After association with iron, the siderophore–iron complexes are bound by receptor proteins in the outer membrane (e.g., Neilands 1995; Crosa and Walsh 2002; Wandersman and Delepelaire 2004). TBDTs are also able to bind iron-free siderophores, but the biological function of this interaction is still unknown (Schalk et al. 2004). The siderophore-dependent iron transporters are usually subclassified according to their substrate. Siderophores are divided into the classes of hydroxamates (ferrichrome), catecholates (e.g., enterobactin) or ferric citrate. Beside the ones for iron transport, TBDTs for other molecules, e.g., sucrose (SuxA; Blanvillain et al. 2007), maltose (MalA; Neugebauer et al. 2005), oligosaccharides (CsuF; Cheng et al. 1995), polysaccharides (SusC; Reeves et al. 1996) and degradation products of proteins (RagA; Nagano et al. 2007) have been described.

Many TonB-dependent iron transporters have been described and functionally characterized (see Table 6.3). They all posses similar structural units, namely a β-barrel spanning the outer membrane, a siderophore-binding pocket formed by the extracellular loops, a plug domain within the barrel and a TonB box which accomplishes the interaction with the periplasmic TonB (Fig. 6.6a). In general, the molecular function and action of all these transporters is comparable and two models for siderophore import have been proposed so far. On one hand, results of fluorescence experiments indicate that the plug domain is totally removed from the β-barrel domain in the case of FepA (Ma et al. 2007). On the other hand, a partial unfolding of the plug domain forming a transient channel was observed for FhuA (Eisenhauer et al. 2005). That such channel formation might be caused by TonB exerting a pulling force via the TonB-box was suggested based on molecular dynamics simulations of BtuB (Gumbart et al. 2007). These in silico experiments revealed that the energy required to totally remove the plug domain is an order of magnitude larger than the energy that can theoretically be provided by TonB.

Table 6.3 List of all experimentally characterized TBDTs. The first column gives the annotated name, the second column the GenBank ID, the third column the species, the fourth column the identified siderophores recognized by the according protein, and the fifth column a representative reference (o.a.: only annotated). A "[T]" after the name indicates a transducer. "[metal]" (column 5) indicates that the type of transported metal ion is known, but the associated metallophore has not yet been identified

Name	gi code	Species	Substrate	Reference
OprC	1498191	*Pseudomonas aeruginosa*	Copper chelate	Yoneyama and Nakae (1996)
BfeA	538279	*Bordetella pertussis*	Enterobactin	Beall and Sanden (1995)
PirA	2981053	*Pseudomonas aeruginosa*	Enterobactin	Ochsner et al. (2000)
PfeA	548479	*Pseudomonas aeruginosa*	Enterobactin	Dean and Poole (1993)
FepA	2507463	*Escherichia coli* K12	Enterobactin	Lundrigan and Kadner (1986)
IroN	2738252	*Salmonella enterica*	Salmochelin	Bäumler et al. (1998)
CfrA	112360090	*Campylobacter jejuni*	Enterobactin	Palyada et al. (2004); Carswell et al. (2008)
CirA	2507462	*Escherichia coli* K12	2,3-Dihydroxybenzoylserine (DHBS)	Nau and Konisky (1989)
IrgA	12644182	*Vibrio cholerae*	Enterobactin	Goldberg et al. (1992)
BfrA	1314835	*Bordetella bronchiseptica*	2,3-Dihydroxybenzoylserine (DHBS)	Beall and Hoenes (1997)
HutR	147671724	*Vibrio cholerae*	Heme	Mey and Payne (2001)
HuvA	12697532	*Listonella anguillarum*	Heme	Henderson and Payne (1994)
HutA	529727	*Vibrio cholerae*	Heme	Mazoy et al. (2003)
PhuR	3044098	*Pseudomonas aeruginosa*	Heme	Ochsner et al. (2000)
PfhR	4838477	*Pseudomonas fluorescens*	Heme	Ochsner et al. (2000)
HpuB	11386826	*Neisseria meningitidis*	Heme	Lewis et al. (1997)
HmbR	687640	*Neisseria meningitidis*	Heme	Stojiljkovic et al. (1995)
HgbA	28194090	*Actinobacillus pleuropneumoniae*	Heme	Srikumar et al. (2004)
HemR	6016198	*Yersinia enterocolitica*	Heme	Stojiljkovic and Hantke (1992)
HmuR	2501236	*Yersinia pestis*	Heme	Hornung et al. (1996)
ChuA	1763009	*Escherichia coli* O157:H7	Heme	Torres and Payne (1997)
ShuA	1655877	*Shigella dysenteriae*	Heme	Mills and Payne (1997)

(*continued*)

Table 6.3 (continued)

Name	gi code	Species	Substrate	Reference
HxuC	1170441	*Haemophilus influenzae*	Heme	Cope et al. (1995)
TdhA	33151615	*Haemophilus ducreyi* 35000HP	Heme	Thomas et al. (1998)
HasR [T]	34787214	*Serratia marcescens*	Heme	Letoffe et al. (1994)
MhuA	50403825	*Moraxella catarrhalis*	Heme	Furano et al. (2005)
FetA_FrpB	4768684	*Neisseria gonorrhoeae*	Enterobactin	Carson et al. (1999)
VctA	18476494	*Vibrio cholerae*	Enterobactin	Mey et al. (2002)
LbpA	915278	*Neisseria gonorrhoeae*	Lactoferrin	Biswas and Sparling (1995)
TbpA	150361	*Neisseria gonorrhoeae*	Transferrin	Cornelissen et al. (1992)
FrpB4	15646121	*Helicobacter pylori* 26695	[Nickel]	Schauer et al. (2007)
BtuB	416728	*Escherichia coli* K12	Vitamin B_{12}	Heller and Kadner (1985)
MxcH	162452159	*Sorangium cellulosum* "So ce 56"	Myxochelin	Silakowski et al. (2000)
IutA	1170593	*Escherichia coli*	Aerobactin	Krone et al. (1985)
RhtA	6685883	*Sinorhizobium meliloti*	Rhizobactin 1021	Lynch et al. (2001)
RumA	1247762	*Morganella morganii*	Diferric dicitrate	Kühn et al. (1996)
FecA [T]	729471	*Escherichia coli* K12	Diferric dicitrate	Pressler et al. (1988)
VciA	147673813	*Vibrio cholerae* O395	Unknown	Mey et al. (2008)
PiuA_Fiu	115587765	*Pseudomonas aeruginosa*	Pyochelin	Ochsner and Vasil (1996)
FoxA	1169726	*Yersinia enterocolitica*	Desferriferrioxamine	Bäumler and Hantke (1992)
FegA	1518696	*Bradyrhizobium japonicum*	Desferriferrioxamine	LeVier and Guerinot (1996)
FctA	871032	*Erwinia chrysanthemi*	Crysobactin	(Sauvage et al. (1996)
FmtA	53719389	*Burkholderia pseudomallei* K96243	Ferric malleobactin	Alice et al. (2006)
OrbA	11230853	*Burkholderia cepacia*	Ferric ornibactin	Sokol et al. (2000)
FhuA	2507464	*Escherichia coli* K12	Ferrichrome	Coulton et al. (1986)
OptS [T]	116050410	*Pseudomonas aeruginosa*	Desferrioxamine	Llamas et al. (2006)
BfrZ [T]	6850914	*Bordetella bronchiseptica*	Unknown	Pradel and Locht (2001)
PrhA [T]	17549099	*Ralstonia solanacearum*	Transducer without transport function	Brito et al. (2002)
PbuA [T]	1172035	*Pseudomonas sp.* M114	Pseudobactin M114	Morris et al. (1994)

FpvA [T]	12230910	*Pseudomonas aeruginosa*	Pyoverdine	Poole et al. (1993)
PupA [T]	45723	*Pseudomonas putida* WCS358	Pseudobactin A	Bitter et al. (1991)
PupB [T]	585759	*Pseudomonas putida* WCS358	Pseudobactin A	Koster et al. (1993)
FauA	4589285	*Bordetella pertussis*	Alcaligin	Brickman and Armstrong (1999)
FhuE	2507465	*Escherichia coli* K12	Coprogen	Sauer et al. (1990)
FptA	1169730	*Pseudomonas aeruginosa*	Pyochelin	Ankenbauer and Quan (1994)
FatA	132510	*Listonella anguillarum*	Anguibactin	Actis et al. (1988)
BauA	49175779	*Acinetobacter baumannii*	Anguibactin	Dorsey et al. (2004)
FcuA	1169655|	*Yersinia enterocolitica*	Anguibactin	Koebnik et al. (1993)
FyuA	517234	*Yersinia enterocolitica*	Yersiniabactin	Rakin et al. (1994)
IrpC	17380443	*Yersinia pestis*	Yersiniabactin	Fetherston et al. (1995)
ViuA	267356	*Vibrio cholerae*	Vibriobactin	Butterton et al. (1992)
SftP	6019468	*Pseudomonas putida*	Hexylsulfate	Kahnert and Kertesz (2000)
SuxA	21232787	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913	Sucrose	Blanvillain et al. (2007)
RagA	34540042	*Porphyromonas gingivalis* W83	Digested proteins	Nagano et al. (2007)
SusC	29349110	*Bacteroides thetaiotaomicron* VPI-5482	Malto-oligosaccharides /starch	Reeves et al. (1996)
CsuF	29348741	*Bacteroides thetaiotaomicron* VPI-5482	Chondroitin, sulfate/ hyaluronic acid	Cheng et al. (1995)
MalA	16126526	*Caulobacter crescentus* CB15	Maltodextrins	Neugebauer et al. (2005)
SchE	Alr0397	*Anabaena* sp. PCC 7120	Schizokinen	Nicolaisen et al. (2008)

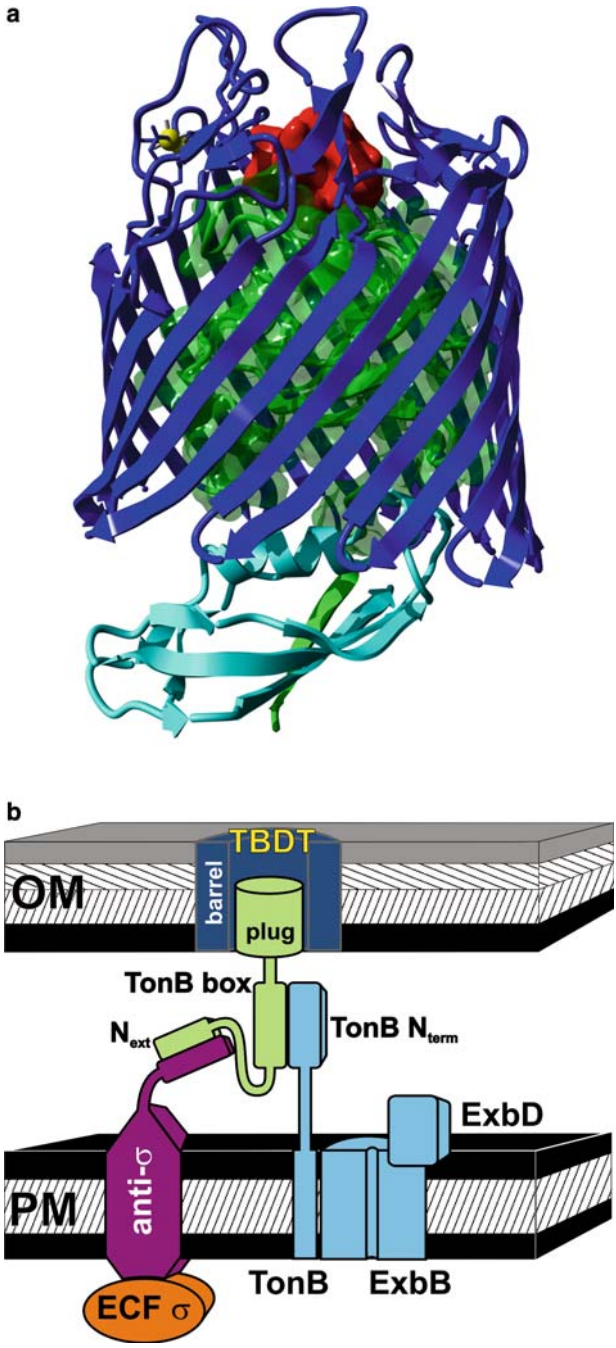

Fig. 6.6 TonB-dependent transporters. (**a**) The structure of BtuB (pdb: 2GSK; Shultis et al. 2006). The β-sheet is shown in *blue*; the plug domain is shown as surface representation in *green* and the TonB box as ribbon in *green*. The transporter is complexed with cobalamin shown in *red* and with

Summarizing, these contradictory results might indicate that both import mechanisms are feasible. However, it remains to be investigated whether any TBDT is capable of using both mechanisms or whether the TBDTs are rather split into two groups either forming a transient channel or removing the plug domain from the channel interior.

The majority of genes coding for TBDTs are expressed only under iron deficiency in a Fur-regulated manner (Braun et al. 2003). At life-supporting intracellular iron concentrations, the Fur protein – the global regulator of iron metabolism – is complexed with Fe^{2+} (Bagg and Neilands 1987; Andrews et al. 2003). Fur–Fe^{2+} binds to promoter regions of genes/operons containing a specific binding motif (Fur-box, e.g., Escolar et al. 1998) resulting in the downregulation of expression. Under low intracellular iron conditions the Fe^{2+} dissociates from Fur protein leading to its dissociation from the promoter and thereby to transcription of the appropriate genes (Le Cam et al. 1994; Escolar et al. 1999). Hence, the cell enriches TBDTs in the outer membrane in times of iron starvation.

A subclass of TBDTs also functions as signal transducers, which participate in the transcriptional regulation of iron transport genes encoding the components of their own iron transport system (Fig. 6.6b; Koebnik 2005). They are characterized by a unique N-terminal extension or an N-domain upstream of the TonB box reaching into the periplasm (Kim et al. 1997), which transduces information about the extracellular binding of the substrate. In *E. coli,* only the FecA transporter is such a signal transducer (Koebnik 2005). When ferric citrate binds to the FecA receptors present in the outer membrane and when at the same time iron becomes limited inside the cell, the inner membrane-spanning FecR antisigma factor is synthesized (Enz et al. 2000). FecR then interacts in the periplasm with the N-domain of FecA receiving a signal that ferric citrate siderophore is present in the medium and bound to FecA. The signal is transferred from the FecR antisigma factor to FecI, an extracytoplasmic function (FEC) σ-factor (Lonetto et al. 1994). The *fecI* gene is cotranscribed in the same Fur-regulated operon with *fecR* under iron limitation (Braun 1997). FecI σ-factor binds to an RNA polymerase, which then transcribes the *fecABCDE* operon encoding the ferric citrate transport system (Luck et al. 2001). This results in the coordination of the information about iron limitation and siderophore supply.

Fig. 6.6 (continued) the N-terminal domain of TonB (*cyan*). (**b**) A model of the organization of TonB-dependent transducer systems. A TonB-dependent transducer (*dark blue*) contains a plug domain and the TonB box (*green*), as in all typical TBDTs, but in addition an N-terminal extension. The TonB box interacts with the N-terminus of TonB (*blue*), which is part of a complex together with the plasma membrane-inserted protein ExbB and the periplasmic protein ExbD, which together form the "energizing complex." The N-terminal extension of the transducer interacts with a plasma membrane-localized antisigma factor (*violet*), which forms a complex with a cytosolic ECF-subfamily sigma factor (*orange*). The figure was adapted from Koebnik (2005). Abbreviations: PM, plasma membrane; OM, outer membrane

6.5 Noncoding RNA's: An Important Theme in Solute Transport Regulation

Considering the importance of the outer membrane as the permeability barrier of the cell it is logical that expression of many genes coding for OMPs is regulated in an environmentally dependent manner. On the one hand, the folding status of OMPs is monitored by the σ^E signaling system. The system is required to regulate gene expression of *omp* genes both under envelope stress conditions and during normal growth (Alba and Gross 2004; Ruiz and Silhavy 2005). Envelope stress is accompanied by the accumulation of misfolded OMPs upon envelope damage or excessive OMP synthesis. In response to the accumulation of these nonfunctional OMPs the σ^E system induces up-regulation of expression of factors that will counteract damage. Further, many different kinds of nutrient limitations cause the production of guanosine 3′,5′-bispyrophosphate (Magnusson et al. 2005). On the other hand, the expression of several porins is regulated by the EnvZ–OmpR two-component system composed of the sensor component EnvZ and the signal transducer OmpR. For instance, under high osmotic conditions OmpR becomes phosphorylated, which subsequently leads to the association with *ompF* and its expression (Pratt et al. 1996).

Regulation by both the σ^E-system and the EnvZ–OmpR two-component system at least partially involves so-called small noncoding RNAs. Small noncoding RNAs (sRNAs) play an important role in the regulation of gene expression in responses to environmental stimuli (e.g., Storz et al. 2005; Gottesman 2005). In Gram-negative bacteria these sRNAs are trans-acting and they inhibit translation by masking the translation initiation region and thereby disturbing the ribosome binding (Marzi et al. 2008). The base pairing of the sRNA with the target RNA is assisted by the bacterial Sm-like protein, Hfq (Valentin-Hansen et al. 2004). Indeed, the first discovered trans-encoded antisense sRNA was MicF (Mizuno et al. 1984) which regulates the expression of the porin-encoding gene *ompF* (Schmidt et al. 1995) by targeting the 5′-region of *ompF* under high temperature, leading to the inhibition of its translation (Delihas 1995). Since the discovery of MicF, the regulatory network involving sRNA elements in regulation of the OMPs has been expanded. At least eight different sRNA have been identified in *E. coli* and one additional in *Salmonella enterica* serovar Typhimurium (Fig. 6.7). For many, but not all, even a relation to the two main sensor systems has already been established.

At least three OmpR-dependent sRNA regulation regimes exist involving OmrA/OmrB, MicF and MicC. The latter is about 100 base pairs long and encoded between the *ompN* and *ydbK* genes (Chen et al. 2004). This sRNA specifically interacts with the *ompC* mRNA leading to the repression of translation. However, in contrast to OmrA/OmrB and MicF which are activated in an OmpR-dependent manner, the action of MicC is repressed by OmpR (Chen et al. 2004). MicF specifically targets *ompF* in response to many external stimuli. Beside the OmpR-mediated response to osmolarity, a reaction in response to weak acids via MarA, oxidative stress via SoxS and cationic peptides and antibiotics via RoB was manifested (for more details see, e.g., Delihas and Forst 2001). Both sRNAs,

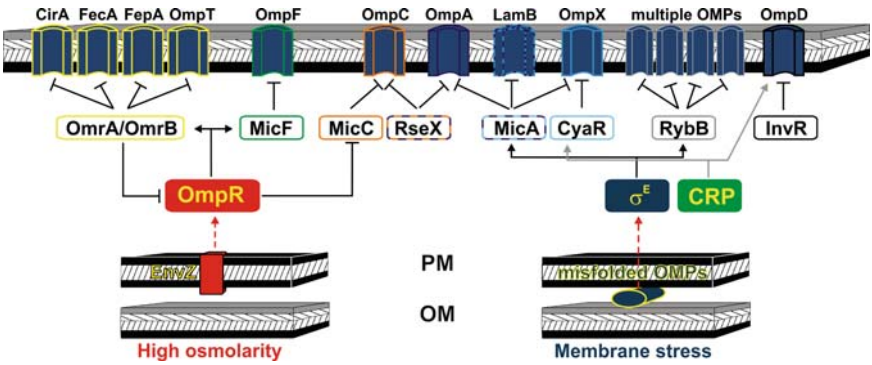

Fig. 6.7 The sRNA-based regulatory network of outer membrane proteins. The regulation of genes encoding for outer membrane proteins is indicated. The *top row* shows the outer membrane proteins which are targeted, the *second row* gives the names of the sRNAs involved in the regulation of the appropriate genes. The regulatory imprint of the three regulatory systems, namely OmpR, the σ^E-system and the cyclic AMP-dependent receptor CRP system, is shown, where *arrows* indicate positive regulation and *bars* negative regulation. The relation of the OmpR and the σ^E-system to the membrane integrity is depicted at the *bottom*

MicF and MicC, were found to be highly specific for their targeted gene (Urban and Vogel 2007). In contrast, OmrA and OmrB regulate several genes as determined by the analysis of the changes in the global transcript level, namely the genes coding for the outer membrane protease OmpT and the TBDTs CirA, FecA and FepA (Guillier and Gottesman 2006). All targeted genes are negatively regulated by mRNA destabilization (Guillier and Gottesman 2008). The existence of two almost redundant sRNAs was discussed as an additionally regulatory circuit, as it is not unique to an individual bacterial species (Papenfort and Vogel 2009).

The sRNAs known today to be regulated by the σ^E-system are, discovered at stage, are MicA and RybB (Figueroa-Bossi et al. 2006; Johansen et al. 2006). RybB is a global regulator of at least ten genes coding for OMPs (Papenfort et al. 2006), which accumulates in the stationary phase (e.g., Wassarman et al. 2001). This accumulation, however, is consistent with the observed increase in activity of the σ^E-system during this phase (Testerman et al. 2002). However, RybB appears also to be important for the "fine-tuning" of OMP production under all conditions of life, as its deletion results in overproduction of the relevant proteins and hyperactivation of the σ^E-system (e.g., Papenfort et al. 2006). Hence, the function of the σ^E-system appears to be regulated by the RybB action, but in contrast to the OmrA/OmrB system rather indirectly. MicA, which is also controlled by the σ^E-system, has higher target specificity than RybB as it only targets *ompA, lamB* and *ompX* (Udekwu et al. 2005; Rasmussen et al. 2005; Bossi and Figueroa-Bossi 2007; Johansen et al. 2008). In line with this, decoupled expression of MicA reduces the level of OmpA. The molecular mode of MicA action involves the formation of a RNA duplex between a 17-nucleotide region of the 5′ MicA-RNA and the *ompA* 5′ UTR (Udekwu et al. 2005). This interaction results in a disturbance of the RNA ribosome binding and subsequently destabilizes the mRNA. Summing up, these two

sRNAs and thereby the σ^E-system regulate the OMP content in relation to the cell stress and cell development and thereby prevents OMP synthesis in times where they are not needed or when they cannot be assembled into the membrane (Johansen et al. 2006).

Beside the sRNAs directly related to the two sensor systems, three additional sRNAs involved in OMP synthesis have been identified, namely RseX, CyaR and InvR. The latter, however, regulates the occurrence of OmpD and was only identified in *Salmonella* (Pfeiffer et al. 2007). Hence, this sRNA might be specific for this species. RseX was identified by interaction with *ompA* and *ompC* RNA and the sRNA negatively regulates the expression of both genes (Douchin et al. 2006). Strikingly, RseX was initially discovered as a multicopy suppressor of an RseP-depleted strain. RseP is a plasma membrane protease involved in degradation of the anti-σ^E factor RseA and subsequently in the regulation of the σ^E-system. Hence it can be speculated that RseX serves as a fine-tuning or back-up element of the σ^E-system. The sRNA CyaR is regulated by the cAMP receptor Crp and regulates the expression of *ompX* (Johansen et al. 2008; Papenfort et al. 2008), but also of genes coding for cytosolic proteins such as the quorum sensor LuxS and NadE involved in NAD synthesis (De Lay and Gottesman 2009). Hence, this sRNA might interlink the nutrition status of the cell with outer membrane expression.

Summarizing, the research of recent years has unraveled a complex regulatory network of *omp* expression involving small RNAs. The understanding of this regulation is variable as MicF-mediated regulation is quite well understood and CyaR-mediated regulation is only just discovered. Hence, future work on the regulatory network will be important for understanding the biogenesis of the outer membrane proteome.

6.6 Outer Membrane Proteins Involved in Membrane Biogenesis

6.6.1 Transporter Involved in Lipopolysaccharide Layer Biogenesis

The lipid A transporter Imp/OstA. Two essential components of the asymmetric outer membrane bilayer are synthesized on the cytoplasmic side of the plasma membrane, namely lipid A and the capsular polysaccharides (CPS) (see below). The synthesis of both components is well understood, but their transport to the outer rim of the cell remained for a long time in the dark. However, recent discoveries have unveiled the molecular players of these pathways.

Initially, it was discovered that lipid A and glycerophospholipids accumulate in the inner membrane of *E. coli* carrying a temperature-sensitive *msbA* mutant at the restrictive temperature (Zhou et al. 1998). This gene encodes for an ABC transporter. The accumulated lipid A form was not modified by periplasmic enzymes, indicating a defect of transport across the inner membrane (Doerrler et al. 2004).

Currently it is proposed that the (soluble) periplasmic protein LptA (for LPS transport) facilitates the transfer of lipid A across the periplasmic space (Sperandeo et al. 2007), at least in *E. coli*. In the outer membrane the protein known as Imp or OstA (organic solvent tolerance) is thought to export the lipid A to the outer face of the outer membrane. Imp/OstA is essential in *Escherichia coli* and was first discovered to modulate the outer membrane permeability while overexpressing the gene (Sampson et al. 1989). In a conditional *imp* mutant, OMP abundance was enhanced, which is indicative of an altered lipid/protein ratio (Braun and Silhavy 2002). In the *imp* deletion mutant of *Neisseria meningitidis* (where Imp is not essential) less than 10% of wild-type levels of LPS were produced but not exported to the outside of the cell (Bos et al. 2004). A similar result was observed with an *E. coli imp* depletion strain, where newly synthesized LPS can no longer be exported after depletion of the gene (Wu et al. 2006). Based on these observations, a function of Imp in the transport of LPS across the outer membrane can be proposed. Furthermore, in *E. coli*, Imp forms a complex with the essential lipoprotein RlpB. Depletion of RlpB resulted in a phenotype similar to that of depletion of Imp, for instance in an increased total cellular LPS content (Wu et al. 2006). Even though the exact function of the individual components of this cascade remains partially unknown and no molecular details for the function of the Imp transporter can be provided, initial steps have been taken to understand lipid A export.

Wza – the exporter of CPS. Many Gram-negative bacteria are covered by CPS, which on one hand cloak the bacterial cellular surface, e.g. to evade the immune system of the host, and which on the other hand form a hydro-gel layer essential for maintaining the hydrated state of the cell (Whitfield and Roberts 1999). The synthesis of the CPS starts at the cytosolic surface of the plasma membrane. Consequently, the synthesized molecules have to be transported across the inner and the outer membrane. This transport involves a molecular machine embedded in both membranes (Fig. 6.8). The plasma membrane-localized enzyme WbaP catalyses the transfer of galactose-1-phosphate from UDP-galactose to und-P. The und-P-bound polysaccharide unit is then "flipped" from the cytoplasmic to the periplasmic side of the inner membrane in a Wzx-dependent manner. Subsequently, the capsule polymer is assembled by addition of new repeat units to the polysaccharide chain by the plasma membrane-localized polymerase Wzy (Drummelsmith and Whitfield 1999). Two additional proteins are involved in this process, namely Wzb and Wzc (e.g., Reid and Whitfield 2005). Here, the phosphorylation of Wzc by its autophosphorylation activity and its dephosphorylation by Wzb are regulatory circuits of the polymerization and export process. Afterwards, high-molecular-weight carbohydrate polymers are translocated across the outer membrane through the outer membrane lipoprotein Wza (Beis et al. 2004).

Wza forms a multimer that remains stable in SDS and decomposes only after additional incubation at temperatures >60°C (Drummelsmith and Whitfield 2000). Wza assembles to an octamer which forms an elongated cylindrical structure, which can further be dissected into four structural rings (Dong et al. 2006). A remarkable and so far unique feature of Wza is its transmembrane segment, which forms an α-helical barrel. The helices within the barrel are amphipathic to permit interaction

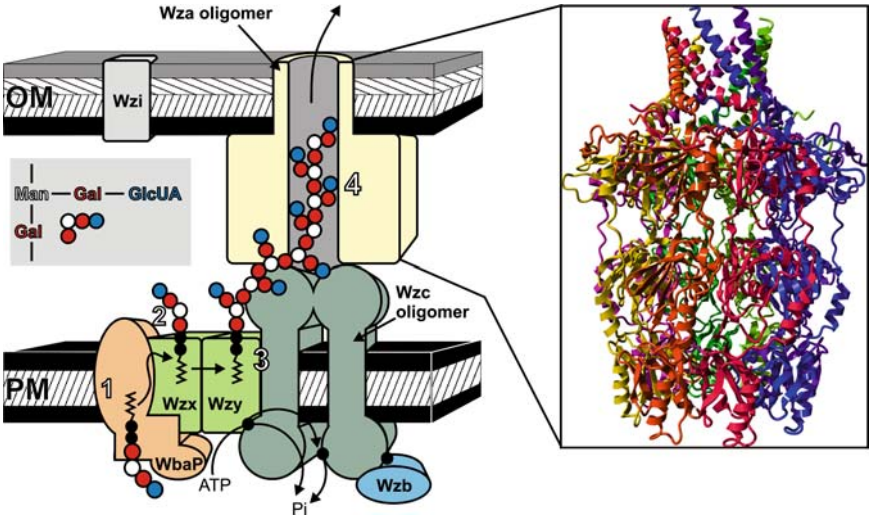

Fig. 6.8 A functional model of the Wza translocon. The model of the Wza translocon is shown on the *left*. Step 1 – und-PP-linked repeats are assembled by WbaP. Step 2 – they are subsequently transferred to the outer leaflet of the plasma membrane and this process is utilized by Wzx. Further polymerization is catalyzed by Wzy. Step 3 – transphosphorylation of Wzc and dephosphorylation by Wzb are required for further polymerization. Step 4 – the polymer is subsequently translocated by Wza, which acts as a channel. Wzi appears to be involved in surface association of group 1 capsules. On the *right side* the high resolution image of Wza (pdb: 2J58, Dong et al. 2006) is shown. The reaction scheme was adapted from Whitfield (2006) and Collins and Derrick (2007)

with the outer membrane on one hand and the export of polysaccharides on the other hand. Consistent with this notion, the α-barrel diameter is 1.7 nm and therefore sufficient to accommodate CPS in an extended conformation. Furthermore, the cavity formed by the Wza octamer is completely sealed and it was argued that this reduces the need for desolvation of the polysaccharide during export. In line with this, Wza forms a stable complex with Wzc initially identified by chemical crosslinking (Nesper et al. 2003) and later confirmed by reconstitution and electron microscopy analysis (Collins et al. 2007). The latter revealed a complex of 17 nm (length) × 12 nm (diameter), which is sufficient to span the periplasm. Therefore, to export the CPLs, both proteins have to act in concert, similar to the TolC system (Sect. 6.7.1). Even though the first structural insights into this fantastic machinery have been obtained, the molecular mechanism of LPS export remains to be established.

6.6.2 Porin Insertion into the Outer Membrane Catalyzed by Omp85

Omp85 proteins are involved in outer membrane biogenesis by mediating the insertion of porins into the outer membrane, a function which can even be identified

in endosymbiotically derived organelles (e.g., Schleiff and Soll 2005). The proteins will first be translocated across the SecYEG channel hosted in the plasma membrane, and periplasmic chaperones deliver them to the outer membrane. In *E. coli*, SurA was identified to be the periplasmic chaperone involved in this process, which can be chemically crosslinked to the *E. coli* Omp85 BamA (Sklar et al. 2007a). Omp85-like proteins were initially discovered in *Haemophilus influenzae* as D15 protective surface antigen (D-15-Ag; Thomas et al. 1990), as Toc75 in plants (Tranel et al. 1995) or as Oma87 in *Pasteurella multocida* (Ruffolo and Adler 1996). Subsequently, the protein was discovered in all branches of life (e.g., Moslavac et al. 2005; Bredemeier et al. 2007) and renaming was enforced by the discovery of the protein in *Neisseria meningitidis* (Genevrois et al. 2003; Voulhoux et al. 2003). The Omp85 protein belongs to the old inventory of the outer membrane (e.g., Bredemeier et al. 2007), which is explained by its essential function during insertion of outer membrane porins into the lipid bilayer (e.g., Genevrois et al. 2003; Voulhoux et al. 2003; Gerdes et al. 2003; Wu et al. 2005). It is proposed that the Omp85 from *E. coli* forms a complex with additional lipoproteins (Wu et al. 2005; Malinverni et al. 2006; Charlson et al. 2006; Sklar et al. 2007b) and that the protein is glycosylated in the outer membrane, at least in *Porphyromonas gingivalis* (Nakao et al. 2008). However, both properties cannot yet be extended to the Omp85 homologs of other species.

The protein is composed of a 16-stranded β-barrel (postulated based on the structure of FhaC; Clantin et al. 2007) and N-terminal polypeptide transport-associated (POTRA) domains (Kim et al. 2007). The function of the β-barrel domain remains partially unknown. On the one hand the function of the Omp85 in the insertion was proposed to be catalytic (Löffelhardt et al. 2007), because SecA, a component of the plasma membrane translocon, is a multicopy suppressor of a BamA mutant (Omp85 in *E. coli;* Doerrler and Raetz 2005). Hence, the accelerated export of OMPs across the plasma membrane by enhanced levels of SecA leads to a higher concentration of these proteins in the periplasm resulting in bypassing the Omp85 action. On the other hand, the channel properties differ between proteobacterial and cyanobacterial Omp85 homologs such that the channel diameter of the proteobacterial Omp85 is considered too small to translocate a polypeptide across the outer membrane (Bredemeier et al. 2007). Hence, further molecular investigations are needed to distinguish between a chaperone-like function and a translocation across the membrane as seen in the case of autotransporters.

The POTRA domain is proposed to serve as a receptor for the incoming OMP and as a docking site for complex components. An interaction between the PhoE signal and the POTRA domain was demonstrated by NMR studies (Knowles et al. 2008). In line with this, this domain of the cyanobacterial Omp85 is able to recognize precursor proteins in a phenylalanine-dependent manner (Wunder et al. 2007), which coincides with the requirement of a C-terminal-positioned phenylalanine for the insertion of porins into the outer membrane (Struyvé et al. 1991). Furthermore, the N-terminal domain was required for oligomerization of the protein within the membrane (Ertel et al. 2005; Bredemeier et al. 2007). Hence, the periplasmic chaperone SurA binds to OMP after its transfer into the periplasm. This interaction

is not directed toward the C-terminus of the OMP but rather to regions containing clusters of aromatic residues (Hennecke et al. 2005). Therefore, the C-terminus of the OMP remains accessible and after recognition of SurA by the Omp85 (complex) the C-terminal phenylalanine will be recognized by Omp85. Indeed, in an elegant study Ureta and coworkers demonstrated that the initial folding rate in vivo of the porin LamB was reduced by a factor of 20 in a *surA* mutant and by a factor of 5 in a mutant of *yfgL* which is a component of the Omp85 complex in *E. coli* (Ureta et al. 2007). Assembly of a trimeric LamB complex, however, was not significantly altered in both mutants. This suggests that Omp85 itself catalyzes the protein insertion into the outer membrane by an as yet unknown mechanism.

Strikingly, recent discoveries have revealed a species-specific complexity of the Omp85 family. For example, in *Anabaena* sp. PCC 7120 at least three distinct genes encoding for an Omp85 have been discovered (e.g., Moslavac et al. 2005) and in *E. coli* a second gene coding for an Omp85-like protein was described (Stegmeier et al. 2007). However, in contrast to YtfM, the Omp85 in *E. coli*, which is not essential for cell viability, all three cyanobacterial genes are essential (Schleiff, unpublished). Hence, it remains to be explored what the function of these proteins might be.

6.7 One Two Three Four Five Six Seven ... The Secretion Systems

So far we have described the uptake of small organic compounds across membranes (Sects. 6.3 and 6.4). However, solutes have also to be exported, e.g., for the biogenesis of the outer membrane (Sect. 6.6). But export is not only limited to biogenesis but also plays a role in pathogenicity or for the assembly of, e.g., flagella or pili. Hence, in the following we will focus on such systems. In general, seven protein secretion systems ranging from type I to type VII are known to date. Further nonflagellar export systems besides types I to VII are the chaperone/usher and curli systems. We will only focus on the type I secretion system (Sect. 6.7.1) and give a brief overview of the other systems in Sect. 6.7.2; for more information, the reader should refer to the literature cited in Sect. 6.7.2.

6.7.1 The TolC-Dependent System – Type I

The TolC-protein family of Gram-negative bacteria, first described by de Zwaig and Luria as "tolerant to colicin mutant" (TolC) (de Zwaig and Luria 1967) has a low sequence identity but is highly conserved on the structural level. In contrast to porins it consists of a homotrimer forming a single channel-tunnel with a membrane-inserted β-barrel and a periplasm-spanning α-helical barrel, which is a unique structural feature among membrane proteins (Fig. 6.9) (Koronakis et al. 2000).

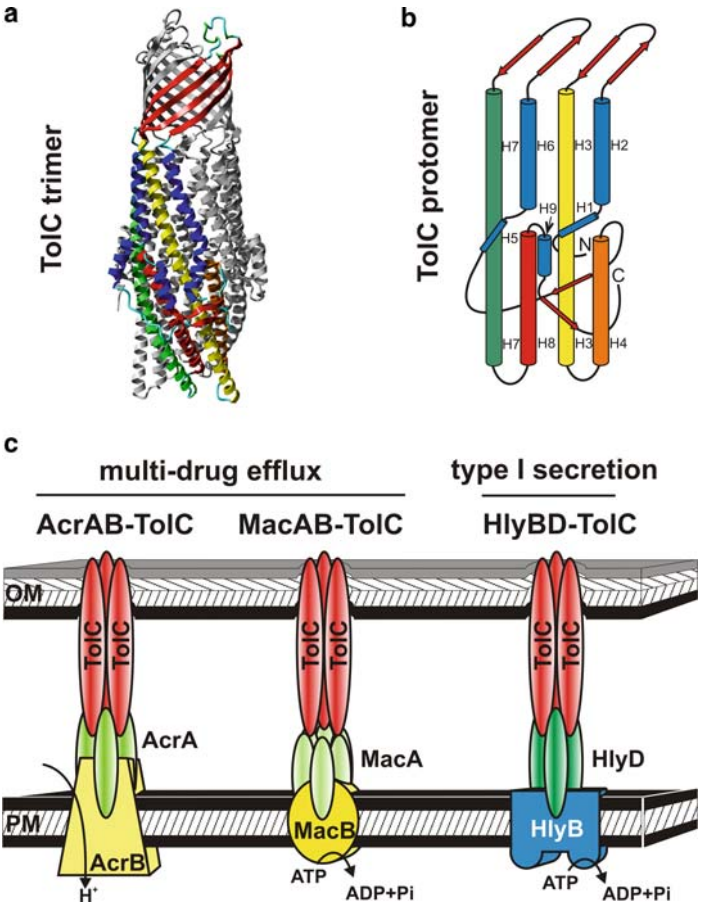

Fig. 6.9 Type I secretion system – TolC. (**a**) Crystal structure of the *E. coli* TolC homotrimer with the outer membrane-inserted β-barrel (*top*) and the periplasm-reaching α-helical barrel (*bottom*). The contribution of α-helices and β-sheets from one monomer to the full homotrimer is highlighted. (**b**) Schematic representation of the *E. coli* TolC monomer (protomer). The α-helices H3/H4 (*yellow*/*orange*) and H7/H8 (*green*/*red*) form the interface for complex assembly of the AcrAB-secretion complex and are involved in the iris-like gating mechanism from the closed-to-open state of TolC. (**c**) Members of the TolC protein family are involved in the formation of different secretion complexes. The ArcAB–TolC and the MacAB–TolC belong to a superfamily of multidrug secretion systems and are involved in secretion of toxic substances such as membrane-permeable antibiotics. For both systems a different arrangement of the corresponding membrane fusion protein (MFP) was proposed. While AcrA interacts with the binding groove of H3/H4 and H7/H8 of the TolC α-barrel, MacA is believed to form a hexameric ring structure, which has to interact (due to its diameter) in a tip-to-tip-like fashion with TolC. Depending on the subclass (RND, MF, SMR or ABC) of efflux pumps, either a proton motive force (PMF) or ATP hydrolysis is utilized as energy source. In contrast, type I secretion systems are involved in secretion of protein toxins like hemolysin A for the HlyBD–TolC secretion system and have ATP-binding cassettes (ABC), which are in many cases expressed as an operon with the transmembrane protein and the MFP of the secretion complex

Each protomer contributes four antiparallel β-sheets and four α-helices (H3, H4, H7, H8) to form the barrel-shaped channel-tunnel. Even though the α-helical part of the TolC protomer is sufficient to promote cytosolic trimerization, the Omp85-homolog BamA (see Sect. 6.5.2) is needed for OM insertion in *E. coli* (Masi et al. 2009). TolC proteins have been described as an outer membrane factor (OMF) as part of three different secretion systems which bypass the periplasm to release substrates directly into the media. Firstly, as part of the type I secretion system, TolC interacts with different inner-membrane ABC transporters and is involved in secretion of protein toxins such as *E. coli* haemolysin A, extra cellular proteases, lipases or cell wall components (Duong et al. 1994; Akatsuka et al. 1995; Awram and Smit 1998). Recently, a TolC homologue in cyanobacteria has also been described to be involved in heterocyst development (Moslavac et al. 2007). Secondly, it functions as OMF for different superfamilies of multidrug efflux pumps such as the resistance–nodulation–cell division type (RND) AcrAB in *E. coli* (Saier et al. 1994; Touzé et al. 2004). They contribute to multidrug resistance by extrusion of membrane-soluble cytotoxic substances like acridin or different antibiotics (Seeger et al. 2008; Pos 2009). Thirdly, it has been described as part of cation efflux pumps for extrusion of toxic metal ions (Goldberg et al. 1999). All these systems have in common a tripartite arrangement of the secretion complex comprising the plasma membrane-localized transporter TolC as the OMF and a periplasmic membrane fusion protein (MFP) which stabilizes the complex. The energy – in terms of ATP hydrolysis or by utilizing an ion-gradient – as well as the substrate specificity is provided by the plasma membrane transporter (Saier et al. 1999). In contrast to previous assumptions, recent molecular dynamics simulations suggest that TolC is not just a passive pore in the outer membrane but rather contributes to substrate secretion by undergoing a peristaltic constriction along the whole channel-tunnel which supports substrate extrusion in a "swallowing out" fashion (Vaccaro et al. 2008). In addition, extended loops between β-sheets facing the external environment might function as a gating mechanism to regulate substrate secretion or prevent intrusion of toxins.

To meet the requirements of a general OMF in different secretion systems, TolC must have a flexible docking interface to facilitate the transient interaction with different inner membrane transporters. The best-described model by far for TolC-secretion complex assembly is the tripartite AcrAB–TolC system (Fralick 1996). The currently favored model by Lobedanz and coworkers suggests a transient recruitment of TolC by the transporter AcrB which is enforced by the plasma membrane-anchored MFP AcrA upon substrate interaction (Lobedanz et al. 2007). Based on chemical crosslinking experiments and crystallization of TolC mutants it could be demonstrated that a weak interaction of AcrB with TolC α-helices H3/H4 induces a twisting movement of the iris-like α-barrel. This leads to a partially open state with the formation of a binding groove between H3/H4 and H7/H8 for AcrA interaction. AcrA acts as an energy transducer and facilitates the closed-to-open-state transition of the iris-like α-helical barrel by a further twisting movement of the α-helices (Bavro et al. 2008; Symmons et al. 2009). Based on this data an equimolar stoichiometry of TolC protomer and MFPs is favored. In contrast

to this model, Xu and Yum describe, based on crystallization packaging and electron microscopy data, the formation of a hexameric ring structure for MacA, the MFP of the MacAB–TolC complex, and suggest a tip-to-tip arrangement of the MacA-ring and TolC which would exclude a MacB–TolC interaction as described for the AcrB–TolC complex (Xu et al. 2009; Yum et al. 2009). Even though the understanding of complex formation has significantly increased in the last few years, it is still unclear how TolC recruitment by the right transporter at the right situation is regulated.

6.7.2 *The Other Secretion Systems*

The nonflagellar protein secretion systems are divided into two groups: one group depends on the Sec translocon to translocate their substrates; the other group provides their own translocation system in the plasma membrane. Type II (T2SS) and V secretion systems (T5SS) and the chaperone/usher and curli systems are Sec-dependent (Fig. 6.10). T2SS is also termed the main terminal branch of the Sec-dependent secretion pathway (Sandkvist 2001). Folded proteins are translocated across the outer membrane. The inner membrane component of T2SS assembles a pilus, which probably pushes substrates, delivered by the Sec complex, through the outer membrane complex (Chen and Dubnau 2004). An example of T2SS is

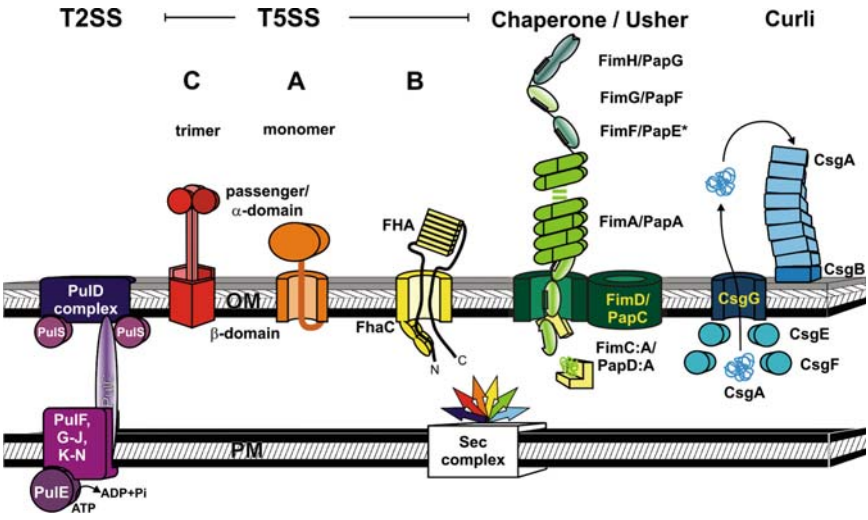

Fig. 6.10 An overview of known Sec-dependent secretion systems. Sec-dependent secretion systems of types II, V and the chaperone/usher and curli pathway are shown schematically. The *asterisk* indicates that PapE of the type P pilus occurs in 5–10 copies in the tip fibrillum, whereas only one FimF copy is present in the type 1 pilus. The figure was adapted from Henderson et al. (2004), Gerlach and Hensel (2007), and Fronzes et al. (2008). Abbreviations: OM, outer membrane; PM, plasma membrane

pullulanase (PulA) secretion from *Klebsiella oxytoca* (Takizawa and Murooka 1985; Cao and Cover 2002). It has been demonstrated that T2SS also secretes proteins, which were transported into the periplasm via the twin-arginine translocation (Tat) system (Voulhoux et al. 2001; De Buck et al. 2008).

Three subtypes of T5SS exist. Type A and C are the autotransporter pathways. An autotransporter contains a transmembrane β-barrel (β-domain) at the C-terminal end and a so-called passenger domain as the N-terminal part (α-domain). Several models for the translocation of the passenger domain across the outer membrane exist (Dautin and Bernstein 2007). A typical example for T5SS-A is the monomeric autotransporter NalP from *Neisseria meningitidis* (Oomen et al. 2004). In contrast, T5SS-C autotransporters form a trimeric pore, which has been shown for bacterial adhesion-mediating autotransporter Hia from *Haemophilus influenzae* (Meng et al. 2006). T5SS-B is the two-partner secretion (TPS) pathway (Jacob-Dubuisson et al. 2004, 2009; Mazar and Cotter 2007). In general, the translocation pore is termed TpsB and the translocated protein TpsA. A prominent example is FhaC, which mediates the secretion of *Bordetella pertussis* filamentous hemagglutinin (FHA, Clantin et al. 2007).

Adhesive fibers, called pili or fimbriae, are assembled via the chaperone/usher (CU) pathway (Sauer et al. 2004). These fibers are virulence factors and mediate host recognition and attachment, invasion, and biofilm formation. The OMP PapC, which secretes these fibers, has recently been crystallized (Remaut et al. 2008). With its 24 transmembrane β-strands it is the largest β-barrel known to date. The active form of PapC is a dimer, but only one monomer secretes the fiber subunits while the other one catches the fiber subunits bound to chaperones (PapD:A complex) in the periplasm and passes them on to the secreting monomer. Another class of bacterial filaments called curli at the outer membrane of Enterobacteriaceae (Olsén et al. 1989; Barnhart and Chapman 2006) contributes to bacterial pathogenesis (Gophna et al. 2001; Lundmark et al. 2005). Curli are the major proteinaceous constituent of the extracellular matrix of Enterobacteriaceae (Barnhart and Chapman 2006). CsgA and CsgB are secreted by the outer membrane lipoprotein CsgG (Robinson et al. 2006). The minor curli subunit, CsgB, is associated with the membrane and multiple copies of the major subunit, CsgA, stack onto CsgB to form curli (Hammar et al. 1995, 1996; Chapman et al. 2002). The periplasmic factors CsgE and CsgF are required for fiber assembly (Robinson et al. 2006).

The Sec-independent secretion systems are type I (see Sect. 6.7.1), III, IV, VI, and VII (Fig. 6.11). T3SS, T4SS and T6SS assemble needle-like structures, which puncture the target membrane and provide a channel for the injection of substances into the host cell. More than 20 proteins are involved in assembly of the injectisome of T3SSs (Cornelis 2006). A hollow structure (PrgJ) spans the periplasmic space linking the complexes in the plasma membrane and outer membrane (Kubori et al. 1998; Marlovits et al. 2004). The extracellular "needle" is a hollow, filamentous structure formed by PrgI (Marlovits et al. 2004). A distinct tip complex is located at the distal end of the needle, which is involved in pore formation in the target membrane, for which additional components are assembled upon membrane contact (Derewenda et al. 2004; Mueller et al. 2005; Deane et al. 2006). In the

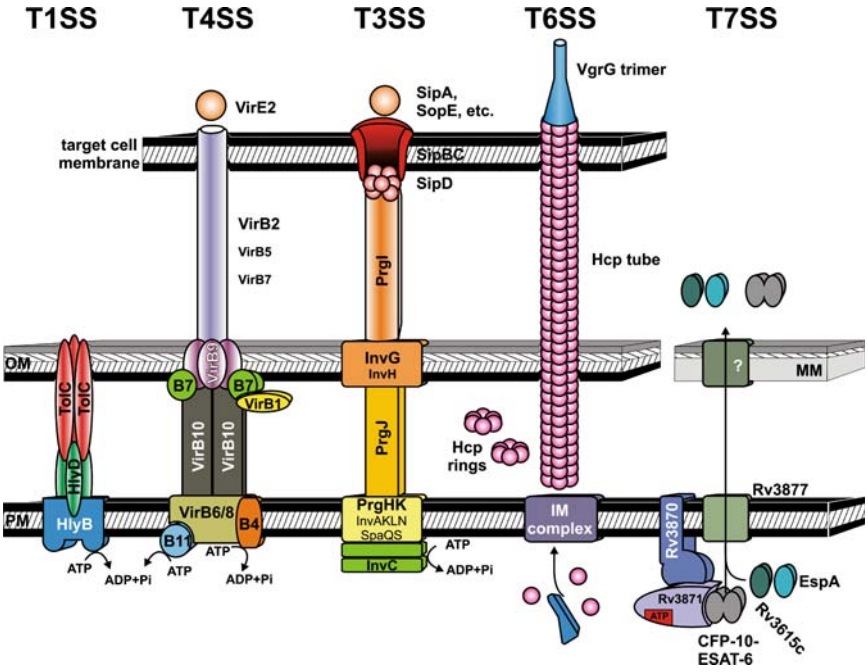

Fig. 6.11 An overview of known Sec-independent secretion systems. The Sec-independent secretion systems of types I, III, IV, VI and VII are shown schematically. T3SS, T4SS and T6SS penetrate the host membrane (target cell membrane) with needle-like structures. The T7SS of mycobacteria is separated from the other systems of Gram-negative bacteria; the *light grey* layer represents the mycolic acid layer of the mycomembrane, the *dark grey* layer on top represents the outer leaflet (more precisely the headgroup region) of the mycomembrane containing various free, mostly mycobacteria-specific lipids intercalated with mycolic acids (Abdallah et al. 2007). A working model of the mycobacterial ESX1 system is shown on the *right*. A transporter of T7SS in the mycomembrane has not so far been identified (marked by *question mark,* Abdallah et al. 2007). The figure was adapted from Abdallah et al. (2007), Gerlach and Hensel (2007), Pukatzki et al. (2009). Abbreviations: MM, mycomembrane (Bayan et al. 2003); OM, outer membrane; PM, plasma membrane

pathogen *Yersinia* spp. T3SS is responsible for secretion of Yop proteins (Michiels et al. 1990). T4SSs are ancestrally related to bacterial conjugation systems (Fronzes et al. 2008) and are utilized for protein and nucleoprotein complex secretion (Juhas et al. 2008). The pili of T4SSs are classified into IncF-like (long) and IncP-like (short) pili (Bradley 1980; Lawley et al. 2003). Using these two pili types, the T4SSs are grouped into IncP-type (IVa) and IncF-type T4SSs (IVb). Typical examples are the *Agrobacterium tumefaciens* nucleoprotein T-DNA transfer system and the Ptl (pertussis toxin) system from *Bordetella pertussis* (Farizo et al. 2000). T6SSs gene clusters contain *IcmF*, a gene known from T4SSs, but the other genes of T6SSs share no homology with known secretion systems (Das and Chaudhuri 2003). The T6SS has so far been found in many pathogens such as

Salmonella typhimurium, *Vibrio cholera* and *Yersinia pestis* (Cascales 2008; Pukatzki et al. 2009). Hcp proteins probably form a needle-like structure for puncturing the target membrane. Proteins of the VgrG family, which contains effector domains (e.g., actin cross-linking, Sheahan et al. 2004), might form the tip of the needle. Hcp and VgrG are translocated in an ATP-dependent manner by the plasma membrane complex of T6SSs (Filloux et al. 2008). Putative T6SS effector proteins identified to date are EvpP (Zheng and Leung 2007), RbsB (Bladergroen et al. 2003; James et al. 2006) and TssM (Schell et al. 2007). The T7SSs (ESX systems) have so far been identified in mycobacteria and Gram-positive bacteria only (Abdallah et al. 2007). Some T7SSs are involved in pathogenicity of, e.g., *Mycobacterium marinum* and *Staphylococcus aureus* (Abdallah et al. 2006; Burts et al. 2005). Although T7SSs share no homologous sequences with other secretion systems, interesting functional parallels to T4SSs were discovered (Abdallah et al. 2007 and references therein). CFP-10 and ESAT-6, forming a tight dimer, seem to depend on each other for translocation. CFP-10 – like substrates of T4SSs (Nagai et al. 2005; Vergunst et al. 2005) – contains an unstructured C-terminal secretion signal, which is recognized by Rv3871. In the same way, EspA might use Rv3615c, which contains the aforementioned C-terminal signal, to translocate in tandem across the plasma membrane (Abdallah et al. 2007). A transporter of T7SS in the mycomembrane has so far not been identified (marked by "?" in Fig. 6.11, Abdallah et al. 2007).

6.8 Conclusions

The general genetic and structural base for many – but still not all – OMPs is now available. At present we are approaching a molecular understanding of porin-like systems and improved bioinformatic procedures in particular will allow new insights in the molecular mechanism of substrate processing. However, many variations on a common theme exist and the transfer of knowledge for one species is not always valid. In particular, the reason for the variation of the number of porin-encoding genes in different species is not yet understood. A further unsolved question concerns the integration of proteins into the outer membrane. Even though the identification of the OMPs of the Omp85 family (Sect. 6.4.2) and of proteins involved in the folding (Mogensen and Otzen 2005) resulted in models for protein insertion, we are far from understanding the molecular mechanism behind this process. In addition, even though it has been investigated for a long time, the regulatory network behind outer membrane biogenesis appears to be rather complex and many issues have to be clarified. Hence, more work is required to provide a detailed picture of outer membrane proteome function. Nevertheless, there is no doubt that we have reached a structural and molecular understanding of the processes, which enables us to approach specific drug development for individual targets at the outer membrane, and this will be one major aspect of future investigations.

References

Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, Eisenberg D, Musters RJ, Vandenbroucke-Grauls CM, Appelmelk BJ, Luirink J, Bitter W (2006) A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. Mol Microbiol 62:667–679

Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmelk BJ, Bitter W (2007) Type VII secretion–mycobacteria show the way. Nat Rev Microbiol 5:883–891

Actis LA, Tolmasky ME, Farrell DH, Crosa JH (1988) Genetic and molecular characterization of essential components of the *Vibrio anguillarum* plasmid-mediated iron-transport system. J Biol Chem 263:2853–2860

Ahn VE, Lo EI, Engel CK, Chen L, Hwang PM, Kay LE, Bishop RE, Privé GG (2004) A hydrocarbon ruler measures palmitate in the enzymatic acylation of endotoxin. EMBO J 23:2931–2941

Akatsuka H, Kawai E, Omori K, Shibatani T (1995) The three genes lipB, lipC, and lipD involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. J Bacteriol 177:6381–6389

Alba BM, Gross CA (2004) Regulation of the *Escherichia coli* σ^E-dependent envelope stress response. Mol Microbiol 52:613–619

Alcaraz A, Nestorovich EM, Aguilella-Arzo M, Aguilella VM, Bezrukov SM (2004) Salting out the ionic selectivity of a wide channel: the asymmetry of OmpF. Biophys J 87:943–957

Alice AF, López CS, Lowe CA, Ledesma MA, Crosa JH (2006) Genetic and transcriptional analysis of the siderophore malleobactin biosynthesis and transport genes in the human pathogen *Burkholderia pseudomallei* K96243. J Bacteriol 188:1551–1566

Andersen C, Jordy M, Benz R (1995) Evaluation of the rate constants of sugar transport through maltoporin (LamB) of *Escherichia coli* from the sugar-induced current noise. J Gen Physiol 105:385–401

Andersen C, Cseh R, Schulein K, Benz R (1998) Study of sugar binding to the sucrose-specific ScrY channel of enteric bacteria using current noise analysis. J Membr Biol 164:263–274

Andersen C, Rak B, Benz R (1999) The gene bgIH present in the bgl operon of *Escherichia coli*, responsible for uptake and fermentation of beta-glucosides encodes for a carbohydrate-specific outer membrane porin. Mol Microbiol 31:499–510

Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237

Ankenbauer RG, Quan HN (1994) FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. J Bacteriol 176:307–319

Arora A, Rinehart D, Szabo G, Tamm LK (2000) Refolded outer membrane protein A of *Escherichia coli* forms ion channels with two conductance states in planar lipid bilayers. J Biol Chem 275:1594–1600

Arora A, Abildgaard F, Bushweller JH, Tamm LK (2001) Structure of outer membrane protein A transmembrane domain by NMR spectroscopy. Nat Struct Biol 8:334–338

Awram P, Smit J (1998) The *Caulobacter crescentus* paracrystalline S-layer protein is secreted by an ABC transporter (type I) secretion apparatus. J Bacteriol 180:3062–3069

Bagg A, Neilands JB (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry 26:5471–5477

Barnhart MM, Chapman MR (2006) Curli biogenesis and function. Annu Rev Microbiol 60: 131–147

Bauer K, Struyve M, Bosch D, Benz R, Tommassen J (1989) One single lysine residue is responsible for the special interaction between polyphosphate and the outer membrane porin PhoE of *Escherichia coli*. J Biol Chem 264:16393–16398

Bäumler AJ, Hantke K (1992) Ferrioxamine uptake in Yersinia enterocolitica: characterization of the receptor protein FoxA. Mol Microbiol 6:1309–1321

Bäumler AJ, Norris TL, Lasco T, Voight W, Reissbrodt R, Rabsch W, Heffron F (1998) IroN, a novel outer membrane siderophore receptor characteristic of *Salmonella enterica*. J Bacteriol 180:1446–1453

Bavro VN, Pietras Z, Furnham N, Pérez-Cano L, Fernández-Recio J, Pei XY, Misra R, Luisi B (2008) Assembly and channel opening in a bacterial drug efflux machine. Mol Cell 30:114–121

Bayan N, Houssin C, Chami M, Leblon G (2003) Mycomembrane and S-layer: two important structures of Corynebacterium glutamicum cell envelope with promising biotechnology applications. J Biotechnol 104:55–67

Bayrhuber M, Meins T, Habeck M, Becker S, Giller K, Villinger S, Vonrhein C, Griesinger C, Zweckstetter M, Zeth K (2008) Structure of the human voltage-dependent anion channel. Proc Natl Acad Sci USA 105:15370–15375

Beall B, Hoenes T (1997) An iron-regulated outer-membrane protein specific to *Bordetella bronchiseptica* and homologous to ferric siderophore receptors. Microbiology 143:135–145

Beall B, Sanden GN (1995) A *Bordetella pertussis* fepA homologue required for utilization of exogenous ferric enterobactin. Microbiology 141:3193–3205

Beis K, Collins RF, Ford RC, Kamis AB, Whitfield C, Naismith JH (2004) Three-dimensional structure of Wza, the protein required for translocation of group 1 capsular polysaccharide across the outer membrane of *Escherichia coli*. J Biol Chem 279:28227–28232

Bellido F, Martin NL, Siehnel RJ, Hancock REW (1992) Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. J Bacteriol 174:5196–5203

Benz R, Ishii J, Nakae T (1980) Determination of ion permeability through the channels made of porins from the outer membrane of *Salmonella typhimurium* in lipid bilayer membranes. J Membr Biol 56:19–29

Birch RG, Pemberton JM, Basnayake WVS (1990) Stable albicidin resistance in *Escherichia coli* involves in altered outer-membrane nucleoside uptake system. J Gen Microbiol 136:51–58

Bishop RE (2005) The lipid A palmitoyltransferase PagP: molecular mechanisms and role in bacterial pathogenesis. Mol Microbiol 57:900–912

Bishop RE (2008) Structural biology of membrane-intrinsic beta-barrel enzymes: sentinels of the bacterial outer membrane. Biochim Biophys Acta 1778:1881–1896

Biswas GD, Sparling PF (1995) Characterization of lbpA, the structural gene for a lactoferrin receptor in *Neisseria gonorrhoeae*. Infect Immun 63:2958–2967

Bitter W, Marugg JD, de Weger LA, Tommassen J, Weisbeek PJ (1991) The ferric-pseudobactin receptor PupA of *Pseudomonas putida* WCS358: homology to TonB-dependent *Escherichia coli* receptors and specificity of the protein. Mol Microbiol 5:647–655

Black PN (1990) Characterization of FadL-specific fatty acid binding in *Escherichia coli*. Biochim Biophys Acta 1046:97–105

Black PN (1991) Primary sequence of the *Escherichia coli* fadL gene encoding an outer membrane protein required for long-chain fatty acid transport. J Bacteriol 173:435–442

Black PN, Dirusso CC (1994) Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli*. Biochim Biophys Acta 1210:123–145

Black PN, Said B, Ghosn CR, Beach JV, Nunn WD (1987) Purification and characterization of an outer membrane-bound protein involved in long-chain fatty acid transport in *Escherichia coli*. J Biol Chem 262:1412–1419

Bladergroen MR, Badelt K, Spaink HP (2003) Infection-blocking genes of a symbiotic Rhizobium leguminosarum strain that are involved in temperature-dependent protein secretion. Mol Plant Microbe Interact 16:53–64

Blanvillain S, Meyer D, Boulanger A, Lautier M, Guynet C, Denancé N, Vasse J, Lauber E, Arlat M (2007) Plant carbohydrate scavenging through tonb-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. PLoS ONE 2:e224

Blot N, Berrier C, Hugouvieux-Cotte-Pattat N, Ghazi A, Condemine G (2002) The oligogalacturonate-specific porin KdgM of *Erwinia chrysanthemi* belongs to a new porin family. J Biol Chem 277:7936–7944

Bond PJ, Faraldo-Gomez JD, Sansom MS (2002) OmpA: a pore or not a pore? Simulation and modeling studies. Biophys J 83:763–775

Bos MP, Robert V (2007) Tommassen J (2007) Biogenesis of the gram-negative bacterial outer membrane. Annu Rev Microbiol 61:191–214

Bos MP, Tefsen B, Geurtsen J, Tommassen J (2004) Identification of an outer membrane protein required for lipopolysaccharide transport to the bacterial cell surface. Proc Natl Acad Sci USA 101:9417–9422

Bossi L, Figueroa-Bossi N (2007) A small RNA downregulates LamB maltoporin in *Salmonella*. Mol Microbiol 65:799–810

Bradley DE (1980) Morphological and serological relationships of conjugative pili. Plasmid 4:155–169

Braun V (1997) Surface signaling: novel transcription initiation mechanism starting from the cell surface. Arch Microbiol 167:325–331

Braun V, Killmann H (1999) Bacterial solutions to the iron-supply problem. Trends Biochem Sci 24:104–109

Braun M, Silhavy TJ (2002) Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. Mol Microbiol 45:1289–1302

Braun V, Mahren S, Ogierman M (2003) Regulation of the FecI-type ECF sigma factor by transmembrane signalling. Curr Opin Microbiol 6:173–180

Bredemeier R, Schlegel T, Ertel F, Vojta A, Borissenko L, Bohnsack MT, Groll M, von Haeseler A, Schleiff E (2007) Functional and phylogenetic properties of the pore-forming beta-barrel transporters of the Omp85 family. J Biol Chem 282:1882–1890

Bremer E, Cole ST, Hindennach I, Henning U, Beck E, Kurz C, Schaller H (1982) Export of a protein into the outer membrane of Escherichia coli K12. Stable incorporation of the OmpA protein requires less than 193 amino-terminal amino acid residues. Eur J Biochem 122: 223–231

Bremer E, Middendorf A, Martinussen J, Valentin-Hansen P (1990) Analysis of the tsx gene, which encodes a nucleoside-specific channel-forming protein (Tsx) in the outer membrane of *Escherichia coli*. Gene 96:59–65

Brickman TJ, Armstrong SK (1999) Essential role of the iron-regulated outer membrane receptor FauA in alcaligin siderophore-mediated iron uptake in *Bordetella* species. J Bacteriol 181:5958–5966

Brito B, Aldon D, Barberis P, Boucher C, Genin S (2002) A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum* hrp genes. Mol Plant Microbe Interact 15:109–119

Buchanan SK (2001) Type I secretion and multidrug efflux: transport through the TolC channel-tunnel. Trends Biochem Sci 26:3–6

Buchanan SK, Smith BS, Venkatramani L, Xia D, Esser L, Palnitkar M, Chakraborty R, van der Helm D, Deisenhofer J (1999) Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. Nat Struct Biol 6:56–63

Burts ML, Williams WA, DeBord K, Missiakas DM (2005) EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. Proc Natl Acad Sci USA 102:1169–1174

Butterton JR, Stoebner JA, Payne SM, Calderwood SB (1992) Cloning, sequencing, and transcriptional regulation of viuA, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J Bacteriol 174:3729–3738

Cao P, Cover TL (2002) Two different families of hopQ alleles in *Helicobacter pylori*. J Clin Microbiol 40:4504–4511

Carson SD, Klebba PE, Newton SM, Sparling PF (1999) Ferric enterobactin binding and utilization by *Neisseria gonorrhoeae*. J Bacteriol 181:2895–2901

Carswell CL, Rigden MD, Baenziger JE (2008) Expression, purification, and structural characterization of CfrA, a putative iron transporter from *Campylobacter jejuni*. J Bacteriol 190:5650–5662

Cascales E (2008) The type VI secretion toolkit. EMBO Rep 9:735–741

Chai TJ, Foulds J (1977) Purification of protein A, an outer membrane component missing in *Escherichia coli* K-12 ompA mutants. Biochim Biophys Acta 493:210–215

Chapman MR, Robinson LS, Pinkner JS, Roth R, Heuser J, Hammar M, Normark S, Hultgren SJ (2002) Role of Escherichia coli curli operons in directing amyloid fiber formation. Science 295:851–855

Charlson ES, Werner JN, Misra R (2006) Differential effects of yfgL mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. J Bacteriol 188:7186–7194

Chart H, Frost JA, Rowe B (1993) Expression of a new porin "OmpE" by strains of *Salmonella enteritidis*. FEMS Microbiol Lett 109:185–188

Chen I, Dubnau D (2004) DNA uptake during bacterial transformation. Nat Rev Microbiol 2: 241–249

Chen S, Zhang A, Blyn LB, Storz G (2004) MicC, a second small-RNA regulator of Omp protein expression in *Escherichia coli*. J Bacteriol 186:6689–6697

Cheng Q, Yu MC, Reeves AR (1995) Salyers AA (1995) Identification and characterization of a Bacteroides gene, csuF, which encodes an outer membrane protein that is essential for growth on chondroitin sulfate. J Bacteriol 177:3721–3727

Clantin B, Delattre AS, Rucktooa P, Saint N, Méli AC, Locht C, Jacob-Dubuisson F, Villeret V (2007) Structure of the membrane protein FhaC: a member of the Omp85-TpsB transporter superfamily. Science 317:957–961

Collins RF, Derrick JP (2007) Wza: a new structural paradigm for outer membrane secretory proteins? Trends Microbiol 15:96–100

Collins RF, Beis K, Dong C, Botting CH, McDonnell C, Ford RC, Clarke BR, Whitfield C, Naismith JH (2007) The 3D structure of a periplasm-spanning platform required for assembly of group 1 capsular polysaccharides in *Escherichia coli*. Proc Natl Acad Sci USA 104: 2390–2395

Conlan S, Bayley H (2003) Folding of a monomeric porin, OmpG, in detergent solution. Biochemistry 42:9453–9465

Conlan S, Zhang Y, Cheley S, Bayley H (2000) Biochemical and biophysical characterization of OmpG: a monomeric porin. Biochemistry 39:11845–11854

Cope LD, Yogev R, Müller-Eberhard U, Hansen EJ (1995) A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. J Bacteriol 177:2644–2653

Cornelis GR (2006) The type III secretion injectisome. Nat Rev Microbiol 4:811–825

Cornelissen CN, Biswas GD, Tsai J, Paruchuri DK, Thompson SA, Sparling PF (1992) Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J Bacteriol 174:5788–5797

Coulton JW, Mason P, Cameron DR, CarmelG JeanR, Rode HN (1986) Protein fusions of beta-galactosidase to the ferrichrome-iron receptor of *Escherichia coli* K-12. J Bacteriol 165:181–192

Cowan SW, Schirmer T, Rummel G, Steiert M, Ghosh R, Pauptit RA, Jansonius JN, Rosenbusch JP (1992) Crystal structures explain functional properties of two *Escherichia coli* porins. Nature 358:727–733

Cowan SW, Garavito RM, Jansonius JN, Jenkins JA, Karlsson R, König N, Pai EF, Pauptit RA, Rizkallah PJ, Rosenbusch JP (1995) The structure of OmpF porin in a tetragonal crystal form. Structure 3:1041–1050

Crosa JH, Walsh CT (2002) Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev 66:223–249

Danielli JF, Davson H (1935) A contribution to the theory of permeability of thin films. J Cell Physiol 5:495–508

Das S, Chaudhuri K (2003) Identification of a unique IAHP (IcmF associated homologous proteins) cluster in *Vibrio cholerae* and other proteobacteria through in silico analysis. In Silico Biol 3:287–300

Dautin N, Bernstein HD (2007) Protein secretion in gram-negative bacteria via the autotransporter pathway. Annu Rev Microbiol 61:89–112

De Buck E, Lammertyn E, Anné J (2008) The importance of the twin-arginine translocation pathway for bacterial virulence. Trends Microbiol 16:442–453

De Lay N, Gottesman S (2009) The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. J Bacteriol 191:461–476

de Zwaig N, Luria SE (1967) Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J Bacteriol 94:1112–1123

Dean CR, Poole K (1993) Cloning and characterization of the ferric enterobactin receptor gene (pfeA) of *Pseudomonas aeruginosa*. J Bacteriol 175:317–324

Deane JE, Roversi P, Cordes FS, Johnson S, Kenjale R, Daniell S, Booy F, Picking WD, Picking WL, Blocker AJ, Lea SM (2006) Molecular model of a type III secretion system needle: implications for hostcell sensing. Proc Natl Acad Sci USA 103:12529–12533

Dekker N, Tommassen J, Lustig A, Rosenbusch JP, Verheij HM (1997) Dimerization regulates the enzymatic activity of *Escherichia coli* outer membrane phospholipase A. J Biol Chem 272:3179–3184

delaVega AL, Delcour AH (1995) Cadaverine induces closing of *E. coli* porins. EMBO J 14:6058–6065

delaVega AL, Delcour AH (1996) Polyamines decrease *Escherichia coli* outer membrane permeability. J Bacteriol 178:3715–3721

Delcour AH (2009) Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta 1794:808–816

Delihas N (1995) Regulation of gene expression by trans-encoded antisense RNAs. Mol Microbiol 15:411–414

Delihas N, Forst S (2001) MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. J Mol Biol 313:1–12

dePetris S (1967) Ultrastructure of the cell wall of *Escherichia coli* and chemical nature of its constituent layers. J Ultrastruct Res 19:45–83

Derewenda U, Mateja A, Devedjiev Y, Routzahn KM, Evdokimov AG, Derewenda ZS, Waugh DS (2004) The structure of Yersinia pestis V-antigen, an essential virulence factor and mediator of immunity against plague. Structure 12:301–306

Dirusso CC, Black PN (2004) Bacterial long chain fatty acid transport: gateway to a fatty acid-responsive signaling system. J Biol Chem 279:49563–49566

Doerrler WT, Raetz CR (2005) Loss of outer membrane proteins without inhibition of lipid export in an *Escherichia coli* YaeT mutant. J Biol Chem 280:27679–27687

Doerrler WT, Gibbons HS, Raetz CRH (2004) MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*. J Biol Chem 276:45102–45109

Dong C, Beis K, Nesper J, Brunkan-Lamontagne AL, Clarke BR, Whitfield C, Naismith JH (2006) Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. Nature 444:226–229

Dorsey CW, Tomaras AP, Connerly PL, Tolmasky ME, Crosa JH, Actis LA (2004) The siderophore-mediated iron acquisition systems of *Acinetobacter baumannii* ATCC 19606 and *Vibrio anguillarum* 775 are structurally and functionally related. Microbiology 150:3657–3667

Douchin V, Bohn C, Bouloc P (2006) Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*. J Biol Chem 281:12253–12259

Dowhan W (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? Annu Rev Biochem 66:199–232

Drummelsmith J, Whitfield C (1999) Gene products required for surface expression of the capsular form of the group 1 K antigen in *Escherichia coli* (09a: K30). Mol Microbiol 31:1321–1332

Drummelsmith J, Whitfield C (2000) Translocation of group 1 capsular polysaccharide to the surface of *Escherichia coli* (O9a:K30) requires a multimeric complex in the outer membrane. EMBO J 19:57–66

Duong F, Soscia C, Lazdunski A, Murgier M (1994) The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. Mol Microbiol 11:1117–1126

Dutzler R, Wang YF, Rizkallah P, Rosenbusch JP, Schirmer T (1996) Crystal structures of various maltooligosaccharides bound to maltoporin reveal a specific sugar translocation pathway. Structure 4:127–134

Dutzler R, Rummel G, Albertí S, Hernández-Allés S, Phale P, Rosenbusch J, Benedí V, Schirmer T (1999) Crystal structure and functional characterization of OmpK36, the osmoporin of *Klebsiella pneumoniae*. Structure 7:425–434

Eaton RW (1997) p-Cymene catabolic pathway in *Pseudomonas putida* F1: cloning and characterization of DNA encoding conversion of p-Cymene to p-cumate. J Bacteriol 179:3171–3180

Edidin M (2003) Lipids on the frontier: a century of cell-membrane bilayers. Nat Rev Mol Cell Biol 4:414–418

Eisenberg D, Weiss RM, Terwilliger TC (1982) The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature 299:371–374

Eisenberg D, Weiss RM, Terwilliger TC (1984) The hydrophobic moment detects periodicity in protein hydrophobicity. Proc Natl Acad Sci USA 81:140–144

Eisenhauer HA, Shames S, Pawelek PD, Coulton JW (2005) Siderophore transport through *Escherichia coli* outer membrane receptor FhuA with disulfide-tethered cork and barrel domains. J Biol Chem 280:30574–30580

Enz S, Mahren S, Stroeher UH, Braun V (2000) Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. J Bacteriol 182: 637–646

Ertel F, Mirus O, Bredemeier R, Moslavac S, Becker T, Schleiff E (2005) The evolutionarily related beta-barrel polypeptide transporters from *Pisum sativum* and *Nostoc* PCC 7120 contain two distinct functional domains. J Biol Chem 280:28281–28289

Escolar L, Pérez-Martín J, de Lorenzo V (1998) Binding of the fur (ferric uptake regulator) repressor of *Escherichia coli* to arrays of the GATAAT sequence. J Mol Biol 283:537–547

Escolar L, Perez-Martin J, de Lorenzo V (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. J Bacteriol 181:6223–6229

Fajardo DA, Cheung J, Ito C, Sugawara E, Nikaido H, Misra R (1998) Biochemistry and regulation of a novel *Escherichia coli* K-12 porin protein, OmpG, which produces unusually large channels. J Bacteriol 180:4452–4459

Faraldo-Gómez JD, Sansom MSP (2003) Acquisition of siderophores in gram-negative bacteria. Nat Rev Mol Cell Biol 4:105–116

Farizo KM, Huang T, Burns DL (2000) Importance of holotoxin assembly in Ptl-mediated secretion of pertussis toxin from *Bordetella pertussis*. Infect Immun 68:4049–4054

Ferenci T, Schwentorat M, Ullrich S, Vilmart J (1980) Lambda receptor in the outer membrane of *Escherichia coli* as a binding protein for maltodextrins and starch polysaccharides. J Bacteriol 142:521–526

Ferguson AD, Deisenhofer J (2002) TonB-dependent receptors-structural perspectives. Biochim Biophys Acta 1565:318–332

Fetherston JD, Lillard JW Jr, Perry RD (1995) Analysis of the pesticin receptor from *Yersinia pestis:* role in iron-deficient growth and possible regulation by its siderophore. J Bacteriol 177:1824–1833

Fiedler G, Pajatsch M, Boeck A (1996) Genetics of a novel starch utilisation pathway present in *Klebsiella oxytoca*. J Mol Biol 256:279–291

Figueroa-Bossi N, Lemire S, Maloriol D, Balbontin R, Casadesus J, Bossi L (2006) Loss of Hfq activates the σ^E-dependent envelope stress response in *Salmonella enterica*. Mol Microbiol 62:838–852

Filloux A, Hachani A, Bleves S (2008) The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiology 154:1570–1583

Forst D, Welte W, Wacker T, Diederichs K (1998) Structure of the sucrosespecific porin ScrY from *Salmonella typhimurium* and its complex with sucrose. Nat Struct Biol 5:37–46

Fralick JA (1996) Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J Bacteriol 178:5803–5805

Fronzes R, Remaut H, Waksman G (2008) Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. EMBO J 27:2271–2280

Furano K, Luke NR, Howlett AJ, Campagnari AA (2005) Identification of a conserved *Moraxella catarrhalis* haemoglobin-utilization protein, MhuA. Microbiology 151:1151–1158

Gehring KB, Nikaido H (1989) Existence and purification of porin heterotrimers of *Escherichia coli* K12 OmpC, OmpF, and PhoE proteins. J Biol Chem 264:2810–2815

Genevrois S, Steeghs L, Roholl P, Letesson JJ, van der Ley P (2003) The Omp85 protein of *Neisseria meningitidis* is required for lipid export to the outer membrane. EMBO J 22: 1780–1789

Gerdes SY, Scholle MD, Campbell JW, Balázsi G, Ravasz E, Daugherty MD, Somera AL, Kyrpides NC, Anderson I, Gelfand MS, Bhattacharya A, Kapatral V, D'Souza M, Baev MV, Grechkin Y, Mseeh F, Fonstein MY, Overbeek R, Barabási AL, Oltvai ZN, Osterman AL (2003) Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. J Bacteriol 185:5673–5684

Gerlach RG, Hensel M (2007) Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int J Med Microbiol 297:401–415

Gil-Cruz C, Bobat S, Marshall JL, Kingsley RA, Ross EA, Henderson IR, Leyton DL, Coughlan RE, Khan M, Jensen KT, Buckley CD, Dougan G, MacLennan IC, López-Macías C, Cunningham AF (2009) The porin OmpD from nontyphoidal *Salmonella* is a key target for a protective B1b cell antibody response. Proc Natl Acad Sci USA 106:9803–9808

Goldberg MB, Boyko SA, Butterton JR, Stoebner JA, Payne SM, Calderwood SB (1992) Characterization of a *Vibrio cholerae* virulence factor homologous to the family of TonB-dependent proteins. Mol Microbiol 6:2407–2418

Goldberg M, Pribyl T, Juhnke S, Nies DH (1999) Energetics and topology of CzcA, a cation/proton antiporter of the resistance-nodulation-cell division protein family. J Biol Chem 274:26065–26070

Gophna U, Barlev M, Seijffers R, Oelschlager TA, Hacker J, Ron EZ (2001) Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. Infect Immun 69:2659–2665

Gottesman S (2005) Micros for microbes: non-coding regulatory RNAs in bacteria. Trends Genet 21:399–404

Grizot S, Buchanan SK (2004) Structure of the OmpA-like domain of RmpM from *Neisseria meningitidis*. Mol Microbiol 51:1027–1037

Guillier M, Gottesman S (2006) Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. Mol Microbiol 59:231–247

Guillier M, Gottesman S (2008) The 50 end of two redundant sRNAs is involved in the regulation of multiple targets, including their own regulator. Nucleic Acids Res 36: 6781–6794

Gumbart J, Wiener MC, Tajkhorshid E (2007) Mechanics of force propagation in TonB-dependent outer membrane transport. Biophys J 93:496–504

Guo L, Lim KB, Poduje CM, Daniel M, Gunn JS, Hackett M, Miller SI (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95:189–198

Habe H, Kasuga K, Nojiri H, Yamane H, Omori T (1996) Analysis of cumene (isopropylbenzene) degradation genes from *Pseudomonas fluorescens* IPO1. Appl Environ Microbiol 62: 4471–4477

Hammar M, Arnqvist A, Bian Z, Olsén A, Normark S (1995) Expression of two csg operons is required for production of fibronectin- and congo red-binding curli polymers in Escherichia coli K-12. Mol Microbiol 18:661–670

Hammar M, Bian Z, Normark S (1996) Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. Proc Natl Acad Sci USA 93:6562–6566

Hancock REW, Decad GM, Nikaido H (1979) Identification of the protein producing transmembrane diffusion pores in the outer membrane of *Pseudomonas aeruginosa* PA01. Biochim Biophys Acta 554:323–331

Hantke K, Braun V (1975) Membrane receptor dependent iron transport in *Escherichia coli*. FEBS Lett 49:301–305

Hardesty C, Ferran C, DiRienzo JM (1991) Plasmid-mediated sucrose metabolism in *Escherichia coli:* characterization of scrY, the structural gene for a phosphoenolpyruvate-dependent sucrose phosphotransferase system outer membrane porin. J Bacteriol 173:449–456

Hearn EM, Patel DR, Lepore BW, Indic M, van den Berg B (2009) Transmembrane passage of hydrophobic compounds through a protein channel wall. Nature 458:367–370

Heller K, Kadner RJ (1985) Nucleotide sequence of the gene for the vitamin B12 receptor protein in the outer membrane of *Escherichia coli*. J Bacteriol 161:904–908

Henderson DP, Payne SM (1994) Characterization of the Vibrio cholerae outer membrane heme transport protein HutA: sequence of the gene, regulation of expression, and homology to the family of TonB-dependent proteins. J Bacteriol 176:3269–3277

Henderson R, Unwin PNT (1975) Three-dimensional model of purple membrane obtained by electron microscopy. Nature 257:28–32

Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D (2004) Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev 68:692–744

Hennecke G, Nolte J, Volkmer-Engert R, Schneider-Mergener J, Behrens S (2005) The periplasmic chaperone SurA exploits two features characteristic of integral outer membrane proteins for selective substrate recognition. J Biol Chem 280:23540–23548

Hiller S, Garces RG, Malia TJ, Orekhov VY, Colombini M, Wagner G (2008) Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. Science 321:1206–1210

Hong H, Szabo G, Tamm LK (2006) Electrostatic couplings in OmpA ion-channel gating suggest a mechanism for pore opening. Nat Chem Biol 2:627–635

Hornung JM, Jones HA, Perry RD (1996) The hmu locus of Yersinia pestis is essential for utilization of free haemin and haem–protein complexes as iron sources. Mol Microbiol 20:725–739

Horrevoets AJ, Hackeng TM, Verheij HM, Dijkman R, de Haas GH (1989) Kinetic characterization of *Escherichia coli* outer membrane phospholipase A using mixed detergent-lipid micelles. Biochemistry 28:1139–1147

Hwang PM, Choy WY, Lo EI, Chen L, Forman-Kay JD, Raetz CR, Prive GG, Bishop RE, Kay LE (2002) Solution structure and dynamics of the outer membrane enzyme PagP by NMR. Proc Natl Acad Sci USA 99:13560–13565

Im W, Roux B (2002) Ions and counterions in a biological channel: a molecular dynamics simulation of OmpF porin from *Escherichia coli* in an explicit membrane with 1 M KCl aqueous salt solution. J Mol Biol 319:1177–1197

Jacob-Dubuisson F, Fernandez R, Coutte L (2004) Protein secretion through autotransporter and two-partner pathways. Biochim Biophys Acta 1694:235–257

Jacob-Dubuisson F, Villeret V, Clantin B, Delattre AS, Saint N (2009) First structural insights into the TpsB/Omp85 superfamily. Biol Chem 390:675–684

James D, Shao H, Lamont RJ, Demuth DR (2006) The *Actinobacillus actinomycetemcomitans* ribose binding protein RbsB interacts with cognate and heterologous autoinducer 2 signals. Infect Immun 74:4021–4029

Johansen J, Rasmussen AA, Overgaard M, Valentin-Hansen P (2006) Conserved small non-coding RNAs that belong to the σ^E regulon: role in down-regulation of outer membrane proteins. J Mol Biol 364:1–8

Johansen J, Eriksen M, Kallipolitis B, Valentin-Hansen P (2008) Down-regulation of outer membrane proteins by noncoding RNAs: unraveling the cAMP-CRP- and sigmaE-dependent CyaR-ompX regulatory case. J Mol Biol 383:1–9

Jones RM, Pagmantidis V, Williams PM (2000) sal genes determining the catabolism of salicylate esters are part of a supraoperonic cluster of catabolic genes in *Acinetobacter* sp. strain ADP1. J Bacteriol 182:2018–2025

Juhas M, Crook DW, Hood DW (2008) Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. Cell Microbiol 10:2377–2386

Kahnert A, Kertesz MA (2000) Characterization of a sulfur-regulated oxygenative alkylsulfatase from *Pseudomonas putida* S-313. J Biol Chem 275:31661–31667

Kahng HY, Byrne AM, Olsen RH, Kukor JJ (2000) Characterization and role of tbuX in utilization of toluene by *Ralstonia pickettii* PKO1. J Bacteriol 182:1232–1242

Kasai Y, Inoue J, Harayama S (2001) The TOL plasmid pWW0 xylN gene product from *Pseudomonas putida* is involved in m-xylene uptake. J Bacteriol 183:6662–6666

Kim I, Stiefel A, Plantor S, Angerer A, Braun V (1997) Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. Mol Microbiol 23:333–344

Kim S, Malinverni JC, Sliz P, Silhavy TJ, Harrison SC, Kahne D (2007) Structure and function of an essential component of the outer membrane protein assembly machine. Science 317: 961–964

Klebba PE (2002) Mechanism of maltodextrin transport through LamB. Res Microbiol 153: 417–424

Knowles TJ, Jeeves M, Bobat S, Dancea F, McClelland D, Palmer T, Overduin M, Henderson IR (2008) Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. Mol Microbiol 68:1216–1227

Koebnik R (2005) TonB-dependent trans-envelope signalling: the exception or the rule? Trends Microbiol 13:343–347

Koebnik R, Kramer L (1995) Membrane assembly of circularly permuted variants of the *E. coli* outer membrane protein OmpA. J Mol Biol 250:617–626

Koebnik R, Hantke K, Braun V (1993) The TonB-dependent ferrichrome receptor FcuA of *Yersinia enterocolitica*: evidence against a strict co-evolution of receptor structure and substrate specificity. Mol Microbiol 7:383–393

Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature 405:914–919

Koski P, Vaara M (1991) Polyamines as constituents of the outer membranes of Escherichia coli and Salmonella typhimurium. J Bacteriol 173:3695–3699

Koster M, van de Vossenberg J, Leong J, Weisbeek PJ (1993) Identification and characterization of the pupB gene encoding an inducible ferric-pseudobactin receptor of Pseudomonas putida WCS358. Mol Microbiol 8:591–601

Krone WJA, Stegehuis F, Koningstein G, van Doorn C, Roosendaal B, de Graaf FK, Oudega B (1985) Characterization of the pColV-K30 encoded cloacin DF13/aerobactin outer membrane receptor protein of *Escherichia coli;* isolation and purification of the protein and analysis of its nucleotide sequence and primary structure. FEMS Microbiol Lett 26:153–161

Kubori T, Matsushima Y, Nakamura D, Uralil J, Lara-Tejero M, Sukhan A, Galán JE, Aizawa SI (1998) Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science 280:602–605

Kühn S, Braun V, Köster W (1996) Ferric rhizoferrin uptake into *Morganella morganii:* characterization of genes involved in the uptake of a polyhydroxycarboxylate siderophore. J Bacteriol 178:496–504

Kumar GB, Black PN (1993) Bacterial long-chain fatty acid transport: identification of amino acid residues within the outer membrane protein FadL required for activity. J Biol Chem 268:15469–15476

Lakey JH, Lea EJ, Pattus F (1991) OmpC mutants which allow growth on maltodextrins show increased channel size and greater voltage sensitivity. FEBS Lett 278:31–34

Lawley TD, Klimke WA, Gubbins MJ, Frost LS (2003) F factor conjugation is a true type IV secretion system. FEMS Microbiol Lett 224:1–15

Le Cam E, Frechon D, Barray M, Fourcade A, Delain E (1994) Observation of binding and polymerization of Fur repressor onto operator-containing DNA with electron and atomic force microscopes. Proc Natl Acad Sci USA 91:11816–11820

Lee BC (1995) Quelling the red menace: haem capture by bacteria. Mol Microbiol 18:383–390

Lenard J, Singer SJ (1966) Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. Proc Natl Acad Sci USA 56:1828–1835

Letoffe S, Ghigo JM, Wandersman C (1994) Iron acquisition from heme and hemoglobin by a *Serratia marcescens* extracellular protein. Proc Natl Acad Sci USA 91:9876–9880

LeVier K, Guerinot ML (1996) The *Bradyrhizobium japonicum* fegA gene encodes an iron-regulated outer membrane protein with similarity to hydroxamate-type siderophore receptors. J Bacteriol 178:7265–7275

Lewis LA, Gray E, Wang YP, Roe BA, Dyer DW (1997) Molecular characterization of hpuAB, the haemoglobin-haptoglobin-utilization operon of *Neisseria meningitidis*. Mol Microbiol 23:737–749

Liu WM (1998) Shear numbers of protein β-barrels: definition refinements and statistics. J Mol Biol 275:541–545

Llamas MA, Sparrius M, Kloet R, Jiménez CR, Vandenbroucke-Grauls C, Bitter W (2006) The heterologous siderophores ferrioxamine B and ferrichrome activate signaling pathways in *Pseudomonas aeruginosa*. J Bacteriol 188:1882–1891

Lobedanz S, Bokma E, Symmons MF, Koronakis E, Hughes C, Koronakis V (2007) A periplasmic coiled-coil interface underlying TolC recruitment and the assembly of bacterial drug efflux pumps. Proc Natl Acad Sci 104:4612–4617

Löffelhardt W, von Haeseler A, Schleiff E (2007) The β-barrel shaped polypeptide transporter, an old concept for precursor protein transfer across membranes. Symbiosis 44:33–42

Lonetto MA, Brown KL, Rudd KE, Buttner MJ (1994) Analysis of the *Streptomyces coelicolor* sigE gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. Proc Natl Acad Sci USA 91: 7573–7577

Luck SN, Turner SA, Rajakumar K, Sakellaris H, Adler B (2001) Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. Infect Immun 69:6012–6021

Lundmark K, Westermark GT, Olsén A, Westermark P (2005) Protein fibrils in nature can enhance amyloid protein A amyloidosis in mice: Cross-seeding as a disease mechanism. Proc Natl Acad Sci USA 102:6098–6102

Lundrigan MD, Kadner RJ (1986) Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*. Homology among outer membrane receptors that interact with TonB. J Biol Chem 261:10797–10801

Lynch D, O'Brien J, Welch T, Clarke P, Cuiv PO, Crosa JH, O'Connell M (2001) Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti*. J Bacteriol 183:2576–2585

Ma L, Kaserer W, Annamalai R, Scott DC, Jin B, Jiang X, Xiao Q, Maymani H, Massis LM, Ferreira LC, Newton SM, Klebba PE (2007) Evidence of ball-and-chain transport of ferric enterobactin through FepA. J Biol Chem 282:397–406

Magnusson LU, Farewell A, Nyström T (2005) ppGpp a global regulator in *Escherichia coli*. Trends Microbiol 13:236–242

Malinverni JC, Werner J, Kim S, Sklar JG, Kahne D, Misra R, Silhavy TJ (2006) YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. Mol Microbiol 61:151–164

Marlovits TC, Kubori T, Sukhan A, Thomas DR, Galán JE, Unger VM (2004) Structural insights into the assembly of the type III secretion needle complex. Science 306:1040–1042

Marsh D (2003) Lipid interactions with transmembrane proteins. Cell Mol Life Sci 60:1575–1580

Marzi S, Fechter P, Chevalier C, Romby P, Geissmann T (2008) RNA switches regulate initiation of translation in bacteria. Biol Chem 389:585–598

Masi M, Duret G, Delcour AH, Misra R (2009) Folding and trimerization of signal sequence-less mature TolC in the cytoplasm of *Escherichia coli*. Microbiology 55:1847–1857

Mazar J, Cotter PA (2007) New insight into the molecular mechanisms of two-partner secretion. Trends Microbiol 15:508–515

Mazoy R, Osorio CR, Toranzo AE, Lemos ML (2003) Isolation of mutants of *Vibrio anguillarum* defective in haeme utilisation and cloning of huvA, a gene coding for an outer membrane protein involved in the use of haeme as iron source. Arch Microbiol 179:329–338

Meng G, Surana NK, St. Geme JW III, Waksman G (2006) Structure of the outer membrane translocator domain of the *Haemophilus influenzae* Hia trimeric autotransporter. EMBO J 25:2297–2304

Mey AR, Payne SM (2001) Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. Mol Microbiol 42:835–849

Mey AR, Wyckoff EE, Oglesby AG, Rab E, Taylor RK, Payne SM (2002) Identification of the *Vibrio cholerae* Enterobactin Receptors VctA and IrgA: IrgA Is Not Required for Virulence. Infect Immun 70:3419–3426

Mey AR, Wyckoff EE, Hoover LA, Fisher CR, Payne SM (2008) *Vibrio cholerae* VciB promotes iron uptake via ferrous iron transporters. J Bacteriol 190:5953–5962

Michiels T, Wattiau P, Brasseur R, Ruysschaert JM, Cornelis G (1990) Secretion of Yop proteins by *Yersiniae*. Infect Immun 58:2840–2849

Mills M, Payne SM (1997) Identification of shuA, the gene encoding the heme receptor of *Shigella dysenteriae*, and analysis of invasion and intracellular multiplication of a shuA mutant. Infect Immun 65:5358–5363

Mirus O, Schleiff E (2005) Prediction of beta-barrel membrane proteins by searching for restricted domains. BMC Bioinformatics 6:254

Misra R, Benson SA (1989) A novel mutation, cog, which results in production of a new porin protein (OmpG) of *Escherichia coli* K-12. J Bacteriol 171:4105–4111

Mizuno T, Chou MY, Inouye M (1984) A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc Natl Acad Sci USA 81:1966–1970

Mogensen JE, Otzen DE (2005) Interactions between folding factors and bacterial outer membrane proteins. Mol Microbiol 57:326–346

Morris J, Donnelly DF, O'Neill E, McConnell F, O'Gara F (1994) Nucleotide sequence analysis and potential environmental distribution of a ferric pseudobactin receptor gene of *Pseudomonas* sp. strain M114. Mol Gen Genet 242:9–16

Moslavac S, Mirus O, Bredemeier R, Soll J, von Haeseler A, Schleiff E (2005) Conserved pore-forming regions in polypeptide-transporting proteins. FEBS J. 272:1367–1378

Moslavac S, Nicolaisen K, Mirus O, Al Dehni F, Pernil R, Flores E, Maldener I, Schleiff E (2007) A TolC-like protein is required for heterocyst development in *Anabaena* sp. strain PCC 7120. J Bacteriol 189:7887–7895

Mueller CA, Broz P, Muller SA, Ringler P, Erne-Brand F, Sorg I, Kuhn M, Engel A, Cornelis GR (2005) The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles. Science 310:674–676

Nagai H, Cambronne ED, Kagan JC, Amor JC, Kahn RA, Roy CR (2005) A C-terminal translocation signal required for Dot/Icm-dependent delivery of the Legionella RalF protein to host cells. Proc Natl Acad Sci USA 102:826–831

Nagano K, Murakami Y, Nishikawa K, Sakakibara J, Shimozato K, Yoshimura F (2007) Characterization of RagA and RagB in *Porphyromonas gingivalis:* study using gene-deletion mutants. J Med Microbiol 56:1536–1548

Nakae T (1975) Outer membrane of *Salmonella typhimurium:* Reconstitution of sucrose-permeable membrane vesicles. Biochem Biophys Res Commun 64:1224–1230

Nakao R, Tashiro Y, Nomura N, Kosono S, Ochiai K, Yonezawa H, Watanabe H, Senpuku H (2008) Glycosylation of the OMP85 homolog of *Porphyromonas gingivalis* and its involvement in biofilm formation. Biochem Biophys Res Commun 365:784–789

Nau CD, Konisky J (1989) Evolutionary relationship between the TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequences of the *Escherichia coli* colicin I receptor gene. J Bacteriol 171:1041–1047

Neilands JB (1995) Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 270:26723–26726

Nesper J, Hill CM, Paiment A, Harauz G, Beis K, Naismith JH, Whitfield C (2003) Translocation of group 1 capsular polysaccharide in *Escherichia coli* serotype K30. Structural and functional analysis of the outer membrane lipoprotein Wza. J Biol Chem 278:49763–49772

Neugebauer H, Herrmann C, Kammer W, Schwarz G, Nordheim A, Braun V (2005) ExbBD-dependent transport of maltodextrins through the novel MalA protein across the outer membrane of *Caulobacter crescentus*. J Bacteriol 187:8300–8311

Nicolaisen K, Moslavac S, Samborski A, Valdebenito M, Hantke K, Maldener I, Muro-Pastor AM, Flores E, Schleiff E (2008) Alr0397 is an outer membrane transporter for the siderophore schizokinen in *Anabaena* sp. strain PCC 7120. J Bacteriol 190:7500–7507

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Nikaido H, Rosenberg EY (1983) Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J Bacteriol 153:241–252

Nikaido H, Rosenberg EY, Foulds J (1983) Porin channels in *Escherichia coli:* studies with beta-lactams in intact cells. J Bacteriol 153:232–240

Ochsner UA, Vasil ML (1996) Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc Natl Acad Sci USA 93:4409–4414

Ochsner UA, Johnson Z, Vasil ML (2000) Genetics and regulation of two distinct haem-uptake systems, phu and has, in *Pseudomonas aeruginosa*. Microbiology 146:185–198

Olsén A, Jonsson A, Normark S (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature 338:652–655

Oomen CJ, van Ulsen P, van Gelder P, Feijen M, Tommassen J, Gros P (2004) Structure of the translocator domain of a bacterial autotransporter. EMBO J 23:1257–1266

Pajatsch M, Andersen C, Mathes A, Boeck A, Benz R, Engelhardt H (1999) Properties of a cyclodextrin-specific, unusual porin from *Klebsiella oxytoca*. J Biol Chem 274: 25159–25166

Palyada K, Threadgill D, Stintzi A (2004) Iron acquisition and regulation in *Campylobacter jejuni*. J Bacteriol 186:4714–4729

Papenfort K, Vogel J (2009) Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level. Res Microbiol 160:278–287

Papenfort K, Pfeiffer V, Mika F, Lucchini S, Hinton JC, Vogel J (2006) Sigma(E)-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global omp mRNA decay. Mol Microbiol 62:1674–1688

Papenfort K, Pfeiffer V, Lucchini S, Sonawane A, Hinton JC, Vogel J (2008) Systematic deletion of *Salmonella* small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. Mol Microbiol 68:890–906

Pautsch A, Schulz GE (1998) Structure of the outer membrane protein A transmembrane domain. Nat Struct Biol 5:1013–1017

Pellinen T, Ahlfors H, Blot N, Condemine G (2003) Topology of the *Erwinia chrysanthemi* oligogalacturonate porin KdgM. Biochem J 372:329–334

Perkins-Balding D, Ratliff-Griffin M, Stojiljkovic I (2004) Iron transport systems in Neisseria meningitidis. Microbiol Mol Biol Rev 68:154–171

Pfeiffer V, Sittka A, Tomer R, Tedin K, Brinkmann V, Vogel J (2007) A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the *Salmonella* core genome. Mol Microbiol 66:1174–1191

Poole K, Neshat S, Krebes K, Heinrichs DE (1993) Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene fpvA of *Pseudomonas aeruginosa*. J Bacteriol 175: 4597–4604

Pos KM (2009) Drug transport mechanism of the AcrB efflux pump. Biochim Biophys Acta 1794:782–793

Pradel E, Locht C (2001) Expression of the putative siderophore receptor gene bfrZ is controlled by the extracytoplasmic-function sigma factor BupI in *Bordetella bronchiseptica*. J Bacteriol 183:2910–2917

Pratt LA, Hsing W, Gibson KE, Silhavy TJ (1996) From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. Mol Microbiol 20:911–917

Pressler U, Staudenmaier H, Zimmermann L, Braun V (1988) Genetics of the iron dicitrate transport system of *Escherichia coli*. J Bacteriol 170:2716–2724

Prince SM, Achtman M, Derrick JP (2002) Crystal structure of the OpcA integral membrane adhesin from *Neisseria meningitidis*. Proc Natl Acad Sci USA 99:3417–3421

Pukatzki S, McAuley SB, Miyata ST (2009) The type VI secretion system: translocation of effectors and effector-domains. Curr Opin Microbiol 12:11–17

Rakin A, Saken E, Harmsen D, Heesemann J (1994) The pesticin receptor of *Yersinia enterocolitica*: a novel virulence factor with dual function. Mol Microbiol 13:253–263

Ranquin A, Van Gelder P (2004) Maltoporin: sugar for physics and biology. Res Microbiol 155:611–616

Rasmussen AA, Eriksen M, Gilany K, Udesen C, Franch T, Petersen C, Valentin-Hansen P (2005) Regulation of ompA mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. Mol Microbiol 58:1421–1429

Reeves AR, D'Elia JN, Frias J, Salyers AA (1996) A *Bacteroides thetaiotaomicron* outer membrane protein that is essential for utilization of maltooligosaccharides and starch. J Bacteriol 178:823–830

Reid AN, Whitfield C (2005) Functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. J Bacteriol 187:5470–5548

Remaut H, Tang C, Henderson NS, Pinkner JS, Wang T, Hultgren SJ, Thanassi DG, Waksman G, Li H (2008) Fiber formation across the bacterial outer membrane by the chaperone/usher pathway. Cell 133:640–652

Remsen C, Lundgren DG (1966) Electron microscopy of the cell envelope of *Ferrobacillus ferrooxidans* prepared by freeze-etching and chemical fixation techniques. J Bacteriol 92:1765–1771

Richardson JS (1977) beta-Sheet topology and the relatedness of proteins. Nature 268:495–500

Robinson LS, Ashman EM, Hultgren SJ, Chapman MR (2006) Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein. Mol Microbiol 59:870–881

Rosenbusch JP (1974) Characterization of the major envelope protein from *Escherichia coli* – Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. J Biol Chem 249:8019–8029

Ruffolo CG, Adler B (1996) Cloning, sequencing, expression, and protective capacity of the oma87 gene encoding the *Pasteurella multocida* 87-kilodalton outer membrane antigen. Infect Immun 64:3161–3167

Ruiz N, Silhavy TJ (2005) Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. Curr Opin Microbiol 8:122–126

Ruiz N, Kahne D, Silhavy TJ (2006) Advances in understanding bacterial outer-membrane biogenesis. Nat Rev Microbiol 4:57–66

Saier MH, Tam R, Reizer A, Reizer J (1994) Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol 11:841–847

Saier MH Jr, Beatty JT, Goffeau A, Harley KT, Heijne WH, Huang SC, Jack DL, Jähn PS, Lew K, Liu J, Pao SS, Paulsen IT, Tseng TT, Virk PS (1999) The major facilitator superfamily. J Mol Microbiol Biotechnol 1:257–279

Saint N, Lou KL, Widmer C, Luckey M, Schirmer T, Rosenbusch JP (1996) Structural and functional characterization of OmpF porin mutants selected for larger pore size. II. Functional characterization. J Biol Chem 271:20676–20680

Samartzidou H, Delcour AH (1999) Excretion of endogenous cadaverine leads to a decrease in porin-mediated outer membrane permeability. J Bacteriol 181:791–798
Sampson BA, Misra R, Benson SA (1989) Identification and characterization of a new gene of *Escherichia coli* K-12 involved in outer membrane permeability. Genetics 122:491–501
Sandkvist M (2001) Biology of type II secretion. Mol Microbiol 40:271–283
Santiviago CA, Toro CS, Hidalgo AA, Youderian P, Mora GC (2003) Global regulation of the *Salmonella enterica* serovar typhimurium major porin, OmpD. J Bacteriol 185:5901–5905
Sauer M, Hantke K, Braun V (1990) Sequence of the fhuE outer-membrane receptor gene of *Escherichia coli* K12 and properties of mutants. Mol Microbiol 4:427–437
Sauer FG, Remaut H, Hultgren SJ, Waksman G (2004) Fiber assembly by the chaperone-usher pathway. Biochim Biophys Acta 1694:259–267
Sauvage C, Franza T, Expert D (1996) Analysis of the Erwinia chrysanthemi ferrichrysobactin receptor gene: resemblance to the *Escherichia coli* fepA-fes bidirectional promoter region and homology with hydroxamate receptors. J Bacteriol 178:1227–1231
Scandella CJ, Kornberg A (1971) A membrane-bound phospholipase A1 purified from *Escherichia coli*. Biochemistry 10:4447–4456
Schalk IJ, Yue WW, Buchanan SK (2004) Recognition of iron-free siderophores by TonB-dependent irontransporters. Mol Microbiol 54:14–22
Schauer K, Gouget B, Carrière M, Labigne A, de Reuse H (2007) Novel nickel transport mechanism across the bacterial outer membrane energized by the TonB/ExbB/ExbD machinery. Mol Microbiol 63:1054–1068
Schell MA, Ulrich RL, Ribot WJ, Brueggemann EE, Hines HB, Chen D, Lipscomb L, Kim HS, Mrázek J, Nierman WC, Deshazer D (2007) Type VI secretion is a major virulence determinant in *Burkholderia mallei*. Mol Microbiol 64:1466–1485
Schirmer T, Keller TA, Wang YF, Rosenbusch JP (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution. Science 267:512–514
Schleiff E, Soll J (2005) Membrane protein insertion: mixing eukaryotic and prokaryotic concepts. EMBO Rep 6:1023–1027
Schleiff E, Eichacker LA, Eckart K, Becker T, Mirus O, Stahl T, Soll J (2003) Prediction of the plant beta-barrel proteome: a case study of the chloroplast outer envelope. Protein Sci 12: 748–759
Schmid K, Ebner R, Jahreis K, Lengeler JW, Titgemeyer F (1991) A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. Mol Microbiol 5:941–950
Schmidt M, Zheng P, Delihas N (1995) Secondary structures of *Escherichia coli* antisense micF RNA, the 50-end of the target ompF mRNA, and the RNA/RNA duplex. Biochemistry 34:3621–3631
Schuelein K (1991) The sugar-specific outer membrane channel ScrY contains functional characteristics of general diffusion pores and substratespecific porins. Mol Microbiol 5:2233–2241
Schulz GE (2000) beta-Barrel membrane proteins. Curr Opin Struct Biol 10:443–447
Schulz GE (2002) The structure of bacterial outer membrane proteins. Biochim Biophys Acta 1565:308–317
Seeger MA, Diederichs K, Eicher T, Brandstätter L, Schiefner A, Verrey F, Pos KM (2008) The AcrB efflux pump: conformational cycling and peristalsis lead to multidrug resistance. Curr Drug Targets 9:729–749
Sheahan KL, Cordero CL, Satchell KJ (2004) Identification of a domain within the multifunctional Vibrio cholerae RTX toxin that covalently cross-links actin. Proc Natl Acad Sci USA 101:9798–9803
Shultis DD, Purdy MD, Banchs CN, Wiener MC (2006) Outer membrane active transport: structure of the BtuB:TonB complex. Science 312:1396–1399
Silakowski B, Kunze B, Nordsiek G, Blöcker H, Höfle G, Müller R (2000) The myxochelin iron transport regulon of the myxobacterium *Stigmatella aurantiaca* Sg a15. Eur J Biochem 267:6476–6485
Singer SJ (1962) The properties of proteins in non-aqueous solvents. Adv Protein Chem 17:1–68

Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720–731

Sklar JG, Wu T, Kahne D, Silhavy TJ (2007a) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. Genes Dev 21:2473–2484

Sklar JG, Wu T, Gronenberg LS, Malinverni JC, Kahne D, Silhavy TJ (2007b) Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. Proc Natl Acad Sci USA 104:6400–6405

Smith SG, Mahon V, Lambert MA, Fagan RP (2007) A molecular Swiss army knife: OmpA structure, function and expression. FEMS Microbiol Lett 273:1–11

Snijder HJ, Ubarretxena-Belandia I, Blaauw M, Kalk KH, Verheij HM, Egmond MR, Dekker N, Dijkstra BW (1999) Structural evidence for dimerization-regulated activation of an integral membrane phospholipase. Nature 401:717–721

Sokol PA, Darling P, Lewenza S, Corbett CR, Kooi CD (2000) Identification of a siderophore receptor required for ferric ornibactin uptake in *Burkholderia cepacia*. Infect Immun 68:6554–6560

Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, Gouaux JE (1996) Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science 274:1859–1866

Sperandeo P, Cescutti R, Villa R, Di Benedetto C, Candia D, Dehò G, Polissi A (2007) Characterization of lptA and lptB, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. J Bacteriol 189:244–253

Srikumar R, Mikael LG, Pawelek PD, Khamessan A, Gibbs BF, Jacques M, Coulton JW (2004) Molecular cloning of haemoglobin-binding protein HgbA in the outer membrane of *Actinobacillus pleuropneumoniae*. Microbiology 150:1723–1734

Stanley AM, Chuawong P, Hendrickson TL, Fleming KG (2006) Energetics of outer membrane phospholipase A (OMPLA) dimerization. J Mol Biol 358:120–131

Stanley AM, Treubrodt AM, Chuawong P, Hendrickson TL, Fleming KG (2007) Lipid chain selectivity by outer membrane phospholipase A. J Mol Biol 366:461–468

Stathopoulos C (1996) An alternative topological model for *Escherichia coli* OmpA. Protein Sci 5:170–173

Stegmeier JF, Glück A, Sukumaran S, Mäntele W, Andersen C (2007) Characterisation of YtfM, a second member of the Omp85 family in Escherichia coli. Biol Chem 388:37–46

Stojiljkovic I, Hantke K (1992) Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J 11:4359–4367

Stojiljkovic I, Hwa V, de Saint Martin L, O'Gaora P, Nassif X, Heffron F, So M (1995) The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. Mol Microbiol 15:531–541

Storz G, Altuvia S, Wassarman KM (2005) An abundance of RNA regulators. Annu Rev Biochem 74:199–217

Struyvé M, Moons M, Tommassen J (1991) Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J Mol Biol 218:141–148

Sugawara E, Nikaido H (1992) Pore-forming activity of OmpA protein of *Escherichia coli*. J Biol Chem 267:2507–2511

Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V (2009) The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc Natl Acad Sci 106:6893–6984

Szmelcman S, Hofnung M (1975) Maltose transport in Escherichia coli K-12: Involvement of the bacteriophage lambda receptor. J Bacteriol 124:112–118

Takizawa N, Murooka Y (1985) Cloning of the pullulanase gene and overproduction of pullulanase in *Escherichia coli* and *Klebsiella aerogenes*. Appl Environ Microbiol 49: 294–298

Tamm LK, Hong H, Liang B (2004) Folding and assembly of beta-barrel membrane proteins. Biochim Biophys Acta 1666:250–263

Tarshis LC, Proteau PJ, Kellogg BA, Sacchettini JC, Poulter CD (1996) Regulation of product chain length by isoprenyl diphosphate synthases. Proc Natl Acad Sci USA 93:15018–15023

Testerman TL, Vazquez-Torres A, Xu Y, Jones-Carson J, Libby SJ, Fang FC (2002) The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella virulence* and stationary-phase survival. Mol Microbiol 43:771–782

Thomas WR, Callow MG, Dilworth RJ, Audesho AA (1990) Expression in *Escherichia coli* of a high-molecular weight protective surface antigen found in nontypeable and type b *Haemophilus influenzae*. Infect Immun 58:1909–1913

Thomas CE, Olsen B, Elkins C (1998) Cloning and characterization of tdhA, a locus encoding a TonB-dependent heme receptor from *Haemophilus ducreyi*. Infect Immun 66:4254–4262

Tommassen J (1988) Biogenesis and membrane topology of outer membrane proteins in *Escherichia coli*. In: op den Kamp JAF (ed) Membrane biogenesis. NATO ASI Series, vol H16. Springer, Berlin

Tommassen J, Lugtenberg B (1982) PHO-regulon of *Escherichia coli* K12: a minireview. Ann Microbiol 133:243–249

Torres AG, Payne SM (1997) Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. Mol Microbiol 23:825–833

Touzé T, Eswaran J, Bokma E, Koronakis E, Hughes C, Koronakis V (2004) Interactions underlying assembly of the *Escherichia coli* AcrAB-TolC multidrug efflux system. Mol Microbiol 53:697–706

Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. EMBO J 14:2436–2446

Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. Genes Dev 19:2355–2366

Ujwal R, Cascio D, Colletier JP, Faham S, Zhang J, Toro L, Ping P, Abramson J (2008) The crystal structure of mouse VDAC1 at 2.3 A resolution reveals mechanistic insights into metabolite gating. Proc Natl Acad Sci USA 105:17742–17747

Ulmke C, Kreth J, Lengeler JW, Welte W, Schmid K (1999) Site-directed mutagenesis of loop L3 of sucrose porin ScrY leads to changes in substrate selectivity. J Bacteriol 181: 1920–1923

Urban JH, Vogel J (2007) Translational control and target recognition by *Escherichia coli* small RNAs in vivo. Nucleic Acids Res 35:1018–1037

Ureta AR, Endres RG, Wingreen NS, Silhavy TJ (2007) Kinetic analysis of the assembly of the outer membrane protein LamB in *Escherichia coli* mutants each lacking a secretion or targeting factor in a different cellular compartment. J Bacteriol 189:446–454

Vaccaro L, Scott KA, Sansom MS (2008) Gating at both ends and breathing in the middle: conformational dynamics of TolC. Biophys J 95:5681–5691

Valentin-Hansen P, Eriksen M, Udesen C (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. Mol Microbiol 51:1525–1533

van den Berg B (2005) The FadL family: unusual transporters for unusual substrates. Curr Opin Struct Biol 15:401–407

Van den Berg B, Black PN, WMJr C, Rapoport TA (2004) Crystal structure of the long-chain fatty acid transporter FadL. Science 304:1506–1509

Van Gelder P, Dutzler R, Dumas F, Koebnik R, Schirmer T (2001) Sucrose transport through maltoporin mutants of *Escherichia coli*. Protein Eng 14:943–948

Vandeputte-Rutten L, Kramer RA, Kroon J, Dekker N, Egmond MR, Gros P (2001) Crystal structure of the outer membrane protease OmpT from *Escherichia coli* suggests a novel catalytic site. EMBO J 20:5033–5039

Vandeputte-Rutten L, Bos MP, Tommassen J, Gros P (2003) Crystal structure of *Neisserial surface* protein A (NspA), a conserved outer membrane protein with vaccine potential. J Biol Chem 278:24825–24830

Vergunst AC, van Lier MC, den Dulk-Ras A, Stüve TA, Ouwehand A, Hooykaas PJ (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of Agrobacterium. Proc Natl Acad Sci USA 102:832–837

Vogel H, Jähnig F (1986) Models for the structure of outer-membrane proteins of Escherichia coli derived from raman spectroscopy and prediction methods. J Mol Biol 190:191–199
Vogt J, Schulz GE (1999) The structure of the outer membrane protein OmpX from *Escherichia coli* reveals possible mechanisms of virulence. Struct Fold Des 7:1301–1309
Voulhoux R, Ball G, Ize B, Vasil ML, Lazdunski A, Wu LF, Filloux A (2001) Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J 20:6735–6741
Voulhoux R, Bos MP, Geurtsen J, Mols M, Tommassen J (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. Science 299:262–265
Wandersman C, Delepelaire P (2004) Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol 58:611–647
Wang J, Betton JM, Michel V, Hofnung M, Charbit A (1997) Identification of a cryptic porin gene in the *Escherichia coli* genome: expression and insertion of a monomeric form of the protein into the outer membrane. Mol Microbiol 26:1141–1143
Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S (2001) Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev 15:1637–1651
Weiss MS, Abele U, Weckesser J, Welte W, Schiltz E, Schulz GE (1991) Molecular architecture and electrostatic properties of a bacterial porin. Science 254:1627–1630
Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem 75:39–68
Whitfield C, Roberts IS (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol 31:1307–1319
Wimley WC (2002) Toward genomic identification of beta-barrel membrane proteins: composition and architecture of known structures. Protein Sci 11:301–312
Wimley WC (2003) The versatile beta-barrel membrane protein. Curr Opin Struct Biol 13: 404–411
Wimley WC, White SH (1996) Experimentally determined hydrophobicity scale for proteins at membrane interfaces. Nat Struct Biol 3:842–848
Wu T, Malinverni J, Ruiz N, Kim S, Silhavy TJ, Kahne D (2005) Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. Cell 121:235–245
Wu T, McCandlish AC, Gronenberg LS, Chng SS, Silhavy TJ, Kahne D (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. Proc Natl Acad Sci USA 103:11754–11759
Wunder T, Martin R, Löffelhardt W, Schleiff E, Steiner JM (2007) The invariant phenylalanine of precursor proteins discloses the importance of Omp85 for protein translocation into cyanelles. BMC Evol Biol 7:236
Wyckoff TJ, Lin S, Cotter RJ, Dotson GD, Raetz CR (1998) Hydrocarbon rulers in UDP-N-acetylglucosamine acyltransferases. J Biol Chem 273:32369–32372
Xu Y, Sim SH, Nam KH, Jin XL, Kim HM, Hwang KY, Lee K, Ha NC (2009) Crystal structure of the periplasmic region of MacB, a noncanonic ABC transporter. Biochemistry 48:5218–5225
Yau WM, Wimley WC, Gawrisch K, White SH (1998) The preference of tryptophan for membrane interfaces. Biochemistry 37:14713–14718
Ye J, van den Berg B (2004) Crystal structure of the bacterial nucleoside transporter Tsx. EMBO J 23:3187–3195
Yoneyama H, Nakae T (1996) Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. Microbiology 142:2137–2144
Yoshimura F, Zalman LS, Nikaido H (1983) Purification and properties of *Pseudomonas aeruginosa* porin. J Biol Chem 258:2308–2314
Yum S, Xu Y, Piao S, Sim SH, Kim HM, Jo WS, Kim KJ, Kweon HS, Jeong MH, Jeon H (2009) Crystal structure of the periplasmic component of a tripartite macrolide-specific efflux pump. J Mol Biol 387:1286–1297
Zakharian E, Reusch RN (2005) Kinetics of folding of *Escherichia coli* OmpA from narrow to large pore conformation in a planar bilayer. Biochemistry 44:6701–6707

Zheng J, Leung KY (2007) Dissection of a type VI secretion system in *Edwardsiella tarda*. Mol Microbiol 66:1192–1206

Zhou Z, White K, Polissi A, Georgopoulos C, Raetz CRH (1998) Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. J Biol Chem 273:12466–12475

Zou H, Zheng M, Luo X, Zhu W, Chen K, Shen J, Jiang H (2008) Dynamic mechanism of fatty acid transport across cellular membranes through FadL: molecular dynamics simulations. J Phys Chem B 112:13070–13078

Part II
Cell Wall Polymers and Structures of Archaea

Chapter 7
Cell Envelopes of Methanogens

Harald Claus and Helmut König

7.1 Introduction

The domain Archaea is divided into four kingdoms, Euryarchaeota, Crenarchaeota, Korarchaeota and the recently described Nanoarchaeota (Woese 2007). Methane-producing anaerobes (methanogens), which are the main group of the Euryarchaeota, have been shown to be phylogenetically and physiologically distinct from all other cell types, and are the founding members of the Archaea (Woese 1987; Woese et al. 1990). Methane formation is the last step in anoxic biodegradation of organic compounds where electron acceptors other than CO_2 are limiting. The typical growth substrates of methanogens are H_2 and CO_2, acetate, methanol or methylamines. Methane is a greenhouse gas and important in the global carbon cycle. Approximately 80% to 85% of the global annual production of methane is biogenic. Methanogens are involved in the production of methane in biogas plants, an important source of renewable energy. Natural habitats for methanogens include anoxic freshwater swamps, ocean and lake sediments, hydrothermal vents, animal digestive tracts (e.g. cattle, termites, and humans), and within the cytoplasm of anaerobic protozoa. They are morphologically and phylogenetically very diverse and are divided into five orders: Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales (Ferry and Kastead 2007; Kletzin 2007). The phylogenetic diversity of methanogens is also represented in various cell envelope types.

H. Claus (✉)
Institute of Microbiology and Wine Research, Johannes Gutenberg-University, 55099 Mainz, Germany
e-mail: hclaus@uni-mainz.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_7,

7.2 Pseudomurein

The orders Methanobacteriales and Methanopyrales contain different genera with anaerobic rod- or lancet-shaped bacteria or cocci. They live under conditions which range from mesophilic to hyperthermophilic temperatures. The cells of this orders exhibit cell wall sacculi with a thickness of about 15–20 nm, a density of 1.39–1.46 g/cm^3 and a unit cell dimension of 4.5 × 10 × 21–22.5 Å. Conformational energy calculations suggested a similar secondary structure as the eubacterial murein (Leps et al. 1984a, 1984b). Due to its chemical and three-dimensional structure this cell wall polymer has to be classified as a peptidoglycan, which means that glycan and peptides each constitute about 50% of this polymer. The archaeal peptidoglycan was named pseudomurein, and possesses structural similarities, but also differs significantly from the eubacterial murein (Kandler and König 1978, 1985, 1993). The glycan consists of alternating β(1→3)-linked *N*-acetyl-D-glucosamine and *N*-acetyl-L-talosaminuronic acid residues (Fig. 7.1). The glycan strands are cross-linked by peptide subunits, which are often composed of a set of three L-amino acids: glutamic acid, alanine, and lysine. The main differences between pseudomurein and murein are the occurrence of talosaminuronic acid instead of muramic acid, the presence of a β(1→3) linkage instead of a β(1→4) linkage of the glycan components (Leps et al. 1984a, 1984b), the partial replacement of glucosamine by galactosamine, the lack of D-amino acids, and the accumulation of ε- and γ-peptide bonds (Kandler and König 1978; König and Kandler 1979; König et al. 1983). Chemical modifications are found in the glycan as well as in the peptide moiety.

The pathways of the biosynthesis of archaeal cell wall polymers exhibit similarities, but also striking differences to the well-known biosynthetic pathways of structurally related bacterial and eukaryotic glycoconjugates. A pathway for the

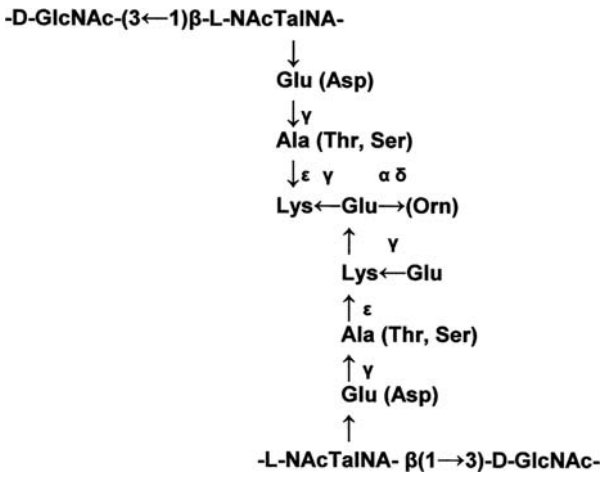

Fig. 7.1 Proposed chemical structure of the pseudomurein (from Kandler and König 1993)

biosynthesis of the pseudomurein has been proposed (König et al. 1994). The biosynthesis of the glycan intermediates starts with UDP-*N*-acetylglucosamine and UDP-*N*-acetyltalosaminuronic acid, which form the disaccharide UDP-GlcNAc (3.1)β-NAcTalNA (Fig. 7.2). The biosynthesis of UDP-*N*-acetyltalosaminuronic acid is achieved by epimerization and oxidation of UDP-*N*-acetylgalactosamine, where UDP-*N*-acetylaltrosaminuronic acid forms an intermediate. In parallel, a

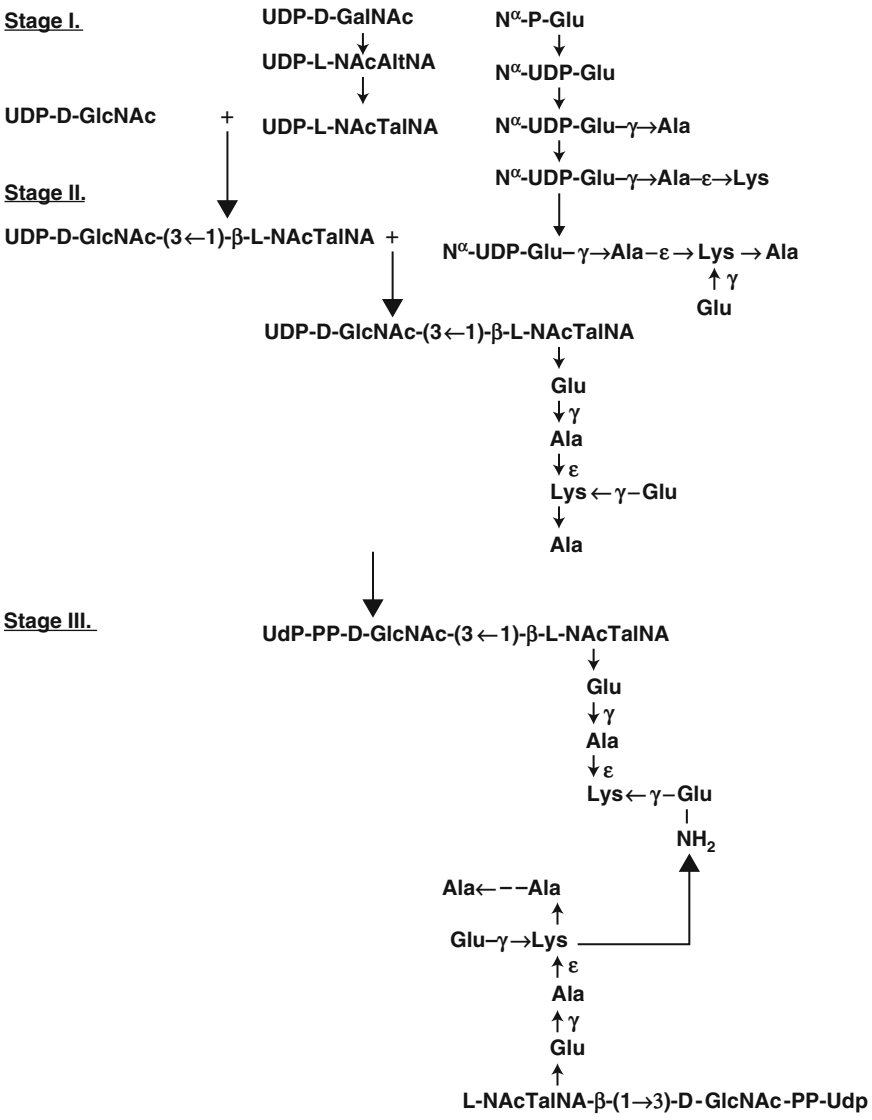

Fig. 7.2 Proposed biosynthetic pathway of the pseudomurein (from König et al. 1994) Udp = undecaprenyl

UDP-activated pentapeptide is formed. The biosynthesis of the pentapeptide starts with N^{α}-P-glutamic acid, wich is transformed to N^{α}-UDP-glutamic acid, to which the amino acid residues of alanine, lysine, and glutamate are subsequently linked. The formation of a nucleotide-activated oligosaccharide and a nucleotide-activated pentapeptide are novel features of oligosaccharide and peptide biosynthesis, showing that a common origin of the archaeal pseudomurein and the eubacterial murein is unlikely. The convergent evolution hypothesis of murein and pseudomurein is supported by the results of genome sequencing projects. In the last decade, extensive data have been published on the enzymes and the encoding genes that are involved in murein biosynthesis and assembly (Scheffers and Pinho 2005). No gene homologues or similar pathways have been identified in genomes of microorganisms that contain a pseudomurein cell wall, which implies that a completely different set of enzymes is involved in the pseudomurein turnover of Archaea.

Prokaryotic cell wall components such as murein or lipopolysaccharides exhibit a series of biological activities. Like murein, the pseudomurein causes antigenic recations in animals. Immunological characterization with monoclonal antibodies has revealed four distinctive determinants, namely the three glycan components GlcNAc, GalNAc and NacTalNA, and the C-terminal γ-glutamyl-alanine bond (Conway de Macario et al. 1983). Pseudomurein also causes somnogenic effects and pyrogenic effects. Intravenous injections with suspensions of pseudomurein in rabbits alter sleep and brain temperature (Johannsen et al. 1990). Acute inflammation was caused in a rat arthritis model (Stimpson et al. 1986).

As a consequence of its composition, which differs from the murein, the pseudomurein is resistant to cell-wall antibiotics such as β-lactams. Furthermore, it was shown that β-lactamases are absent from different species of methanogenic and halophilic Archaea, where glycoprotein S-layers, methanochondroidin, or pseudomurein serve as constituents of the rigid cell wall instead of murein (Martin and König 1996). Pseudomurein is also insensitive to lysozymes and common proteases.

Methanobacterium bryantii undergoes spontaneous lysis when the NH_4^+ or Ni^{2+} ions are exhausted in the culture medium (Jarrel et al. 1982). In the presence of D-sorbitol, *Methanobacterium thermoautotrophicum* forms protoplasts after addition of lysozyme to growing cells (Sauer et al. 1984). The target of the added lysozyme was not determined. An extracellular enzyme from a streptomycete was found to lyse *Methanobacterium formicicum* (Bush 1985).

Lysis of the pseudomurein sacculi also leads to a disruption of the cells. An autolytic enzyme which hydrolyzes the pseudomurein sacculi after depletion of the energy source could be induced in *Methanobacterium wolfei*. The lytic enzyme was found to be a peptidase hydrolysing the ε-bond between an alanine and a lysine residue of the peptide subunit (König et al. 1985; Kiener et al. 1987). At present, two enzymes, PeiW and PeiP, originating from *M. wolfeii* and *Methanothermobacter marbugiensis* prophages are known to hydrolyse pseudomurein and function as autolysins (Luo et al. 2002). Both pseudomurein endoisopeptidases (PEI) contain a cysteine protease catalytic domain that cuts the linker peptide of pseudomurein. PeiW and PeiP contain different N-terminal pseudomurein cell wall binding domains, with homologues found in the genomes of *Methanothermobacter*

thermoautotrophicus and *Methanosphaera stadtmanae* (Steenbakkers et al. 2006). Treatment of cells with PEI has been shown to improve 16S rRNA-targeting fluorescence in situ hybridization (FISH) analysis of methanogens (Nakamura et al. 2006).

7.3 Surface Layer

Regular ordered crystalline surface protein or glycoprotein layers (S-layers) are common structures of the prokaryotic cell envelope (Baumeister et al. 1989; Messner and Sleytr 1992; Sleytr et al. 1994; Sleytr 1997; Sleytr and Beveridge 1999; Sára and Sleytr 2000; Claus et al. 2005). The morphological building blocks are composed of six, four, three or two (glyco)protein subunits. Accordingly, the symmetry axis is p6, p4, p3 or p2. The molecular masses of the surface (glyco) proteins range from 40 to 200 kDa (Messner and Sleytr 1992; Claus et al. 2005).

Many Archaea possess S-layers as the sole cell wall component (see Eichler Chap II.10. and Rachel Chap II.12; König et al. 2004, 2007) which must therefore integrate the basic function of mechanical and osmotic cell stabilization (Engelhard 2007). In most Crenarchaeota the S-layer is anchored by an elongated, filamentous protrusion, spanning the quasi-periplasmatic space between the S-layer and the cytoplasmic membrane. The centre-to-centre spacings vary between 11 and 30 nm among the known genera and species. Within the methanogenic Archaea, S-layers have been decribed for the genera *Methanococcus* (Kandler and König 1978, 1985, 1993; Nußer and König 1987), *Methanocorpusculum* (Zellner et al. 1987), *Methanoculleus* (Bayley and Koval 1994), *Methanogenium* (Zellner et al. 1989), *Methanomicrobium* (Kandler and König 1978), *Methanolacinia* (König and Stetter 1982), *Methanolobus* (König and Stetter 1982), *Methanoplanus* (Wildgruber et al. 1982), *Methanosarcina* (Mayerhofer et al. 1998) and *Methanothermus* (Stetter et al. 1981; Bröckl et al. 1991). In addition, at least a corresponding gene has been found in *Methanobacterium* (Akca et al. 2002).

7.3.1 S-layer from Methanothermus fervidus

The hyperthermophilic methanogen *Methanothermus fervidus* has a double-layered cell envelope. The pseudomurein is covered by an S-layer in hexagonal arrangement (p6) with a 20 nm distance between the subunits (Stetter et al. 1981; Nußer et al. 1988; Bröckl et al. 1991; Kandler and König 1993; Kärcher et al. 1993; König et al. 2007). The S-layer (glyco)protein could be extracted with a solution of trichloroacetic acid and reverse-phase chromatography with aqueous formic acid as eluant. The mature protein consists of 593 amino acids. Compared to mesophilic S-layer (glyco)proteins a significant higher amount of, e.g., isoleucine, asparagine and cysteine and also 14% more β-sheet structure is present. The glycoprotein also

possesses a typical leader peptide and 20 sequon structures as potential *N*-glycosylation sites. One type of oligosaccharide is present consisting of D-3-*O*-MetMan, D-Man and D-GalNAc (Kärcher et al. 1993; Fig. 7.3). It is linked via *N*-acetylgalactosamine to asparagine residues of the peptide moiety.

7.3.2 S-layer from Methanococcales

All species belonging to the order Methanococcales possess hexagonal S-layers with an average centre-to-centre subunit distance of 11 nm as exclusive cell-wall components outside the cytoplasma membrane (Nußer and König 1987). As the Methanococcales include mesophilic, thermophilic and hyperthermophilic species, they represent an ideal model system for studying thermal adaptation of S-layer (glyco)proteins. The special features of these S-layers are described below in more detail (Table 7.1).

α-D-3-O-MetMan*p*-(1→6)-α-D-3-O-MetMan*p*-((1→2)- α-D-Man*p*)$_3$-(1→4)-D-GalNAc

Fig. 7.3 Proposed structure of the oligosaccharide of the S-layer (glyco)protein of *Methanothermus fervidus* (from Kärcher et al. 1993)

Table 7.1 Cell envelopes of selected methanogens

Genus	Cell Envelope Types	References
Methanobacterium	Pseudomurein/gene of S-layer protein	Kandler and König 1978; Akca et al. 2002
Methanobrevibacter	Pseudomurein	Kandler and König 1978
Methanocaldococcus	S-layer	Kandler and König 1978; Nußer and König 1987; Akça et al. 2002
Methanococcus	S-layer	Kandler and König 1978; Nußer and König 1987; Akça et al. 2002
Methanocorpusculum	S-layer	Zellner et al. 1987
Methanoculleus	S-layer	Bayley and Koval 1994
Methanogenium	S-layer	Kandler and König 1978
Methanomicrobium	S-layer	Kandler and König 1978
Methanolacinia	S-layer	Zellner et al. 1989
Methanolobus	S-layer	König and Stetter 1982
Methanoplanus	S-layer	Wildgruber et al. 1982
Methanopyrus	Pseudomurein	Kurr et al. 1991
Methanosaeta	Sheath	Patel and Sprott 1990
Methanothrix	Sheath	Kandler and König 1985
Methanosarcina	Methanochondroitin/ S-layer	Kreisl and Kandler 1986; Mayerhofer et al. 1998
Methanosphaera	Pseudomurein	König 1986
Methanospirillum	Sheath	Southam et al. 1993
Methanothermococcus	S-layer	Kandler and König 1985; Nußer and König 1987; Claus et al. 2005
Methanothermus	Pseudomurein/S-layer	Stetter et al. 1981; Bröckl et al. 1991
Methanotorris	S-layer	Claus et al. 2005

Primary structure and glycosylation. The first Methanococcales S-layer gene sequence was from the mesophile, *Methanococcus voltae* (Konisky et al. 1994). Additional putative S-layer genes have been identified in the complete genome sequences of the mesophile *Methanococcus maripaludis* (Zhang et al. 2004) and the hyperthermophile *Methanocaldococcus jannaschii* (Bult et al. 1996). Based on the conserved N-terminal region, primers were constructed for PCR amplification and sequencing of previously unidentified S-layer genes from the mesophile *Methanococcus vannielii,* the thermophile *Methanothermococcus thermolithotrophicus,* and the hyperthermophile *Methanotorris igneus* (Table 7.2).

The identity of the presumptive S-layer gene was confirmed by purification of the corresponding protein and sequence determination of their N-termini (Akca et al. 2002, Claus et al. 2005; König et al. 2007).

Molecular characteristics of S-layer (glyco)proteins from Methanococcales were deduced from their gene sequences (Table 7.3). They are all slightly acidic proteins with molecular masses ranging from 56 to 61 kDa. Thermal stabilization of S-layer proteins may be attributed to post-translational modifications (e.g. glycosylation, phosphorylation), covalent cross-linking, or salt-bridging (Engelhardt and

Table 7.2 S-layer genes from mesophilic and (hyper)thermophilic Methanococcales

Species	Growth optimum (°C)	Gene	Nucleotide Acc. No.	Protein Acc. No.
Methanotorris igneus	88	*slmi 1*	AJ564995[b]	Q6KEQ4[a]
Methanocaldococcus jannaschii	85	*slmj 1*	AJ311636[b]	Q58232[a]
Methanothermococcus thermolithotrophicus	65	*slmt 1*	AJ308554[b]	Q8X235[a]
Methanococcus vannielii	37	*slmv 1*	AJ308553[b]	Q8X234[a]
Methanococcus voltae	37	*sla*	M59200[b]	Q50833[a]
Methanococcus maripaludis	37	*slp*	NC_005791[c]	NP_987503[c]

[a]UniProt/SwissProt
[b]EMBL database
[c]GenBank/NCBI

Table 7.3 Molecular features of S-layer (glyco)proteins from selected mesophilic and (hyper) thermophilic Methanococcales

Species	Size (aa)	Mass (Daltons)	pI	*N*-glycosylation sites	Cys (mol %)	Ala (mol %)	Asp +Glu (mol %)	Arg +Lys (mol %)
Methanotorris igneus	519	55669	4.68	8	0.4	9.4	15.4	11.2
Methanocaldococcus jannaschii	558	60547	4.27	8	0.4	9.9	20.4	10.8
Methanothermococcus thermolitho-trophicus	559	59225	4.30	5	–	12.2	18.2	10.2
Methanococcus vannielii	566	59064	4.29	–	–	16.3	14.3	8.1
M. voltae	565	59707	4.15	2	–	14.0	18.6	8.7
M. maripaludis	575	58948	3.90	–	–	17.6	16.9	5.7

Peters 1998). Glycosylation of S-layer proteins is generally well characterized (Messner and Schäffer 2003), e.g. for the hyperthermophilic methanogenic species *Methanothemus fervidus* and *Methanothermus sociabilis* (Nußer et al. 1988; Bröckl et al. 1991; Kärcher et al. 1993).

A higher number of *N*-glycan sites are predicted in the primary amino acid sequences of S-layer (glyco)proteins from the hyperthermophilic *Methanocaldococcus jannaschi* compared with their mesophilic relatives (Akca et al. 2002, Claus et al. 2002). The same was found for the S-layer protein of the hyperthermophilic *Methanotorris igneus* (Claus et al. 2005) suggesting a role for glycosylation in the thermostabilization of these proteins. Although conventional staining methods for the detection of glycoproteins (PAS) were negative, positive signals were obtained with more sensitive immunoblots. In addition the S-layer proteins of Methanococcales revealed apparent higher molecular masses in SDS-PAGE than expected from their gene sequences. Additional indirect evidence for post-translational modification is indicated by the smaller size of the S-layer (glyco) protein from *Methanocaldococcus jannaschii* when heterologously expressed in *Escherichia coli* (König et al. 2007).

Recently, it has been demonstrated that flagellin proteins, and probably the S-layer protein of *Methanococcus voltae*, are glycosylated (Jarrel et al. 2007). The glycan structure of the flagellin elucidated by NMR analysis was shown to be a novel trisaccharide composed of β-Man*p*NAcA6Thr-(1-4)-β-Glc*p*NAc3NacA-(1-3)-β-Glc*p*Nac linked to Asn (Voisin et al. 2005). This low degree of glycosylation might not be detected by less sensitive glycoprotein-staining methods.

In addition to post-translational modifications, intrinsic features of the polypeptide chain contribute to thermostabilization of proteins. Sequences of 115 proteins (S-layer proteins were not included) from *Methanocaldococcus jannaschii* were compared with homologues from mesophilic Methanococcales (Haney et al. 1999). Characteristics of the proteins from thermophiles included higher residue volume and hydrophobicity, a higher percentage of charged amino acids (especially Glu, Lys and Arg) and a lower percentage of uncharged polar residues (Ser, Thr, Asn and Gln). In a similar study a large number of proteins from mesophilic and thermophilic to extreme thermophilic *Bacillus* and Methanococcales species were compared (McDonald et al. 1999). An increase of Ile, Glu, Lys and Arg and a decrease in Met, Asn, Gln, Ser and Thr was observed in the thermophilic methanococcal proteins. In several studies the complete genome sequences of mesophiles and thermophiles were analysed (Cambillau and Claverie 2000; Chakravarty and Varadarajan 2000). A large difference between the proportions of charged versus polar (noncharged) amino acids was found to be a common signature of all hyperthermophilic organisms. Ionic interactions may provide a mechanism for thermostabilization (Haney et al. 1999) and the proportional increase of oppositely charged residues in hyperthermophiles may provide a thermodynamic advantage due to the increased stability of coulombic interactions with temperature (Cambillau and Claverie 2000).

These structural features are also present in S-layer (glyco)proteins of Methanococcales. The S-layer (glyco)proteins of the thermophilic and hyperthermophilic

methanococci exhibited an increase in basic amino acids and a reduction of some amino acids with nonpolar side chains, e.g. alanine compared to their mesophilic counterparts. The overall hydrophobicity is higher for the S-layer (glyco)proteins from the mesophilic strains indicating that they do not play a major role in adaptation to high temperatures in Methanococcales.

An increase of solvent-accessible surfaces in proteins of hyperthermophiles has been described (Cambillau and Claverie 2000). Thus, the increase in charged amino acids, especially lysine, in the S-layer (glyco)proteins of *Methanothermococcus thermolithotrophicus, Methanocaldococcus jannaschii* and *Methanotorris igneus* could contribute to their increased thermal stability (Akca et al. 2002, Claus et al. 2002; König et al. 2007). An increase in charged residues is also present in the S-layer (glyco)proteins of *Methanosarcina mazei* (mesophilic) < *Methanothermobacter thermoautotrophicus* (thermophilic) < *Methanothermus fervidus* (hyperthermophilic). Another significant feature of the S-layer (glyco)proteins from the hyperthermophiles *Methanocaldoccus jannaschii* and *Methanotorris igneus* is the occurrence of cysteine, which has only been detected in a few S-layer (glyco) proteins (Sleytr et al. 1994; Sleytr 1997; Sleytr and Beveridge 1999). Intramolecular disulfide bridges may be another factor involved in the thermal stability of this surface (glyco)protein.

Secondary structures. A higher content of ordered structures (e.g. helical conformations), and fewer loops are predicted in S-layer (glyco)proteins from the mesophilic *Methanococcus voltae* and *Methanococcus vannielii* compared to *Methanothermococcus thermolithotrophicus* and *Methanocaldococcus jannaschii (*Table 7.4). In contrast to most common conceptions of factors determinating protein thermostability, the relatively low extent of ordered secondary structures in the S-layer (glyco)proteins of the hyperthermophilic members of the *Methanococcales* suggests that they are flexible molecules. This might explain their unusual behaviour in SDS-PAGE, where they often appear in several conformational states in response to cations, pH and temperature (König et al. 2007).

Signal sequences for secretion. Most S-layer (glyco)proteins contain N-terminal signal sequences which allow their secretion across the cytoplasmic membrane by the general secretory pathway (Boot and Pouwels 1996; Fernandez and Berenguer 2000, Pohlschröder and Dilks 2007). The putative 28-amino-acid-leader-peptides of proteins from the Methanococcales are highly conserved (Akca et al. 2002; Claus et al. 2002; König et al. 2007). They display typical characteristics of a signal

Table 7.4 Secondary structures of S-layer (glyco)proteins from selected methanogens[a]

Species	Helices	Sheets	Loops
Methanotorris igneus	14	41	46
Methanocaldococcus jannaschii	22	25	51
Methanothermococcus thermolithotrophicus	27	28	46
Methanococcus vannielii	45	19	36
Methanococcus voltae	36	27	36
Methanococcus maripaludis	17	37	46

[a]predicted by the PHD program, represented as percentage of total structural features

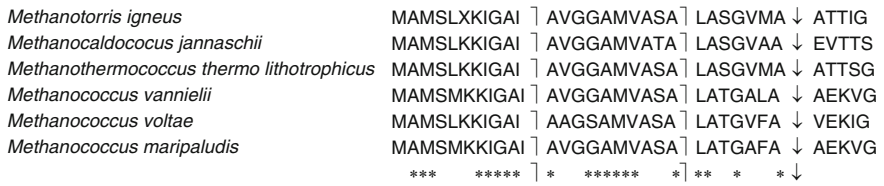

Fig. 7.4 Comparison of the leader peptides of the S-layer (glyco)proteins of Methanococcales (from König et al. 2007)

sequence with a non-hydrophobic (n)-region, a hydrophobic (h)-core, and a charged (c)-region with an alanine residue at the peptide cleavage site (Fig. 7.4; Bendtsen et al. 2004).

The 22- and 30-amino-acid-long signal peptides of *Methanothermus fervidus* (Bröckl et al. 1991) and *Methanosarcina mazei* (Yao et al. 1994), respectively, are not homologous with signal peptides of the Methanococcales. In addition to S-layer (glyco)proteins, archaeal flagellins possess leader peptides (Correia and Jarrell 2000; Albers and Driessen 2002; Bardy et al. 2003). However, these short positively charged leader peptides consist of only 4–14 (in many cases 12) amino acids, with an invariant glycine at the cleavage site, and have little similarity with the signal peptides of S-layer (glyco)proteins.

Several bacteria produce excess amounts of S-layer (glyco)proteins to ensure complete coverage of the cells during all phases of growth. The thermophilic eubacterium *Geobacillus stearothermophilus* appears to produce an S-layer (glyco)protein pool in the peptidoglycan layer (Breitwieser et al. 1992). Several eubacteria have been reported to shed S-layer material into the culture medium (Boot and Pouwels 1996). For the eubacterium, *Acinetobacter*, this occurs as a result of an overproduction of new S-layer protein (Thorne et al. 1976). Considerable quantities of S-layer proteins (between about 14% and 50% of the total S-layer protein) are released into the culture medium from *Methanococcus vannielii*, *Methanothermococcus thermolithotrophicus* and *Methanocaldococcus jannaschii* (Claus et al. 2001). For *Methanothermococcus thermolithotrophicus* this commences early and continues throughout the growth phase. A similar pattern was observed with the other two methanogens (data not shown).

Sequence comparisons. In general, minor structural differences were observed in the primary and secondary structures of the S-layer (glyco)proteins of mesophilic and hyperthermophilic Methanococcales. Most striking differences were found in respect of the occurrence of cysteine, the amount of basic amino acid residues and the degree of hydrophobicity.

Apart from the leader peptides, sequence alignments revealed a notable degree of homology between the S-layer (glyco)proteins of the mesophilic up to the thermophilic and hyperthermophilic Methanococcales especially at the N- and C-termini. The S-layer genes of the Methanococcales shared a significant homology with the presumptive S-layer genes of the hyperthermophilic heterotrophs

Table 7.5 Sequence homology of selected S-layer (glyco)proteins[a]

Species	*M. vol.*	*M. van.*	*M. the.*	*M. jan.*	*M. ign.*	*P. aby.*	*P. hor.*
Methanococcus voltae (*M. vol.*)		44	48	38	31	23	28
Methanococcus vannielii (*M. van.*)	47		49	44	31	24	31
Methanothermococcus thermolithotrophicus (*M. the.*)	50	49		53	40	26	29
Methanocaldococcus jannaschii (*M. jan.*)	40	44	53		35	25	33
Methanotorris igneus (*M. ign.*)	34	35	43	39		n.d.	n.d.
Pyrococcus abyssii (*P. aby.*)	24	25	26	26	n.d.		79
Pyrococcus horikoshi (*P. hor.*)	27	32	29	29	n.d.	79	

[a]Alignments of amino acid sequences (BLAST 2.0), represented as % amino acid identity; n.d. = not determined

Pyrococcus abyssi and *Pyrococcus horikoshii*. No significant similarity was found to any other archaeal S-layer (glyco)protein (Table 7.5).

Another group is formed by the S-layer (glyco)proteins of *Methanosarcina mazei* and the Gram-positive methanogens *Methanothermobacter thermoautotrophicus*, *Methanothermus fervidus* and *Methanothermus sociabilis* which are related to each other and to the sulfate-reducing hyperthermophilic *Archaeoglobus fulgidus* (Claus et al. 2002).

Regulatory sequences. Putative regulatory sequences involved in transcription and translation were examined in S-layer genes from members of the four genera, *Methanococcus*, *Methanothermococcus*, *Methanocaldococcus* and *Methanotorris* (Table 7.6, Claus et al. 2002; König et al. 2007). The TATA box and the "factor B recognition element" (BRE) of the S-layer genes matched the consensus sequence for methanogenic Archaea (Thomm 1996), however it seems they are located at different positions within the S-layer gene sequences of the *Methanococcales* (Claus et al. 2002; König et al. 2007). Several promotors have been described for the S-layer gene of *Methanococcus voltae* (Kansy et al. 1994).

The putative translation start and the Shine–Dalgarno sequences have been identified downstream from the transcription start site. The ribosome binding site of *Methanocaldococcus jannaschi*, usually localized 3–9 nucleotides upstream of the translation start site (Dalgaard and Garrett 1993), was found to be complementary to a region at the 3′ terminus of the corresponding 16S rRNA. Translation is terminated with a series of stop codons, which is a common feature of methanogenic Archaea (Dalgaard and Garrett 1993). They are followed by a poly A/poly T sequence, which probably leads to formation of a hair-pin structure and terminates transcription (Akca et al. 2002; Claus et al. 2002; König et al. 2007.

Crystallization experiments. The limited number of crystal structures from thermophiles and hyperthermophiles has hampered detailed structural comparisons with mesophiles. Up to now it has not been possible to obtain crystals for X-ray studies from complete S-layer (glyco)proteins. We selected the S-layer (glyco) protein of *Methanothermus fervidus* for our crystallization experiments. This species has been isolated from an Icelandic hot solfatara field. It grows optimally

Table 7.6 Putative regulatory sequences for S-layer genes

Region	Sequence	Position[a]
Promotor		
a. BRE-box	–CGAAA–[b]	
Methanocaldococcus jannaschii	–CGTAA–	−33 to −29
Methanotorris igneus	–CGTAA–	−35 to −31
Methanothermococcus thermolithotrophicus	–CGTAA–	−35 to −31
Methanococcus vannielii	–CGTAA–	−40 to −36
M. voltae I	–CGTAA–	−34 to −30
M. voltae II	–CGTAA–	−33 to −29
b. TATA-box		
Methanocaldococcus jannaschii	–TTTATATA–	−26 to −19
Methanotorris igneus	–TTTATATA–	−28 to −29
Methanothermococcus thermolithotrophicus	–TATATATA–	−28 to −21
Methanococcus vannielii	–TATAATAA–	−32 to −25
M. voltae I	–TATATATA–	−27 to −20
M. voltae II	–AATAAAA–	−26 to −19
Transcription start	–A/TTGC–[b]	
Methanocaldococcus jannaschii	–ATAC–	1
Methanotorris igneus	–ATCG–	1
Methanothermococcus thermolithotrophicus	–ATCC–	1
Methanococcus vannielii	–ATAC–	1
M. voltae I	–ATTT–	1
M. voltae II	–ATAC–	1
Shine–Dalgarno sequence		
Methanocaldococcus jannaschii	–AGGTGAT–	33–39
Methanotorris igneus	–AGGTGAT–	37–43
Methanothermococcus thermolithotrophicus	–AGGGTGA–	64–70
Methanococcus vannielii	–AGGTGAA–	60–66
M. voltae I	–AGGTGAT–	444–450
M. voltae II	–AGGTGAT–	204–210
Translation start		
Methanocaldococcus jannaschii	–ATG–	45
Methanotorris igneus	–ATG–	49
Methanothermococcus thermolithotrophicus	–ATG–	77
Methanococcus vannielii	–ATG–	72
M. voltae I	–ATG–	456
M. voltae II	–ATG–	216

[a]position relative to the transcription start
[b]consensus sequence

at 85°C and its S-layer may serve as a model to study the molecular strategies for survival at high temperatures. The gene (*slgA*) encoding the S-layer (glyco)protein has been sequenced (Bröckl et al. 1991) and the chemical structure of the heterosaccharide has been elucidated (Kärcher et al. 1993). The mature peptide is predicted to consist of 593 amino acids resulting in a molecular mass of 65 kDa (Bröckl et al. 1991). With mass spectroscopy (MALDI) a molecular mass of 83 kDa was determined for the the mature glycoprotein indicating that the glycan moiety accounts for 22%. The derived amino acid composition contains high amounts of Asn. The heterosaccharide is composed of 3-*O*-methylmannose, mannose and

N-acetylgalactosamine. For X-ray analysis it is essential to get crystals with high quality. Crystallization experiments were conducted under microgravity conditions (Evrard et al. 1999) using the Advanced Protein Crystallization Facilities developed by Dornier. The crystals were successfully grown during flight STS-05 of the Space Shuttle *Discovery*. Hanging drop reactors (HD-80) were used. One of the crystals with dimensions of 30 × 20 × 5 µm was selected for X-ray analysis. The diffraction experiments were performed at the EMBL DESY synchrotron facility in Hamburg. The crystal system is monoclinic and has the space group C2. In subsequent experiments we also obtained crystals from the S-layer (glyco)protein of the mesophilic bacterium *Bacillus sphaericus* during flight STS-101 (Claus et al. 2001; Debaerdemaeker et al. 2002). Peptide domains from the S-layer (glyco)protein of *Methanocarcina mazei* were also successfully crystallized (Jing et al. 2002).

7.4 Methanochondroitin

Cells of *Methanosarcina* often form large cuboid aggregates. The shape-maintaining component is a fibrillar nonsulfated polymer composed of an uronic acid and two *N*-acetylgalactosamine residues (Fig. 7.5). A trimer was identified as being the building block of the glycan (Kreisl and Kandler 1986). This polymer forms a compact or sometimes loose matrix. Because of its resemblance to eukaryotic tissue, especially to chondroitin sulfate, it was named "methanochondroitin". Modifications of the methanochondroitin are due to the additional occurrence of galacturonic acid in the two species *Methanosarcina mazei* and *Methanosarcina sp.* G1. Electron microscopical investigations indicated that in some species an S-layer may be located between the cytoplasmic membrane and the methanochondroitin matrix. *Methanosarcina mazei* occurs in three morphological forms: single cells, laminae and packets (Mayerhofer et al. 1992, 1998). Since the methanochondroitin matrix is degraded during the life cycle, leading to single disaggregated cells, the methanochondroitin matrix is responsible for the aggregate formation (Boone and Mah 1987).

The biosynthesis of methanochondroitin starts with the formation of UDP-*N*-acetylchondrosine and UDP-glucuronic acid, followed by the transfer of *N*-acetylchondrosine to UDP-*N*-acetylgalactosamine, thus yielding a UDP-activated trisaccharide, which possess the same structure as the repeating unit of methanochondroitin (Fig. 7.6, König et al. 1994). The trisaccharide is subsequently linked to the lipid carrier undecaprenol via pyrophosphate, transferred through the cytoplasmic membrane and polymerized.

In substrate-depleted cultures of *Methanosarcina barkeri*, the cell wall matrix is lost yielding spontaneous protoplasts (Archer and King 1984). During the life cycle of *Methanosarcina mazei*, the matrix material is degraded leading to a

Fig. 7.5 Proposed structure of the repeating units of methanochondroitin (from König et al. 1994)

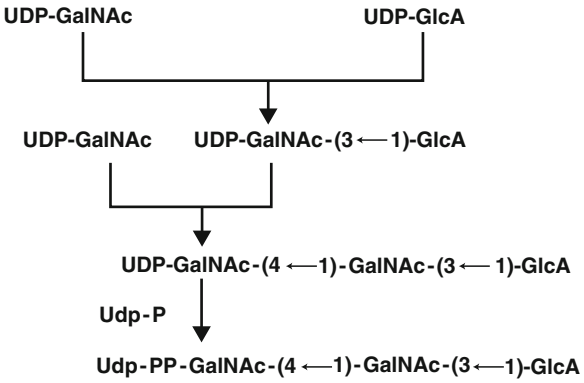

Fig. 7.6 Proposed biosynthetic pathway of methanochondroitin (from König et al. 1994) Udp = undecaprenyl

disaggregation of the cells. For complete disaggregation, elevated concentrations of substrate (100 mmol/L acetate or methanol) and divalent cations (Ca^{++}) were required (Boone and Mah 1987).

7.5 Proteinaceaous Sheaths

The filamentous chains of *Methanospirillum hungatei* and *Methanosaeta concilii* (formerly *Methanothrix soehngenii*) (Patel and Sprott 1990) are held together by a proteinaceous fibrillary sheath (Zeikus and Bowen 1975). Each single cell of *Methanospirillum* is surrounded by a separate electron-dense layer. Standard techniques for cell wall isolation provide pure preparations of sheath material (Kandler and König 1978). Freeze-etched specimens showed each fibril to be composed of two subfibrils. The isolated sheath material is resistant to detergent, chaotropic agents and common proteases.

Isolated sheaths (Kandler and König 1978; Sprott and McKellar 1980) are composed of amino acids and neutral sugars. Computer image processing of tilted-view electron micrographs of isolated, negatively stained sheaths revealed a two-dimensional S-layer-like paracrystalline structure. The structure consisted of subunits with P1 symmetry with cellular dimensions of $\alpha = 12.0$ nm, $\beta = 2.9$ nm, and $\gamma = 93.7°$ (Shaw et al 1985), or subunits arranged on a lattice with P2 symmetry and cells with $\alpha = 5.66$ nm, $\beta = 2.81$ nm, and $\gamma = 85.6°$. Treatment of isolated sheaths at 90°C with a combination of β-mercaptoethanol and sodium dodecyl sulfate under alkaline conditions results in the solubilization of "glue peptides" and the liberation of single hoops, the essential structural component of the cylindrical sheaths (Sprott et al. 1986). Imaging the inner and outer surfaces of isolated sheaths

with a bimorph scanning tunneling electron microscope confirmed that the sheaths form a paracrystalline structure, and that they consist of a series of stacked hoops of c. 2.5 nm in width (Beveridge et al. 1990). This study also showed that the sheath possesses minute pores and therefore is impervious to solutes with a hydrated radius of >0.3 nm.

In cross-sections of *M. concilii*, the envelope appears as a double track about 25 to 30 nm in width, with a very dark inner and a more electron-transparent outer layer. Only the inner layer participates in septum formation during cell division. Hence, it may be assumed that only the outer, electron-transparent layer represents a striated sheath that embraces many cells, whereas the inner layer represents a rigid cell wall sacculus surrounding individual cells (Zeikus and Bowen 1975; Huser et al. 1982). They also show striation not only at the cylindric part of the sacculi, but also at the septa. This indicates that both layers seen in cross-sections of whole cells belong to the same morphological entity, which therefore may not fit the definition of a sheath in the strict sense of the word. Chemical analysis of isolated envelopes revealed a complex amino acid pattern and the presence of neutral sugars (mainly glucose and mannose), resembling the composition of the sheath of *Methanospirillum hungatei* (Kandler and König 1978; Sprott and McKellar 1980). After hydrozinolysis of sheath preparations from *Methanosaeta concilii* strain FE, an asparagine-rich glycoprotein fraction was obtained. This is indicative of the presence of asparaginylrhamnose linkages on Asn-X-Ser glycosylation sites in the sheath glycoprotein.

7.6 Conclusions

The morphological and physiological diversity of methanogens is reflected in a remarkable diversity of their cell envelope types which are composed of different polymers essentially composed of glucans and/or proteins (Fig. 7.7). These cell envelopes are the result of an evolutionary adaptation to their specific environmental conditions which seem to be extreme, at least from a human point of view, for several members of the Methanococcales. Thus, they may serve as models to elucidate survival strategies of natural compounds and may give clues about molecular mechanisms of resistance against high temperature, low and high pH and high salt concentrations.

S-layers represent the most common cell wall structure of Archaea. They are the simplest biological membranes found in nature. Some important structural differences were observed in the primary and secondary structures of the S-layer (glyco) proteins of mesophilic and hyperthermophilic methanococci. Most striking differences were found with respect to the amount of cysteines, charged residues and degree of hydrophobicity. One important point to consider is that the common ancestor of Methanococcales was probably a thermophile (Keswani et al. 1996). This pattern supports the hypothesis that mesophily is a modern adaptation and

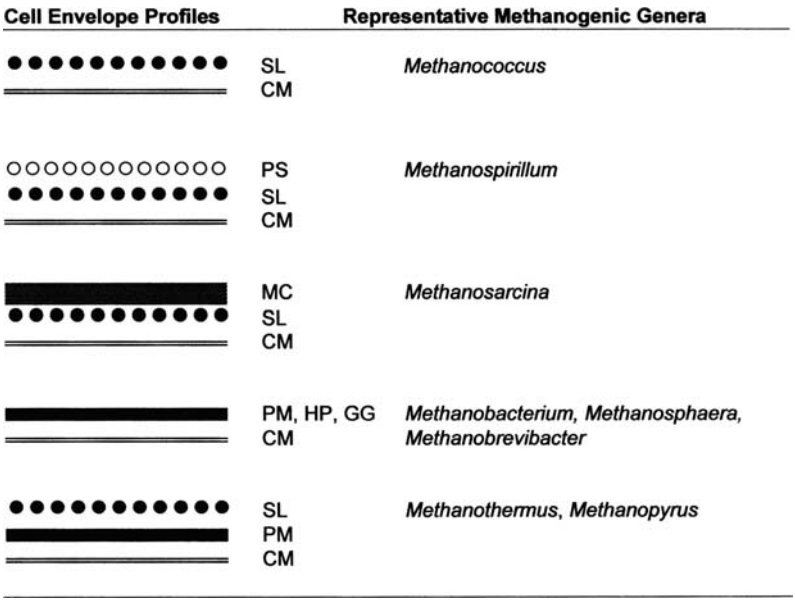

Fig. 7.7 Cell wall profiles of methanogens. *CM* cytoplasmic membrane, *GG* glutaminylglycan, *HP* heteropolysaccharide, *MC* methanochondroitin, *PM* pseudomurein, *PS* protein sheath, *SL* S layer

thermophilic structures are still conserved in mesophilic proteins. An exchange of an amino acid in mesophilic proteins may then be simply the result of a relaxation of selection against this amino acid which may be of importance in the hyperthermophilic counterparts (McDonald et al. 1999).

A wide spectrum of applications for S-layers have already emerged. Isolated S-layer subunits assemble into monomolecular crystalline arrays in suspension, on surfaces and interfaces. These lattices have functional groups on the surface in an identical position and orientation in a nanometer range.These features have led to different applications as ultrafiltration membranes, immobilization matrices for functional molecules, affinity microcarriers and biosensors, conjugate vaccines, carriers for Langmuir–Blodgett films and reconstituted biological membranes and pattering elements in molecular nanotechnology (Sleytr et al. 1994). In the past, the applied investigations have almost exclusively been performed with eubacterial S-layers. Since archaeal S-layer (glyco)proteins resist extreme conditions, a new spectrum of future developments with them should be found.

So far no genes of enzymes involved in the cell wall biosynthesis have been unambigously assigned. The knowledge of the complete genome may be helpful in the identification of special enzymes involved in the biosynthesis and degradation of cell wall polymers.

References

Akca E, Claus H, Schultz N, Karbach G, Schlott B, Debaerdemaeker T, Declercq JP, König H (2002) Genes and derived amino acid sequences of S-layer proteins from mesophilic, thermophilic and extremely thermophilic methanococci. Extremophiles 6:351–358

Albers SV, Driessen AJM (2002) Signal peptides of secreted proteins of the archaeon *Sulfolobus solfataricus*: a genomic survey. Arch Microbiol 177:209–216

Archer DB, King NR (1984) Isolation of gas vesicles from *Methanosarcina barkeri*. J Gen Microbiol 130:167–172

Bardy SL, Ng SYM, Jarrell KF (2003) Prokaryotic motility structures. Microbiology 149:295–304

Baumeister W, Wildhaber I, Phipps BM (1989) Principles of organizazion in eubacterial and archaebacterial surface proteins. Can J Microbiol 35:215–227

Bayley DP, Koval SF (1994) Membrane association and isolation of the S-layer protein of *Methanoculleus marisnigri*. Can J Microbiol 40:237–241

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: signalP 3.0. J Mol Biol 16:783–795

Beveridge TJ, Southam G, Jericho MH, Blackford BL (1990) High resolution topography of the S-layer sheath of the archaebacterium *Methanospirillum hungatei* provided by scanning tunneling microscopy. J Bacteriol 172:6589–6595

Boone DR, Mah RA (1987) Effects of calcium, magnesium, pH, and extent of growth on the morphology of *Methanosarcina barkeri* S-6. Appl Environ Microbiol 53:1699–1700

Boot HJ, Pouwels PH (1996) Expression, secretion and antigenic variation of bacterial S-layer proteins. Mol Microbiol 21:1117–1123

Breitwieser A, Gruber K, Sleytr UB (1992) Evidence of an S-layer protein pool in the peptidoglycan of *Bacillus stearothermophilus*. J Bacteriol 174:8008–8015

Bröckl G, Behr M, Fabry S, Hensel R, Kaudewitz H, Biendl E, König H (1991) Analysis and nucleotide sequence of the genes encoding the surface-layer glycoproteins of the hyperthermophilic methanogens *Methanothermus fervidus* and *Methanothermus sociabilis*. Eur J Biochem 199:147–152

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb JF, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JD, Geoghagen NS, Weidman JF, Fuhrmann JL, Nguyen DT, Utterback T, Kelley JM, Peterson JD, Sadow PW, Hanna MC, Cotton MD, Hurst MA, Roberts KM, Kaine B, Borodovsky M, Klenk HP, Fraser CM, Smith HO, Woese CR, Venter JC (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058–1073

Bush JW (1985) Enzymatic lysis of the pseudomurein containing methanogen *Methanobacterium formicicum*. J Bacteriol 163:27–36

Cambillau C, Claverie JM (2000) Structural and genomic correlates of hyperthermostabilty. J Biol Chem 275:32383–32386

Chakravarty S, Varadarajan R (2000) Elucidation of determinants of protein stability through genome sequence analysis. FEBS Lett 470:65–69

Claus H, Akça E, Schultz N, Karbach G, Schlott B, Debaerdemaeker T, Declercq JP, König H (2001) Surface (glyco-)proteins: primary structure and crystallization under microgravity conditions. In: Proceedings of first European Workshop on Exo-/Astro-Biology, Frascati, 21–23 May 2001, pp 313–320, ESA SP-496, August 2001; ISBN No 92-9092-806-9

Claus H, Akca E, Debaerdemaeker T, Evrard C, Declercq JP, König H (2002) Primary structure of selected archaeal mesophilic and extremely thermophilic outer surface layer proteins. System Appl Microbiol 25:3–12

Claus H, Akca E, Debaerdemaeker T, Evrard C, Declercq JP, Harris JB, Schlott B, König H (2005) Molecular organization of selected prokaryotic S-layer proteins. Can J Microbiol 51:731–743

Conway de Macario E, Macario AJL, Magarinos MC, König H, Kandler O (1983) Dissecting the antigenic mosaic of the archaebacterium *Methanobacterium thermoautotrophicum* by monoclonal antibodies of defined molecular specificity. Proc Natl Acad Sci USA 80:6346–6350

Correia J, Jarrell KF (2000) Posttranslational processing of *Methanococcus voltae* preflagellin by preflagellin peptidases of *M. voltae* and other methanogens. J Bacteriol 182:855–858

Dalgaard JZ, Garrett RA (1993) Archaeal hyperthermophile genes. In: Kates M, Kushner DJ, Matheson AT (eds) The Biochemistry of Archaea (Archaebacteria). Elsevier, Amsterdam, pp 535–563

Debaerdemaeker T, Evrard C, Declercq JP, Claus H, Akca E, König H (2002) The first crystallization of the outer surface (S-layer)glycoprotein of the mesophilic bacterium *Bacillus sphaericus* and the hyperthermophilic archaeon *Methanothermus fervidus*. In: Procedings of 2nd European Workshop on Exo/Astro-Biology, Graz, Austria, 16–19 Sept 2002, pp 441–442, ESA SP-518, November 2002; ISBN No 92-9092-828-X)

Engelhard H (2007) Mechanism of osmoprotection by archaeal S-layer: a theoretical study. J Struct Biol 160:190–199

Engelhardt H, Peters J (1998) Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. J Struct Biol 124:276–302

Evrard C, Declercq JP, Debaerdemaeker T, König H (1999) The first successful crystallization of a prokaryotic extremely thermophilic outer surface layer glycoprotein. Z Kristallogr 214: 427–429

Fernandez LA, Berenguer J (2000) Secretion and assembly of regular surface structures in gram-negative bacteria. FEMS Microbiol Rev 24:21–44

Ferry JG, Kastead KA (2007) Methanogenesis. In: Cavicchioli R (ed) Archaea – molecular and cell biology. ASM Press, Washington, DC, pp 288–314

Haney PJ, Badger JH, Buldak GL, Reich CI, Woese CR, Olsen GJ (1999) Thermal adaption analyzed by comparison of protein sequences from mesophilic and extremely thermophilic *Methanococcus* species. Proc Natl Acad Sci USA 36:3578–3583

Huser BA, Wuhrmann K, Zehnder AJB (1982) *Methanotrix soehngenii* gen. nov. sp. nov., a novel acetotrophic non-hydrogen-oxidizing methane bacterium. Arch Microbiol 132:1–9

Jarrel KF, Colvin JR, Sprott GD (1982) Spontaneous protoplast formation in *Methanobacterium bryantii*. J Bacteriol 149:346–353

Jarrel KF, Ng SYM, Chaban B (2007) Flagellation and chemotaxis. In: Cavicchioli R (ed) Archaea – molecular and cell biology. ASM Press, Washington, DC, pp 385–410

Jing H, Takagi J, Liu J, Lindgren S, Zang R, Joachimiak A, Wang J, Springer TA (2002) Archaeal surface layer proteins contain β propeller, PKD, and β helix domains and are related to metazoan cell surface proteins. Structure 10:1453–1464

Johannsen L, Toth LA, Rosenthal RS, Opp MR, Obál F Jr, Crady AB, Krueger JM (1990) Somnogenic, pyrogenic, and hematologic effects of bacterial peptidoglycan. Am J Physiol 258:182–186

Kandler O, König H (1978) Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch Microbiol 118:141–152

Kandler O, König H (1985) Cell envelopes of archaebacteria. In: Woese CR, Wolfe RS (eds) The Bacteria. A treatise on structure and function. *Archaebacteria*, VIIIth edn. Academic, New York, pp 413–457

Kandler O, König H (1993) Cell envelopes of *Archaea*: structure and chemistry. In: Kates M, Kushner DJ, Matheson T (eds) The Biochemistry of *Archaea* (Archaebacteria). Elsevier Science, Amsterdam, pp 223–259

Kansy WJ, Carinato ME, Monteggia LM, Konisky J (1994) In vivo transcripts of the S-layer encoding structural gene of the archaeon *Methanococcus voltae*. Gene 148:131–135

Kärcher U, Schröder H, Haslinger E, Allmeier G, Schreiner R, Wieland F, Haselbeck A, König H (1993) Primary structure of the heterosaccharide of the surface glycoprotein of *Methanothermus fervidus*. J Biol Chem 268:26821–26826

Keswani J, Orkand S, Premachandran U, Mandelco L, Franklin MJ, Whitman WB (1996) Phylogeny and taxonomy of mesophilic *Methanococcus spp*. and comparison of rRNA, DNA hybridization, and phenotypic methods. Int J Syst Bacteriol 46:727–735

Kiener A, König H, Winter J, Leisinger Th (1987) Purification and use of *Methanobacterium wolfei* pseudomurein endopeptidase for lysis of *Methanobacterium thermoautotrophicum*. J Bacteriol 169:1010–1016

Kletzin A (2007) General characteristics and important model organisms. In: Cavicchioli R (ed) Archaea – molecular and cell biology. ASM Press, Washington, DC, pp 14–92

König H (1986) Chemical composition of cell envelopes of methanogenic bacteria isolated from human and animal feces. Syst Appl Microbiol 8:159–162

König H, Kandler O (1979) The amino acid sequence of the peptide moiety of the pseudomurein from *Methanobacterium thermoautotrophicum*. Arch Microbiol 121:271–275

König H, Stetter KO (1982) Isolation and characterization of *Methanolobus tindarius, sp. nov.,* a coccoid methanogen growing only on methanol and methylamines. Zbl Bakt Hyg I Abt Orig C 3:478–490

König H, Semmler R, Lerp C, Winter J (1985) Evidence for the occurrence of autolytic enzymes in *Methanobacterium wolfei*. Arch Microbiol 141:177–180

König H, Hartmann E, Kärcher U (1994) Pathways and principles of the biosynthesis of methanobacterial cell wall polymers. Syst Appl Microbiol 16:510–517

König H, Claus H, Akca E (2004) Cell wall structures of mesophilic, thermophilic and hyperthermophilic *Archaea*. In: Seckbach J (ed) Origins. Kluwer Academic, Netherlands, pp 281–298

König H, Rachel R, Claus H (2007) Proteinaceous surface layers of Archaea: ultrastructure and biochemistry. In: Cavicchioli R (ed) Archaea – molecular and cell biology. ASM Press, Washington, DC, pp 315–340

König H, Kandler O, Jensen M, Rietschel ET (1983) The primary structure of the glycan moiety of the pseudomurein from *Methanobacterium thermoautotrophicum*. Hoppe Seylers Z Physiol Chem 364:627–636

Konisky J, Lynn D, Hoppert M, Mayer F, Haney P (1994) Identification of the *Methanococcus voltae* S-layer structure gene. J Bacteriol 176:1790–1792

Kreisl P, Kandler O (1986) Chemical structure of the cell wall polymer of *Methanosarcina*. Syst Appl Microbiol 7:293–299

Kurr M, Huber R, König H, Jannasch HW, Fricke H, Trincone A, Kristjannson JK, Stetter KO (1991) *Methanopyrus kandleri*, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. Arch Microbiol 156:239–247

Leps B, Barnickel G, Bradaczek H (1984a) Structural studies on the bacterial cell wall peptidoglycan pseudomurein. I. Conformational energy calculations on the glycan strands in C1 confromation and comparison with murein. J Theor Biol 107:85–114

Leps B, Labischinski H, Barnickel G, Bradaczek H, Giesbrecht P (1984b) A new proposal for the primary and secondary structure of the glycan moiety of pseudomurein. Conformational energy calculations on the glycan strands with talosaminuronic acid in 1C conformation and comparison with murein. Eur J Biochem 144:279–286

Luo YN, Pfister P, Leisinger T, Wasserfallen A (2002) Pseudomurein endoisopeptidases Pei W and Pei P, two moderately related members of a novel family of proteases produced in *Methanothermobacter* strains. FEMS Microbiol Lett 208:47–51

Martin HH, König H (1996) β-Lactamases are absent from Archaea (Archaebacteria). Microb Drug Res 2:269–272

Mayerhofer LE, Macario ALJ, Conway de Macario E (1992) Lamina, a novel multicellular form of *Methanocarcina mazei* S-6. J Bacteriol 174:309–314

Mayerhofer LE, Conway de Macario E, Yao R, Macario AJ (1998) Structure, organization, and expression of genes coding for envelope components in the archaeon *Methanosarcina mazei* S-6. Arch Microbiol 169:339–345

McDonald JH, Grasso AM, Rejto LK (1999) Patterns of temperature adaptation in proteins from *Methanococcus* and *Bacillus*. Mol Biol Evol 16:1785–1790

Messner P, Sleytr UB (1992) Crystalline bacterial cell-surface layers. Adv Microbiol Physiol 33:213–275

Messner P, Schäffer C (2003) Prokaryotic glycoproteins. In: Herz H, Falk H, Kirby GW (eds) Progress in the chemistry of organic natural products. Springer, Heidelberg, pp 51–124

Nakamura K, Terada T, Sekiguchi Y, Shinzato N, Meng XY, Enoki M, Kamagat Y (2006) Application of pseudomurein endoisopeptidase to fluorescence in situ hybridization of methanogens within the family *Methanobacteriaceae*. Appl Environ Microbiol 72:6907–6913

Nußer E, König H (1987) S-layer studies on three species of *Methanococcus* living at different temperatures. Can J Microbiol 33:256–261

Nußer E, Hartmann E, Allmeier H, König H, Paul G, Stetter KO (1988) A glycoprotein surface layer covers the pseudomurein sacculus of the extreme thermophile *Methanothermus fervidus*. In: Sleytr UB, Messner P, Pum D, Sàra M (eds) Crystalline bacterial cell surface layers. Springer, Berlin, pp 21–25

Patel GB, Sprott GD (1990) *Methanosaeta concilii* gen. nov., sp. nov. ("*Methanothrix concilii*") and *Methanosaeta thermoacetophila* nom. rev., comb. nov. Int J Bacteriol 40:79–82

Pohlschröder M, Dilks KC (2007) Protein translocation into and across archaeal cytoplasmic membranes. In: Cavicchioli R (ed) Archaea – molecular and cell biology. ASM Press, Washington, DC, pp 369–384

Sára M, Sleytr UB (2000) S-layer proteins. J Bacteriol 182:859–868

Sauer FD, Mahadevan S, Erfle JD (1984) Methane synthesis by membrane vesicles and a cytoplasmic cofactor isolated from *Methanobacterium thermoautotrophicum*. Biochem J 221:61–69

Scheffers DJ, Pinho MG (2005) Bacterial cell wall synthesis: new insights from localization studies. Microbiol Mol Biol Rev 69:585–607

Shaw PJ, Hills GJ, Henwood JA, Harris JE, Archer OB (1985) Three-dimensional architecture of the cell sheath and septa of *Methanospirillum hungatei*. J Bacteriol 161:750–757

Sleytr UB (1997) Basic and applied S-layer research: an overview. FEMS Microbiol Rev 20:5–12

Sleytr UB, Beveridge TJ (1999) Bacterial S-layers. Trends Microbiol 7:253–260

Sleytr UB, Sára M, Messner P, Pum D (1994) Two-dimensional protein crystals (S-layers): fundamentals and applications. J Cell Biochem 56:171–176

Southam G, Firtel M, Blackford BL, Jericho MH, Xu W, Mulhern PJ, Beveridge TJ (1993) Transmission electron microscopy, scanning tunneling microscopy, and atomic force microscopy of the cell envelope layers of the archaeobacterium *Methanospirillum hungatei* GP1. J Bacteriol 175:1946–1955

Sprott GD, McKellar RC (1980) Composition and properties of the cell wall of *Methanospirillum hungatei*. Can J Microbiol 26:115–120

Sprott GD, Beveridge TJ, Patel BG, Ferrante G (1986) Sheath disassembly in *Methanospirillum hungatei* strain GP1. Can J Microbiol 32:847–854

Steenbakkers PJM, Geerts WJ, Ayman-Oz N, Keitjens JT (2006) Identification of pseudomurein cell wall binding domains. Mol Microbiol 62:1618–1630

Stetter KO, Thomm M, Winter J, Wildgruber G, Huber H, Zillig W, Janecovic D, König H, Palm P, Wunderl S (1981) *Methanothermus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. Zbl Bakt Hyg I Abt Orig C 2:166–178

Stimpson SA, Brown RR, Anderle SK, Klapper DG, Clark RL, Cromartie WJ, Schwab JH (1986) Arthropathic properties of cell wall polymers from normal flora bacteria. Infect Immun 51:240–249

Thomm M (1996) Archael transcription factors and their role in transcription initiation. FEMS Microbiol Rev 18:159–171

Thorne KJI, Oliver RC, Glauert AM (1976) Synthesis and turnover of the regularly arranged surface protein of *Acinetobacter sp.* relative to the other components of the cell envelope. J Bacteriol 127:440–450

Voisin S, Houliston RS, Kelly J, Brisson JB, Watson D, Bardy SL, Jarrell KF, Logan SM (2005) Identification and characterization of the unique N-linked glycan common to the flagellins and S-layer glycoprotein of *Methanococcus voltae*. J Biol Chem 280:16586–16593

Wildgruber G, Thomm M, König H, Ober K, Ricchiuto T, Stetter KO (1982) *Methanoplanus limicola,* a plateshaped methanogen representing a novel family, the *Methanoplanaceae*. Arch Microbiol 132:31–36

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR (2007) The archaea: an invitation to evolution. In: Cavicchioli R (ed) Archaea – molecular and cell biology. ASM Press, Washington, DC, pp 1–13

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, *Eucarya*. Proc Natl Acad Sci USA 87:4576–4579

Yao R, Macario AJ, Conway de Macario E (1994) An archaeal S-layer gene homolog with repetitive units. Biochim Biophys Acta 1219:697–700

Zellner G, Alten C, Stackebrandt E, Conway de Macario E, Winter J (1987) Isolation and characterization of *Methanocorpusculum parvum, gen. nov. spec. nov*., a new tungsten requiring, coccoid methanogen. Arch Microbiol 147:13–20

Zellner G, Messner P, Kneifel H, Tindall BJ, Winter J, Stackebrandt E (1989) Methanolacinia gen. nov., incorporating *Methanomicrobium paynteri* as *Methanolacinia paynteri comb. nov*. J Gen Appl Microbiol 35:185–202

Zeikus JG, Bowen VG (1975) Fine structure of *Methanospirillum hungatei*. J Bacteriol 121:373–380

Zhang Y, Larimer FW, Olson MV, Leigh JA (2004) Complete genome sequence of the genetically tractable hydrogenotrophic methanogen *Methanococcus maripaludis*. J Bacteriol 186: 6956–6969

Chapter 8
The Cell Envelopes of Haloarchaea: Staying in Shape in a World of Salt

Jerry Eichler, Mehtap Abu-Qarn, Zvia Konrad, Hilla Magidovich, Noa Plavner, and Sophie Yurist-Doutsch

8.1 Introduction

The dawning of the genome era served to firmly establish the concept of living organisms as belonging to one of three distinct groups, namely the Eukarya, the Bacteria, or the Archaea (Woese and Fox 1977; Woese et al. 1990; Graham et al. 2000). Previously, when the sequencing of even single genes was considered a significant achievement, the Archaea had been largely distinguished through the analysis of selected molecular markers, including traits associated with their cell envelopes (Kandler and König 1978, 1985, 1993; König 2001). Of the various archaeal phenotypes, the cell surfaces of halophilic archaea were amongst the best studied, possibly due to interest on the part of researchers from a variety of scientific disciplines in the light-driven proton pump, bacteriorhodopsin, and the purple membrane of *Halobacterium halobium* (Oesterhelt and Stoeckenius 1971; Henderson 1975), as well as other haloarchaeal retinal proteins.

Indeed, despite lying on the fringes of mainstream research (probably due to their survival being dependent on the presence of molar salt concentrations), halophilic archaea have served as a rich source of novel biological information. This is particularly true of their membranes and envelopes. The unique nature of archaeal ether-based membrane lipids was first discerned in *Halobacterium cutirubrum* in 1963 (Kates et al. 1963), well before the recognition of Archaea as comprising a distinct life form. Analysis of haloarchaeal cell envelopes offered the first example of a non-eukaryal *N*-glycosylated glycoprotein in the form of the *Halobacterium salinarum* surface (S)-layer glycoprotein (Mescher and Strominger 1976a, b). Studies addressing the *Hbt. salinarum* S-layer glycoprotein (Zeitler et al. 1998), moreover, demonstrated the glycosylation of Asn residues other than those found in the classic Asn-X-Ser/Thr sequon motif, where X is any residue but Pro (Gavel and

J. Eichler (✉)
Department of Life Sciences, Ben Gurion University, P.O. Box 653, Beersheva 84105, Israel
e-mail: jeichler@bgu.ac.il

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_8,

von Heijne 1990). Furthermore, halophilic archaea have provided examples of heterosaccharide-based cell walls not seen elsewhere (Niemetz et al. 1997).

The results of earlier biochemical and structural studies, together with today's availability of complete genome sequences, have served to provide considerable insight into the biogenesis, molecular composition and roles served by the cell envelopes surrounding different halophilic archaea. In this chapter, selected aspects of haloarchaeal cell envelope biology will be considered.

8.2 Surface (S)-Layers

Surface (S)-layers correspond to two-dimensional crystalline arrays that, in several haloarchaea, comprise the sole layer surrounding the cell beyond the plasma membrane. Of the various cell envelope types reported for haloarchaea, S-layers have been studied in most detail.

8.2.1 Structure of the Haloarchaeal S-Layer

As long ago as 1956, electron microscopy had shown the surface of *Hbt. halobium* cells to be covered by a hexagon-patterned monolayer, comprising morphological units with a spacing of approximately 17 nm (Houwink 1956). Subsequent analysis of thin-sectioned haloarchaeal cells revealed the presence of a 17 nm thick cell wall outside the plasma membrane (Stoeckenius and Rowen 1967; Steensland and Larsen 1969; Kirk and Ginzburg 1972). Using X-ray diffraction, Blaurock et al. (1976) demonstrated the presence of an outer protein layer lying some 8 nm from the haloarchaeal plasma membrane, with morphological subunits assuming an inverted-parabola shape, thereby creating a periplasmic-like space. Lactoperoxidase-catalyzed iodination of *Hbt. salinarum* surface proteins, together with proteolytic treatment, revealed the S-layer to be essentially composed of the S-layer glycoprotein (Mescher and Strominger 1976a). Indeed, while the glycoprotein-based composition of the *Halobacterium* cell envelope had been previously suggested (Koncewicz 1972; Mescher et al. 1974), it was Mescher and Strominger (1976b) who purified and characterized the *Hbt. salinarum* S-layer glycoprotein, at the same time presenting the first example of a non-eukaryal *N*-glycosylated protein.

Once the *Hbt. salinarum* S-layer had been shown to be important for maintaining the proper shape of the cell (Mescher and Strominger 1976a), later efforts offered a more refined view of the structure of the haloarcheal S-layer. By considering the primary sequence of the *Hbt. salinarum* S-layer glycoprotein (Lechner and Sumper 1987) and the earlier X-ray diffraction data, and with electron microscopic images of negatively-stained cell envelopes from *Halobacterium* (*Haloferax*) *volcanii*, a three-dimensional reconstruction of the haloarchaeal

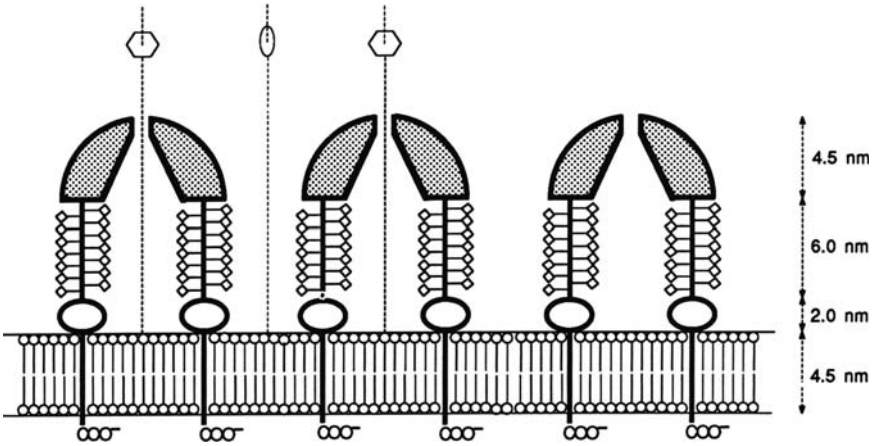

Fig. 8.1 Schematic model of the haloarchaeal S-layer, incorporating data collected from X-ray analysis of cell envelopes, S-layer glycoprotein amino acid sequence information, and three-dimensional electron microscopy reconstruction. The dome-shaped regions formed by the N-terminal regions of the S-layer glycoprotein are separated from the plasma membrane by *O*-glycosylated, threonine-rich domains of the S-layer glycoprotein that serve as "spacers." Reprinted from Kessel et al. (1988), with permission requested from the Nature Publishing Group

S-layer glycoprotein and cell envelope was proposed (Kessel et al. 1988). In this model (Fig. 8.1), based on reconstruction to a resolution of 2 nm, six S-layer glycoproteins are organized into a 4.5 nm thick dome-shaped pore around a narrow opening at its outermost face, widening as one approaches the membrane. The C-terminal transmembrane domain of each S-layer glycoprotein is thought to anchor the structure to the membrane, while an O-glycosylated domain of the S-layer glycoprotein lying upstream of the transmembrane domain is proposed to act as a spacer unit, propping up the domed structure. The forces responsible for maintaining the integrity of these assemblies are not known, although it has been shown that divalent cations are somehow involved (Mescher and Strominger 1976a; Kessel et al. 1988). Subsequent tomographic reconstruction of the *Hbt. salinarum* S-layer offered a more realistic portrayal of the haloarchaeal cell envelope, given the use of intact cells maintained in their growth medium and the high degree of sample preservation afforded by the rapid freezing associated with this experimental technique (Trachtenberg et al. 2000). Such studies showed the *Hbt. salinarum* cell envelope to assume the same basic architecture as the *Hfx. volcanii* S-layer, to a resolution of 2 nm, with both species relying on a hexagonal arrangement of the same basic morphological unit, presenting identical sixfold symmetry and the same domed shape, with identical 15 nm center–center spacing in both cases (Fig. 8.2). Despite these similarities in S-layer architecture, *Hbt. salinarum* and *Hfx. volcanii* cells nonetheless assume very different shapes, with the former appearing as rods and the latter as indented disks, implying that other factors apart from the S-layer affect cell shape.

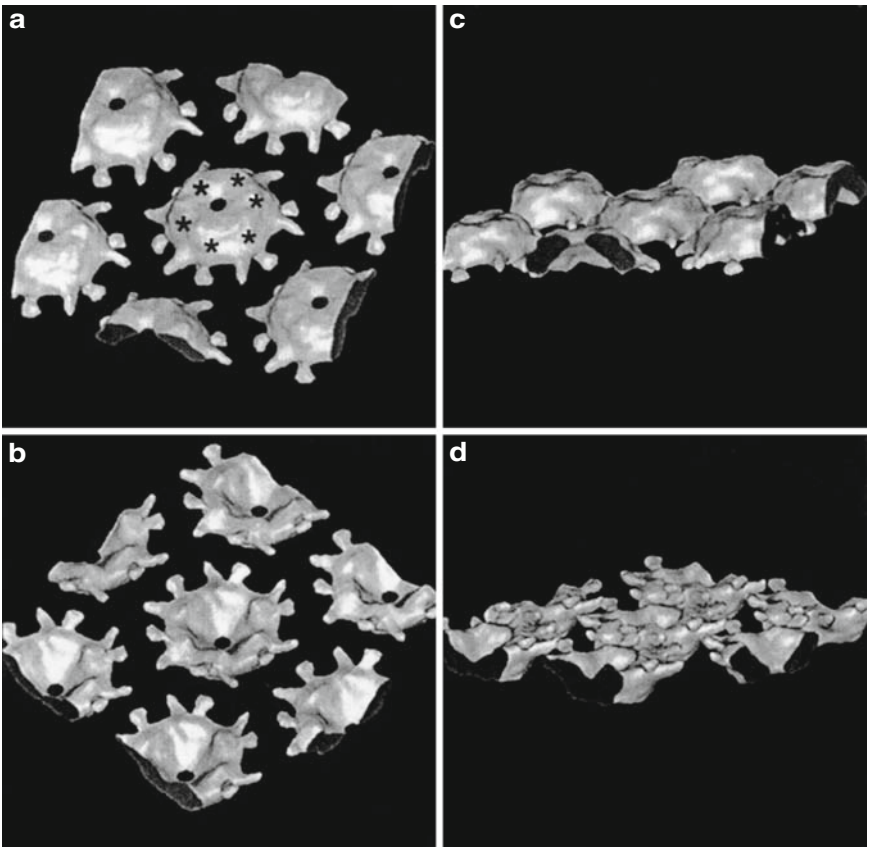

Fig. 8.2 A three-dimensional density map of negatively stained *Hfx. volcanii* envelopes, as calculated from the data of Kessel et al. (1988). (**a**) Top view of the S-layer. Each unit of the six-fold symmetrical structure is marked with an asterisk. (**b**) View of the S-layer domes, looking from the plasma membrane outwards. (**c**) Section through the S-layer domes, looking from the outside. (**d**) Section through the S-layer domes, looking from the plasma membrane. Reprinted from Trachtenberg et al. (2000), with permission from Elsevier

8.2.2 Haloarchaeal S-Layer Glycoproteins

Haloarchaeal S-layers are thought to be formed from a single glycoprotein species, the S-layer glycoprotein (Mescher and Strominger 1976b; Sumper et al. 1990). While genes encoding S-layer glycoproteins have been detected in those haloarchaeal species for which complete genome sequence information is available, only the S-layer glycoproteins from *Hbt. salinarum* and *Hfx. volcanii*, and to a lesser extent, that of *Haloarcula japonica* (Wakai et al. 1997), have been studied in detail (cf. Eichler 2003).

The *Hbt. salinarum* S-layer glycoprotein is an 818 amino acid residue-long polypeptide, synthesized as a preprotein that includes a 34 amino acid signal peptide (Lechner and Sumper 1987). The *Hfx. volcanii* S-layer glycoprotein is a 794 amino acid residue-long polypeptide, and is also synthesized as a preprotein that includes a 34 amino acid signal peptide (Sumper et al. 1990). While these proteins are predicted to have molecular masses of 86.5 and 81.7 kDa, respectively, both migrate at the ~190 kDa position in SDS-PAGE due to an abundance of negatively-charged residues, an adaptation of haloarchaeal proteins that allows proper folding in hypersaline surroundings (Lanyi 1974; Fukuchi et al. 2003). Each protein experiences both *N*- and *O*-glycosylation, although the two polypeptides differ in terms of their glycosylation profiles. The *Hbt. salinarum* S-layer glycoprotein contains 11 putative *N*-glycosylation sites, with only three sites have been experimentally verified as being modified (Paul et al. 1986; Lechner and Sumper 1987). An additional putative *N*-glycosylation site at Asn-17 was shown to be nonmodified (Lechner and Sumper 1987). The glycoprotein is, moreover, modified by two different *N*-linked glycan moieties. While Asn-2 is linked to a repeating sulfated pentasaccharide moiety through an *N*-acetylgalactosamine residue, other Asn residues in the protein are modified by a glucose-linked, sulfated polysaccharide unit (Fig. 8.3). By contrast, the *Hfx. volcanii* S-layer glycoprotein contains seven putative *N*-glycosylation sites, two of which were reported to be modified by a linear string of glucose subunits (Sumper et al. 1990; Mengele and Sumper 1992), although this has recently been brought into question (Abu-Qarn et al. 2007). The glycan profiles of other *Hfx. volcanii* S-layer glycoprotein *N*-glycosylation sites have been partially characterized, with evidence for the presence of glucose, galactose, mannose, and idose being offered (Sumper et al. 1990; Mengele and Sumper 1992). Thus, despite their similarities at the amino acid level (40.5% identity), the S-layer glycoproteins of the two species present very different glycosylation profiles. Accordingly, it had been proposed that the enhanced negative character and subsequent increase in surface charge density resulting from the sulfated nature of the *Hbt. salinarum* S-layer glycoprotein *N*-linked glycans, relative to their nonsulfated counterparts in *Hfx. volcanii*, represents an adaptation to the enhanced saline surroundings encountered by the former species (Mengele and Sumper 1992).

In addition to *N*-glycosylation, both the *Hbt. salinarum* and *Hfx. volcanii* S-layer glycoproteins also experience *O*-glycosylation. In each case, glucose–galactose pairs have been reported as decorating clustered threonine residues lying upstream of the proposed single membrane-spanning domain located just before the C-terminus of each protein (Lechner and Sumper 1987; Sumper et al. 1990).

8.2.2.1 Glycosylation of Haloarchaeal S-Layer Glycoproteins

With the identification of the *Hbt. salinarum* S-layer glycoprotein as a true *N*-glycosylated polypeptide, studies aimed at defining the pathway of haloarchaeal *N*-glycosylation were undertaken. These revealed significant similarities between

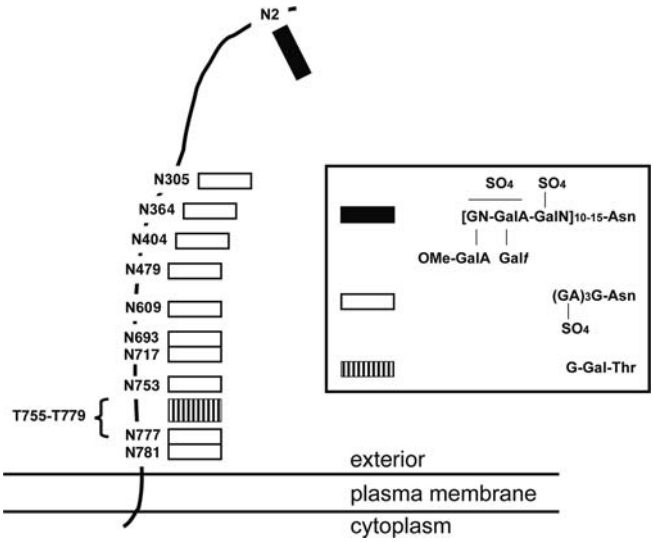

Fig. 8.3 Schematic depiction of the glycosylation experienced by the *Hbt. salinarum* S-layer glycoprotein. Portrayed is the topology of the S-layer glycoprotein, the positions of the 11 Asn residues believed to undergo *N*-glycosylation, as well as the heavily *O*-glycosylated Thr-rich region between Thr-755 and Thr-779, as proposed (Lechner and Sumper 1987; Lechner and Wieland 1989; Mengele and Sumper 1992). The composition of the oligosaccharide moieties bound to the protein are presented in the inset. Abbreviations used: G, glucose; GA, glucaronic acid; Gal, galactose; GalA, galacturonic acid; Galf, galactofuranose; GalN, *N*-acetylgalactosamine; GN, *N*-acetylglucosamine; Ome, O-methyl; SO_4, sulfate. Approximately one third of the glucaronic acid units shown may be replaced by iduronic acid units. Reprinted from Eichler and Adams (2005), with permission from the American Society for Microbiology

the archaeal process and the well-characterized eukaryal *N*-glycosylation pathway (Sumper 1987; Lechner and Wieland 1989).

In higher Eukarya, a dolichol pyrophosphate lipid carrier is sequentially charged with seven soluble nucleotide-activated sugar subunits on the cytoplasmic face of the endoplasmic reticulum (ER) membrane. Following a reorientation of the lipid-linked heptasaccharide to face the ER lumen, seven more sugar subunits are added, each delivered from their own individual dolichol pyrophosphate carrier, previously charged in the cytoplasm and "flipped" to face the ER lumen. Once the 14-member oligosaccharide has been assembled, the entire polymer is transferred, en bloc, to select Asn residues of nascent polypeptide targets translocating across the ER membrane via the Stt3 subunit of the oligosaccharide transferase complex (for review, see Burda and Aebi 1999; Helenius and Aebi 2004).

Like those involved in the eukaryal *N*-glycosylation process, mono- and polysaccharide-charged dolichol carriers have also been detected in *Hbt. salinarum* (Mescher et al. 1976). The involvement of such dolichol pyrophosphate sugar carriers in haloarchaeal S-layer glycoprotein *N*-glycosylation was implied from the inhibition of this post-translational modification by bacitracin, an antibiotic

that selectively interferes with the regeneration of pyrophosphate-containing dolichols (Mescher and Strominger 1976a). The observation that membrane-impermeant bacitracin prevented glycosylation at the Asn-2 position of the *Hbt. salinarum* S-layer glycoprotein points, moreover, to a process involving steps that occur on the outer surface of the plasma membrane, the topological homolog of the lumen-facing leaflet of the ER membrane. Furthermore, the inability of bacitracin to affect *Hfx. volcanii* S-layer glycoprotein *N*-glycosylation fits with the presence of mono- and polysaccharide-charged phosphodolichol rather than pyrophosphodolichol carriers in this species, the former not being susceptible to the effects of bacitracin (Kuntz et al. 1997; Eichler 2001). Further evidence pointing to the outer membrane surface as the site of S-layer glycoprotein *N*-glycosylation came with the modification of membrane-impermeant, synthetic glycosyl acceptor peptides (Lechner et al. 1985a). Later studies employing the glucosyltransferase inhibitors amphomycin, PP36 and PP55 also concluded that glycosylation of *Hfx. volcanii* glycoproteins, including the S-layer glycoprotein, occurs on the outer cell surface (Zhu et al. 1995). By contrast, the sulfation experienced by a lipid-linked oligosaccharide in *Hbt. salinarum*, identical in composition to the sulfated oligosaccharides decorating the S-layer glycoprotein, is thought to take place in the cytoplasm (Lechner et al. 1985a). Similarly, the transient methylation experienced by the lipid-bound sulfated oligosaccharide is also predicted to occur in the cytoplasm. While the role of this transient methylation is not known, its prevention hindered *Hbt. salinarum* S-layer glycoprotein glycosylation (Lechner et al. 1985b). Moreover, such modification of a lipid-linked polysaccharide prior to its assumed transfer to the target protein seems to be unique to Archaea, as such processing does not occur in Eukarya (Varki 1998). Other aspects of the *N*-glycosylation process unique to Archaea include the ability of the *Hbt. salinarum* machinery to modify glycosylation motifs apart from the classic Asn-X-Ser/Thr sequon, where X is any residue but proline (Gavel and von Heijne 1990), demonstrated by the modification of the S-layer glycoprotein Asn-2 position despite the Ser residue found at position 4 being replaced by Val, Leu or Asn residues (Zeitler et al. 1998).

More recently, detailed efforts at deciphering the process of S-layer glycoprotein *N*-glycosylation at the level of individual pathway components have been initiated, using *Hfx. volcanii* as the model system. Abu-Qarn and Eichler (2006) scanned the *Hfx. volcanii* genome sequence for homologs of genes whose products are involved in *N*-glycosylation in Eukarya or in the recently described parallel process in Bacteria. Homologs of genes encoding participants in both systems were thus identified. In some cases, only single *Hfx. volcanii* homologs were detected, whereas in other instances, families of homologous sequences were detected. Reverse-transcriptase polymerase chain reactions confirmed the transcription of the vast majority of these sequences in cells grown to exponential phase in rich medium, indicative of the identified sequences corresponding to true genes. Differential transcription of these genes in response to other growth paradigms suggests that *N*-glycosylation may serve an adaptive role in *Hfx. volcanii* (see below). Employing a gene deletion approach recently developed for use with *Hfx. volcanii*

(Allers et al. 2004), together with a variety of biochemical detection techniques, direct demonstration of the involvement of several of the identified genes in *N*-glycosylation was provided. Finally, the finding that homologs of the various *Hfx. volcanii* sequences shown to be or suspected of being involved in *N*-glycosylation are also present in other haloarchaea, namely *Halobacterium* sp. NRC-1 (Ng et al. 2000), *Haloarcula marismortui* (Baliga et al. 2004), and *Natronomonas pharaonis* (Falb et al. 2005), implies that those gene products implicated in *N*-glycosylation in *Hfx. volcanii* may also serve similar roles in other halophilic archaea.

Of the different *Hfx. volcanii* genes implicated in *N*-glycosylation, experimental evidence for specific roles of several of the encoded proteins is now beginning to emerge. Relying on glycostaining, lectin-binding or radiolabeled sugar incorporation, it could be shown that deletion of *aglD* affected glycosylation of the S-layer glycoprotein (Abu-Qarn and Eichler 2006; Abu-Qarn et al. 2007). Mass spectrometry, however, offers the strongest support for the involvement of AglD in the *N*-glycosylation of the *Hfx. volcanii* S-layer glycoprotein. Mass spectrometry analysis of protease-generated S-layer glycoprotein peptides revealed the presence of a novel pentasaccharide, comprising two hexoses, two hexuronic acids and an additional 190 Da saccharide, decorating at least two of the seven putative *N*-glycosylation sites of the S-layer glycoprotein and not identified in earlier characterization of the protein (Sumper et al. 1990; Mengele and Sumper 1992). When, however, the glycan moiety decorating the same S-layer glycoprotein-derived peptides from cells lacking AglD was considered, the final hexose subunit of the pentasaccharide was shown to be absent (Abu-Qarn et al. 2007). Thus, AglD is involved in adding the ultimate sugar unit to this oligosaccharide. Similarly, mass spectrometry analysis failed to observe *N*-glycosylation of the same S-layer glycoprotein-derived peptides from cells deleted of *aglB*, encoding the *Hfx. volcanii* Stt3 homolog and sole component of the oligosaccharide transferase in Archaea, the eukaryal version of which is responsible for transferring assembled, lipid-linked oligosaccharides to target protein asparagine residues (Abu-Qarn et al. 2007).

Examination of the effects of *aglB* and *aglD* deletion on S-layer architecture and integrity suggests that proper *N*-glycosylation is important for the assembly and stability of the *Hfx. volcanii* cell envelope (Abu-Qarn et al. 2007). In cells lacking AglB and hence unable to perform *N*-glycosylation, the S-layer glycoprotein is released into the growth medium both more rapidly and to a greater extent than what is observed in the background strain. On the other hand, cells lacking AglD present a less-ordered S-layer than do either cells fully able to *N*-glycosylate proteins, i.e., the background strain, or cells incapable of performing this post-translational modification, i.e., cells lacking AglB, as revealed by electron microscopy and tomography (Fig. 8.4). Finally, cells in which either AglB or AglD are absent grow slower than do background cells as the salt concentration in the growth medium rises from 1.7 to 4.5 M NaCl. Taken together, these findings imply that proper *N*-glycosylation of the S-layer glycoprotein represents a molecular adaptation designed to encourage haloarchaeal survival in hypersaline conditions.

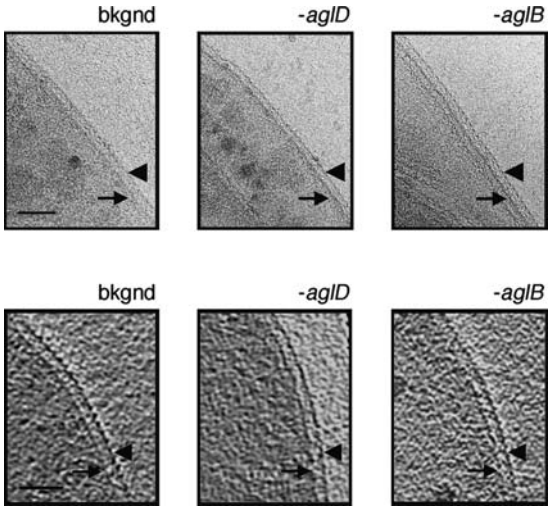

Fig. 8.4 Modified S-layer glycoprotein *N*-glycosylation affects *Hfx. volcanii* S-layer architecture. *Left panels*: *Hfx. volcanii* WR536 cells (bkgnd); middle panels: the same cells lacking AglD (*–aglD*); *right panels*: the same cells lacking AglB (*–aglB*). *Top panels*: Electron micrographs of cells envelopes. *Bottom panels*: X–Y slices, 6.5 nm in thickness, from tomograms of intact cells. In each panel, the *arrow* points to the plasma membrane while the *arrowhead* points to the outer edge of the surface layer. The *bar* in both left panels corresponds to 100 nm. Parts of this figure were previously published in Abu-Qarn et al. (2007) and are reproduced with permission

8.2.2.2 Lipid Modification of Haloarchaeal S-Layer Glycoproteins

In addition to glycosylation, haloarchaeal S-layer glycoproteins also experience post-translational lipid modification. This was clearly shown when incubation of *Hbt. salinarum* with [^{3}H]-mevalonate, as well as with other tritiated lipid tracers, led to selective incorporation of radiolabel into the S-layer glycoprotein (Kikuchi et al. 1999). Mass spectrometric analysis of the linked radioactive entity revealed it to be a novel diphytanylglycerol phosphate. Attempts to define the site of this modification localized the added lipid to a 28 kDa trypsin-generated fragment derived from the C-terminal region of the protein (residues 731–816). While the mode of lipid attachment to the S-layer glycoprotein remains unknown, it is thought to involve phosphodiester-based linkage to either a Ser or Thr residue. As such, it appears that in addition to the single predicted membrane-spanning domain located close to the C-terminus of the haloarchaeal S-layer glycoproteins, a lipid-based moiety also anchors the protein to the membrane. Furthermore, given the sequence similarity of the same C-terminal region in the *Hfx. volcanii* and *Haloarcula japonica* S-layer glycoproteins (Lechner and Sumper 1987; Sumper et al. 1990; Wakai et al. 1997), it was predicted that these two proteins also experience similar lipid modification (Kikuchi et al. 1999). Indeed, in the case in *Hfx. volcanii*, lipid modification was subsequently demonstrated (Eichler 2001; Konrad and Eichler 2002).

Further advances in understanding the process of lipid modification of haloarchaeal S-layer glycoproteins came from later studies addressing the biogenesis of the *Hfx. volcanii* S-layer glycoprotein. Relying on [^{35}S] pulse-chase metabolic radiolabeling and treatment with the ribosome-directed antibiotic, anisomycin, the S-layer glycoprotein was shown to undergo a maturation step on the outer surface of the plasma membrane, reflected as an increase in the hydrophobicity and apparent molecular weight of the protein (Eichler 2001). The subsequent finding (Konrad and Eichler 2002) that (1) growth in the presence of [^{3}H] mevalonic acid resulted in radiolabel being incorporated into the S-layer glycoprotein (Fig. 8.5), and (2) mevinolin, an inhibitor of 3-HMG-CoA reductase (the enzyme responsible for converting acetyl-CoA into mevalonic acid), is able to prevent the maturation of the S-layer glycoprotein as described above, both point to lipid attachment being a late step in the biogenesis of the protein, occuring following its translocation across and insertion into the plasma membrane. Moreover, lipid anchor attachment does not occur in the absence of Mg^{2+} (Eichler 2001), the presence of which is important for maintaining haloarchaeal S-layer integrity (Mescher and Strominger 1976a; Kessel et al. 1988). Finally, the finding that the *Hbt. salinarum* S-layer glycoprotein undergoes a similar lipid modification maturation step (Konrad and Eichler 2002) suggests that this process may be a step common to S-layer glycoprotein biogenesis in haloarchaea.

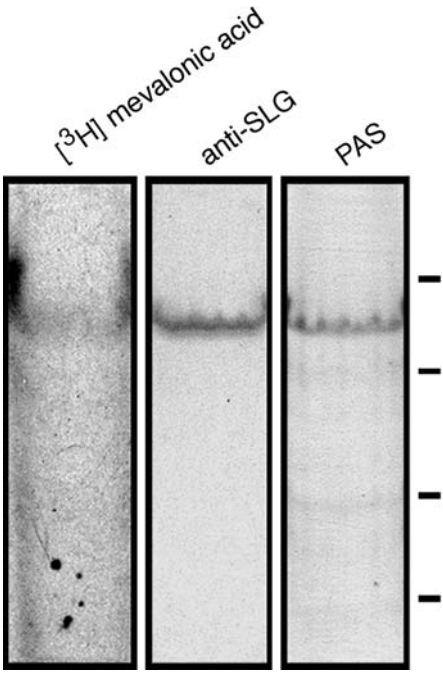

Fig. 8.5 The *Hfx. volcanii* S-layer glycoprotein is lipid-modified. Aliquots of *Hfx. volcanii* cells grown in the presence of [^{3}H] mevalonic acid were separated by SDS-PAGE and examined by fluorography ([^{3}H]mevalonic acid), immunoblotting with anti-S-layer glycoprotein antibodies (anti-SLG) or periodic acid–Schiff reagent glycostaining (PAS). The positions of 250, 150, 100 and 75 kDa molecular weight markers are shown on the *right*

8.2.2.3 A Periplasmic Space in Haloarchaea

As discussed above, the architecture of the haloarchaeal S-layer in at least two species, namely *Hbt. salinarum* and *Hfx. volcanii*, implies the existence of a periplasmic space between the plasma membrane and the inner surface of the S-layer surrounding the cell. The presence of such a structural compartment carries implications for a variety of cell processes in haloarchaea, including the post-translational modification and oligomerization of proteins. Although the presence of multimeric protein machines on the outer surface of the haloarchaeal plasma membrane has not been reported, it is conceivable that such complexes would exploit the existence of a physical barrier, i.e., the S-layer, to prevent the diffusion of any exported complex subunits, thereby encouraging controlled oligomer assembly. The existence of a periplasmic space would also permit the agents of post-translational modification to act on target proteins in an orderly fashion, possibly distinct from the process of protein translocation and folding. As such, the demand for multiple protein processing events to occur simultaneously or within the short window of time before a secreted substrate is released to the extracellular world would be circumvented. Moreover, the existence of a periplasmic space in haloarchaea could represent a compartment where elaborate nutrient capture, breakdown, and uptake machines could work in tandem. Indeed, such coupled strategies in the early phases of nutrient metabolism have been proposed to occur in other archaeal phenotypes (Albers and Driessen 2005).

8.3 Other Haloarchaeal Cell Envelopes

In addition to relatively simple S-layers, other cell envelope structures are used by halophilic archaea, although such structures have been less well studied to date. Nonetheless, what is presently known reveals the great variety possible in haloarchaeal cell envelope composition.

8.3.1 The Heteropolysaccharide Cell Wall of Halococcus morrhuae

Halococcus morrhuae are coccoid haloarchaea surrounded by an electron-dense layer some 50–60 nm wide, forming a rigid cell wall sacculus (cf. Kandler and König 1993). The realization in the early 1970s that these cells walls did not contain peptidoglycan, a major component of bacterial cell walls, was an initial pillar on which the establishment of the Archaea as a distinct phylogenetic kingdom rested (cf. Wolfe 2006). The chemical composition of purified cell walls from *H. morrhuae* stain CCM 859 has since been determined (Steber and Schleifer 1975; Schleifer et al. 1982) and shown to contain glucose, mannose, galactose,

glucuronic acid, galacturonic acid, glucosamine, and gulosaminuronic acid, an aminouronic acid. While its structure has yet to be fully solved (Kandler and König 1998), the heteropolysaccharide is thought to be arranged into three domains (Schleifer et al. 1982) and to include sulfated subunits and *N*-acetylated amino sugars (Fig. 8.6a). In addition, this rigid cell wall also contains significant amounts of glycine, proposed to bridge the amino groups of glucosamines and the carboxyl groups of uronic or gulosaminuronic acid residues in the glycan strands (Steber and Schleifer 1979). Presently, nothing is known of the biosynthesis of this sulfated heterosaccharide.

8.3.2 *The Cell Wall of Natronococcus* occultus *Contains a Novel Glycoconjugate*

Natronococcus occultus is a haloalkalophile, optimally surviving in hypersaline environments with pH values between 8.5 and 10 (Tindall et al. 1984) and surrounded by a cell wall unique amongst the Archaea, comprising L-glutamate, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-galacturonic acid, D-glucuronic acid, and D-glucose in a molar ratio of 5:7:1:8:0.5:0.3 (Niemetz et al. 1997). The novel glycoconjugate comprising this cell wall polymer is formed from a 60 residue-long poly(γ-L-glutamine) chain linked to two distinct heterosaccharides via *N*-amide linkages to the α-carboxylic group of the glutamine residues (Fig. 8.6b). Alternatively, two different poly(γ-L-glutamine) chains may be linked to either heterosaccharide moiety. Here too, nothing is known of the biosynthesis of the cell wall polymer.

8.3.3 *A Water-Enriched Capsule May Surround Haloquadratum walsbyi Cells*

Most thalassic (seawater-derived) NaCl-saturated environments are dominated by *Haloquadratum walsbyi*, the recently isolated and sequenced square, nonmotile pigmented halophilic archaeon (Oren et al. 1996; Anton et al. 1999; Benlloch et al. 2001; Bolhuis et al. 2004, 2006; Burns et al. 2004). With an ability to tolerate up to 2 M $MgCl_2$, *H. walsbyi* can survive conditions of very low water activity and, as such, is the last living organism remaining in crystallizer ponds before they turn into sterile, magnesium-saturated pools (Javor 1984; Oren 2002). The success of *H. walsbyi* in such low water environments may be related to a cell envelope that includes halomucin, a protein reminiscent of animal mucins in terms of amino acid sequence and domain organization (Bolhuis et al. 2006). Halomucin may thus correspond to a specific adaptation to desiccation stress, functioning much like the mucin-based cocoon of lungfish, able to avoid dehydration and stay alive even after prolonged periods outside water (Chew et al. 2004). Consistent with its

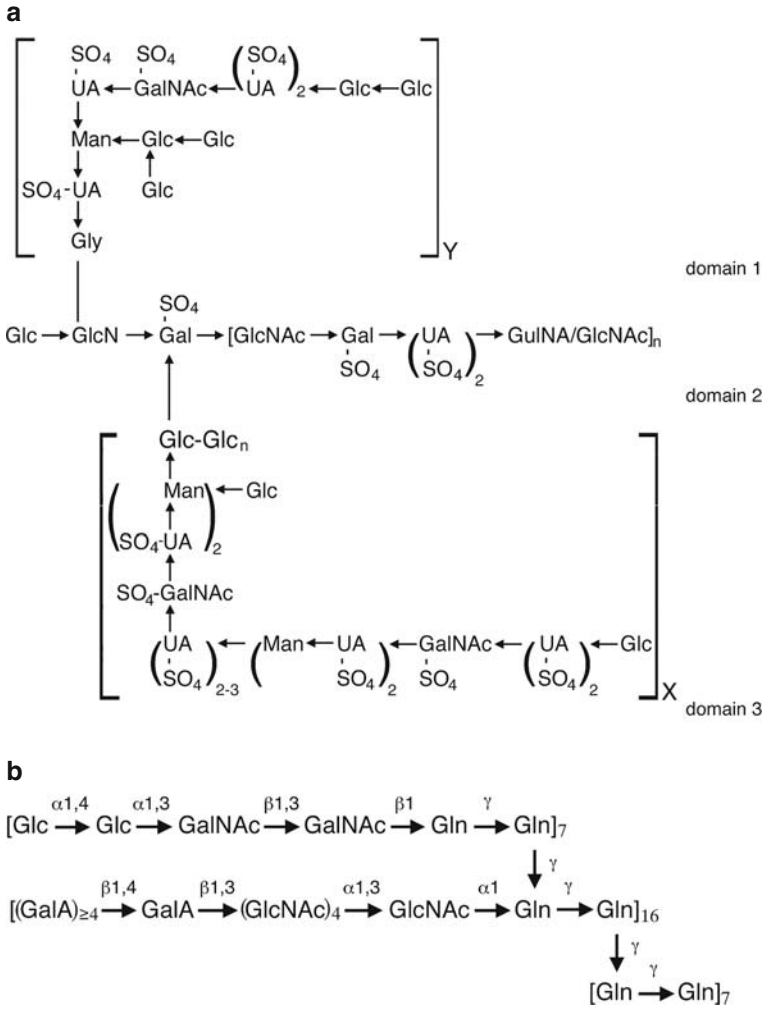

Fig. 8.6 The proposed structures of haloarchaeal cell wall polymers. (**a**) Proposed structure of the sulfated heterosaccharide comprising the *Halococcus morrhuae* cell wall, based on Schleifer et al. (1982) and Kandler and König (1993). Abbreviations used: Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gly, glycine; GulNAc, *N*-acetylgulosaminuronic acid; Man, mannose; UA, uronic acid. The three domains of the polymer are denoted. (**b**) Proposed structure of the polyglutamine-linked heterosaccharide comprising the *Natronococcus occultus* cell wall, based on Niemetz et al. (1997) and Kandler and König (1998), complete with linkages. Abbreviations used: GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GalA, galacturonic acid; Gln, glutamine

sequence and possible functional resemblances to mucins, halomucin contains numerous putative *N*-glycosylation and sulfation sites. It is thought that such modifications would further contribute to the overall negative charge of the protein, thereby encouraging the creation of an aqueous shield to cover *H. walsbyi* cells.

Indeed, a similar role has been attributed to the glycan moieties attached to the major glycosylated membrane-bound protein species coating the cell-wall-lacking thermoacidiphilic archaeon *Thermoplasma acidophilum* (Yang and Haug 1979). As noted above, the increased negative charges emanating from sulfation of *Hbt. salinarum* S-layer glycoprotein glycan moieties has also been cited as contributing to the ability of this species to survive in hypersaline surroundings (Mengele and Sumper 1992). Animal mucins also often contain sialic acids, compounds which form rigid structures capping the end of the polysaccharide side chains decorating mucins (Sheehan et al. 1991). Although widespread in Eukarya (Sheehan et al. 1991; Sampathkumar et al. 2006) and reported in some Bacteria (Vimr et al. 2004), sialic acids have yet to be detected in archaea. Thus, despite the fact that the *H. walsbyi* genome includes homologues of the two essential sialic acid biosynthesis genes, i.e., *neuA* and *neuB* (Bolhuis et al. 2006), it remains to be determined whether halomucin contains sialic acids. *H. walsbyi* also encodes two shorter homologues of halomucin and at least fourteen S-layer glycoprotein homologues that may also participate in the cell envelope of this species. Finally, *H. walsbyi* may also be surrounded by a poly-γ-glutamate capsule, given the presence of genes homologous to bacterial sequences involved in the biosynthesis of this polymer (Ashiuchi and Misono 2002).

8.4 Conclusions

Over some three decades, the haloarchaeal cell envelope has provided novel insight into a wide range of biological processes in archaea, and, indeed, across evolution. There is no reason to doubt that future studies relying on this amazing biological structure as a model system will continue to reveal new information. With the ever-increasing availability of genome- and proteome-wide analytic tools, such investigations will be able to follow changes in the composition, behavior and stability of the haloarchaeal cell envelope in response to a variety of environmental and physiological challenges, thereby providing a deeper understanding of this frontier between the haloarchaeal cell and its hypersaline surroundings.

Acknowledgments Our work is supported by the Israel Science Foundation (grant 30/07), the US Air Force Office for Scientific Research (grant FA9550-07-10057) and the US Army Research Office (grant W911NF-07-1-0260).

References

Abu-Qarn M, Eichler J (2006) Protein N-glycosylation in Archaea: defining *Haloferax volcanii* genes involved in S-layer glycoprotein glycosylation. Mol Microbiol 61:511–525

Abu-Qarn M, Yurist-Doutsch S, Giordano A, Trauner A, Morris HR, Hitchen P, Medalia O, Dell A, Eichler J (2007) *Haloferax volcanii* AglB and AglD are involved in N-glycosylation of the S-layer glycoprotein and proper assembly of the surface layer. J Mol Biol 374:1224–1236

Albers SV, Driessen AJ (2005) Analysis of ATPases of putative secretion operons in the thermoacidophilic archaeon *Sulfolobus solfataricus*. Microbiology 151:763–773

Allers T, Ngo HP, Mevarech M, Lloyd RG (2004) Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. Appl Environ Microbiol 70:943–953

Anton J, Llobet-Brossa E, Rodriguez-Valera F, Amann R (1999) Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. Environ Microbiol 1:517–523

Ashiuchi M, Misono H (2002) Biochemistry and molecular genetics of poly-gamma-glutamate synthesis. Microbiol Biotechnol 59:9–14

Baliga NS, Bonneau R, Facciotti MT, Pan M, Glusman G, Deutsch EW, Shannon P, Chiu Y, Weng RS, Gan RR, Hung P, Date SV, Marcotte E, Hood L, Ng WV (2004) Genome sequence of *Haloarcula marismortui*: a halophilic archaeon from the dead sea. Genome Res 14:2221–2234

Benlloch S, Acinas SG, Anton J, Lopez-Lopez A, Luz SP, Rodriguez-Valera F (2001) Archaeal biodiversity in crystallizer ponds from a solar saltern: culture versus PCR. Microb Ecol 41:12–19

Blaurock AE, Stoeckenius W, Oesterhelt D, Scherfhof GL (1976) Structure of the cell envelope of *Halobacterium halobium*. J Cell Biol 71:1–22

Bolhuis H, te Poele E, Rodriguez-Valera F (2004) Isolation and cultivation of Walsby's square archaeon. Environ Microbiol 6:1287–1291

Bolhuis H, Palm P, Wende A, Falb M, Rampp M, Rodriguez-Valera F, Pfeiffer F, Oesterhelt D (2006) The genome of the square archaeon *Haloquadratum walsbyi*: life at the limits of water activity. BMC Genomics 7:169

Burda P, Aebi M (1999) The dolichol pathway of N-linked glycosylation. Biochim Biophys Acta 1426:239–257

Burns DG, Camakaris HM, Janssen PH, Dyall-Smith ML (2004) Cultivation of Walsby's square haloarchaeon. FEMS Microbiol Lett 238:469–473

Chew SF, Chan NK, Loong AM, Hiong KC, Tam WL, Ip YK (2004) Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land. J Exp Biol 207:777–786

Eichler J (2001) Post-translational modification unrelated to protein glycosylation follows translocation of the S-layer glycoprotein across the plasma membrane of the haloarchaeon *Haloferax volcanii*. Eur J Biochem 268:4366–4373

Eichler J (2003) Facing extremes: archaeal surface-layer (glyco-) proteins. Microbiology 149:3347–3351

Eichler J, Adams MWW (2005) Post-translational protein modification in archaea. Microbiol Mol Biol Rev 69:393–425

Falb M, Pfeiffer F, Palm P, Rodewald K, Hickmann V, Tittor J, Oesterhelt D (2005) Living with two extremes: conclusions from the genome sequence of *Natronomonas pharaonis*. Genome Res 15:1336–1343

Fukuchi S, Yoshimune K, Wakayama M, Moriguchi M, Nishikawa K (2003) Unique amino acid composition of proteins in halophilic bacteria. J Mol Biol 327:347–357

Gavel Y, von Heijne G (1990) Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. Protein Eng 3:433–442

Graham DE, Overbeek R, Olsen GJ, Woese CR (2000) An archaeal genomic signature. Proc Natl Acad Sci USA 97:3304–3308

Helenius A, Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem 73:1019–1049

Henderson R (1975) The structure of the purple membrane from *Halobacterium halobium*: analysis of the X-ray diffraction pattern. J Mol Biol 93:123–128

Houwink AL (1956) Flagella, gas vacuoles and cell-wall structure in *Halobacterium halobium*; an electron microscope study. J Gen Microbiol 15:146–150

Javor B (1984) Growth potential of halophilic bacteria isolated from solar salt environments: carbon sources and salt requirements. Appl Environ Microbiol 48:352–360

Kandler O, König H (1978) Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch Microbiol 118:141–152

Kandler O, König H (1985) Cell envelopes of archaebacteria. In: Woese CR, Wolfe RS (eds) The Bacteria. A treatise on structure and function. Archaebacteria, vol VIII. Academic Press, New York, pp 413–457

Kandler O, König H (1993) Cell envelopes of archaea: structure and chemistry. In: Kates M, Kusher DJ, Matheson AT (eds) The biochemistry of archaea. Elsevier, Amsterdam, pp 223–259

Kandler O, König H (1998) Cell wall polymers in archaea (archaebacteria). Cell Mol Life Sci 54:305–308

Kates M, Sastry PS, Yengoyan LS (1963) Isolation and characterization of a diether analog of phosphatidyl glycerophosphate from *Halobacterium cutirubrum*. Biochim Biophys Acta 70:705–707

Kessel M, Wildehaber I, Cohen S, Baumeiser W (1988) Three-dimensional structure of the regular surface glycoprotein layer of *Halobacterium volcanii* from the Dead sea. EMBO J 7:1549–1554

Kikuchi A, Sagami H, Ogura K (1999) Evidence for covalent attachment of diphytanylglyceryl phosphate to the cell-surface glycoprotein of *Halobacterium halobium*. J Biol Chem 274:18011–18016

Kirk RG, Ginzburg M (1972) Ultrastructure of two species of *Halobacterium*. J Ultrastruct Res 41:80–94

Koncewicz MA (1972) Glycoproteins in the cell envelope of *Halobacterium halobium*. Biochem J 128:124P

König H (2001) Archaeal cell walls. Encyclopedia of life sciences. DOI: 10.1038/npg.els.0000384

Konrad Z, Eichler J (2002) Lipid modification of proteins in archaea: attachment of a mevalonic acid-based lipid moiety to the surface-layer glycoprotein of *Haloferax volcanii* follows protein translocation. Biochem J 366:959–964

Kuntz C, Sonnenbichler J, Sonnenbichler I, Sumper M, Zeitler R (1997) Isolation and characterization of dolichol-linked oligosaccharides from *Haloferax volcanii*. Glycobiology 7:897–904

Lanyi JK (1974) Salt-dependent properties of proteins from extremely halophilic bacteria. Bacteriol Rev 38:272–290

Lechner J, Sumper M (1987) The primary structure of a procaryotic glycoprotein. Cloning and sequencing of the cell surface glycoprotein gene of halobacteria. J Biol Chem 262:9724–9729

Lechner J, Wieland F (1989) Structure and biosynthesis of prokaryotic glycoproteins. Annu Rev Biochem 58:173–194

Lechner J, Wieland F, Sumper M (1985a) Biosynthesis of sulfated saccharides N-glycosidically linked to the protein via glucose. Purification and identification of sulfated dolichyl monophosphoryl tetrasaccharides from halobacteria. J Biol Chem 260:860–866

Lechner J, Wieland F, Sumper M (1985b) Transient methylation of dolichyl oligosaccharides is an obligatory step in halobacterial sulfated glycoprotein biosynthesis. J Biol Chem 260:8984–8989

Mengele R, Sumper M (1992) Drastic differences in glycosylation of related S-layer glycoproteins from moderate and extreme halophiles. J Biol Chem 267:8182–8185

Mescher MF, Strominger JL (1976a) Structural (shape-maintaining) role of the cell surface glycoprotein of *Halobacterium salinarium*. Proc Natl Acad Sci USA 73:2687–2691

Mescher MF, Strominger JL (1976b) Purification and characterization of a prokaryotic glycoprotein from the cell envelope of *Halobacterium salinarium*. J Biol Chem 251:2005–2014

Mescher MF, Strominger JL, Watson SW (1974) Protein and carbohydrate composition of the cell envelope of *Halobacterium salinarium*. J Bacteriol 120:945–954

Mescher MF, Hansen U, Strominger JL (1976) Formation of lipid-linked sugar compounds in *Halobacterium salinarium*. Presumed intermediates in glycoprotein synthesis. J Biol Chem 251:7289–7294

Niemetz R, Karcher U, Kandler O, Tindall BJ, König H (1997) The cell wall polymer of the extremely halophilic archaeon *Natronococcus occultus*. Eur J Biochem 249:905–911

Ng WV, Kennedy SP, Mahairas GG, Berquist B, Pan M, Shukla HD, Lasky SR, Baliga NS, Thorsson V, Sbrogna J, Swartzell S, Weir D, Hall J, Dahl TA, Welti R, Goo YA, Leithauser B, Keller K, Cruz R, Danson MJ, Hough DW, Maddocks DG, Jablonski PE, Krebs MP, Angevine CM, Dale H, Isenbarger TA, Peck RF, Pohlschroder M, Spudich JL, Jung KW, Alam M, Freitas T, Hou S, Daniels CJ, Dennis PP, Omer AD, Ebhardt H, Lowe TM, Liang P, Riley M, Hood L, DasSarma S (2000) Genome sequence of *Halobacterium* species NRC-1. Proc Natl Acad Sci USA 97:12176–12181

Oesterhelt D, Stoeckenius W (1971) Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nat New Biol 233:149–152

Oren A (2002) Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. J Ind Microbiol Biotechnol 28:56–63

Oren A, Duker S, Ritter S (1996) The polar lipid composition of Walsby's square bacterium. FEMS Microbiol Lett 138:135–140

Paul GF, Lottspeich F, Wieland F (1986) Asparaginyl-N-acetylgalactosamine. Linkage unit of halobacterial glycosaminoglycan. J Biol Chem 261:1020–1024

Sampathkumar SG, Li A, Yarema KJ (2006) Sialic acid and the central nervous system: perspectives on biological functions, detection, imaging methods and manipulation. CNS Neurol Disord Drug Targets 5:425–440

Schleifer KH, Steber J, Mayer H (1982) Chemical composition and structure of the cell wall of *Halococcus morrhuae*. Zentralbl Bakt Mikrobiol Hyg Serie C3:171–178

Sheehan JK, Thornton DJ, Somerville M, Carlstedt I (1991) Mucin structure. The structure and heterogeneity of respiratory mucus glycoproteins. Am Rev Respir Dis 144:S4–S9

Steber J, Schleifer KH (1975) *Halococcus morrhuae*: a sulfated heteropolysaccharide as the structural component of the bacterial cell wall. Arch Microbiol 105:173–177

Steber J, Schleifer KH (1979) N-glycyl-glucosamine: a novel constituent in the cell wall of *Halococcus morrhuae*. Arch Microbiol 123:209–212

Steensland H, Larsen H (1969) A study of the cell envelope of the halobacteria. J Gen Microbiol 55:325–336

Stoeckenius W, Rowen R (1967) A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration. J Cell Biol 34:365–393

Sumper M (1987) Halobacterial glycoprotein biosynthesis. Biochim Biophys Acta 906:69–79

Sumper M, Berg E, Mengele R, Strobel I (1990) Primary structure and glycosylation of the S-layer protein of *Haloferax volcanii*. J Bacteriol 172:7111–7118

Tindall BJ, Ross HMN, Grant WD (1984) *Natronobacterium* gen. nov. and *Natronococcus* gen. nov. – two genera of haloalkaliphilic archaebacteria. Syst Appl Microbiol 5:41–57

Trachtenberg S, Pinnick B, Kessel M (2000) The cell surface glycoprotein layer of the extreme halophile *Halobacterium salinarum* and its relation to *Haloferax volcanii*: cryo-electron tomography of freeze-substituted cells and projection studies of negatively stained envelopes. J Struct Biol 130:10–26

Varki A (1998) Factors controlling the glycosylation potential of the golgi apparatus. Trends Cell Biol 8:34–40

Vimr ER, Kalivoda KA, Deszo EL, Steenbergen SM (2004) Diversity of microbial sialic acid metabolism. Microbiol Mol Biol Rev 68:132–153

Wakai H, Nakamura S, Kawasaki H, Takada K, Mizutani S, Aono R, Horikoshi K (1997) Cloning and sequencing of the gene encoding the cell surface glycoprotein of *Haloarcula japonica* strain TR-1. Extremophiles 1:29–35

Woese CR, Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74:5088–5090

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Wolfe RS (2006) The archaea: a personal overview of the formative years. In: Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The Prokaryotes, Vol. 3: Archaea. Bacteria: Firmicutes, Actinomycetes. Springer, Berlin, pp 3–9

Yang LL, Haug A (1979) Purification and partial characterization of a procaryotic glycoprotein from the plasma membrane of *Thermoplasma acidophilum*. Biochim Biophys Acta 556:265–277

Zeitler R, Hochmuth E, Deutzmann R, Sumper M (1998) Exchange of Ser-4 for Val, Leu or Asn in the sequon Asn-Ala-Ser does not prevent N-glycosylation of the cell surface glycoprotein from *Halobacterium halobium*. Glycobiology 8:1157–1164

Zhu CR, Drake RR, Schweingruber H, Laine RA (1995) Inhibition of glycosylation by amphomycin and sugar nucleotide analogs PP36 and PP55 indicates that *Haloferax volcanii* β-glycosylates both glycoproteins and glycolipids through lipid-linked sugar intermediates. Arch Biochem Biophys 319:355–364

Chapter 9
Cell Envelopes of Crenarchaeota and Nanoarchaeota

Reinhard Rachel

9.1 Introduction

Within the domain Archaea, the Crenarchaeota are a well-defined kingdom, based on sequence data and biochemical and physiological investigations. This kingdom comprises, according to our current knowledge, at least three orders: the Sulfolobales, the Thermoproteales, and the Desulfurococcales. Essentially, this classification was suggested more than ten years ago (Burggraf et al. 1997), and was based on phylogenetic analyses by 16S rRNA sequence comparisons. The current phylogenetic status of all microbial species which – based on the same criterion – belong to the Crenarchaeota is perfectly in line with the earlier data and was recently summarized (Huber 2006). In this compilation, six genera were allocated to the order Sulfolobales, six genera to the Thermoproteales, and 13 to the Desulfurococcales. In addition, *Ignisphaera aggregans* (Niederberger et al. 2006) was described and, arbitrarily, affiliated with the Desulfurococcales; in fact, its affiliation to this group is still uncertain, and might be re-evaluated when more isolates of this genus or related to this genus are known and characterized. Preliminary ultrastructural data confirm its similarity to other Crenarchaeota (A. Klingl, H. Morgan, and R. Rachel, unpublished).

In general, phylogenetic comparison of microorganisms, based on ribosomal RNA sequence data, is a reliable and robust method for most genera. It can be performed fairly easily nowadays, a large database is available (Ludwig et al. 2004), and the data correlate well with phylogenetic data based on other genes. Early protein sequence comparisons were performed on the sequence of the α and the β subunits of the H^+-ATPase, providing a first hypothesis of the possible root of the phylogenetic tree (Gogarten et al. 1989; Iwabe et al. 1989). Later, detailed

R. Rachel
Institute of Microbiology and Centre for Electron Microscopy, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany
e-mail: reinhard.rachel@biologie.uni-regensburg.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_9,

analyses were carried out, e.g. with concatenated sequence data of ribosomal proteins or the full sequence of the elongation factor 2 (EF2; Waters et al. 2003; Elkins et al. 2008). More recently, even whole genomes have been compared (Snel et al. 2005). Essentially, the same phylogenetic tree was obtained for the crenarchaeal genera known today, underlining the validity of the current model.

Additional microorganisms have been described which – according to genome data – belong to or are in close vicinity to the Crenarchaeota. At present, a deep branch is emerging, the non-thermophilic terrestrial and marine Crenarchaeota (Schleper et al. 2005; Leininger et al. 2006). These are isolates whose relationship to the Crenarchaeota is solely based on molecular data, e.g. the sequence of the rRNA and of *amoA*, a gene coding for ammonia monooxygenases, enzymes important in ammonium oxidation. The isolates are "*Candidatus* Cenarchaeum symbiosum" (Preston et al. 1996; Hallam et al. 2006), "*Candidatus* Nitrosopumilus marinus" (Könnecke et al. 2005), and "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler et al. 2008). The question was raised whether they are part of the branch of the Crenarchaeota, or whether they form an independent branch or even kingdom, for which a new name was proposed ("Thaumarchaeota"; Brochier-Armanet et al. 2008). At present, this is under discussion. No ultrastructural data for these species are published yet, which would provide a detailed view of their cell wall structure. Therefore, no structural details are included in this review.

Two other isolates are different in their genomes, physiology, and morphology, and are, therefore, not attributed to the Crenarchaeota. Supposedly, they are members of novel kingdoms. These are *Nanoarchaeum equitans*, of the Nanoarchaeota (Huber et al 2002), and "C*andidatus* Korarchaeum cryptofilum", of the Korarchaeota (Elkins et al 2008).

9.2 General Considerations on the Cell Envelope

The focus of this chapter is on the organization of the cell envelope and the molecules involved. According to earlier papers and reviews, we define as cell wall the lipid membrane and all macromolecules outside of the membrane. Since the first studies, some dating back to the 1950s, it has become evident that Archaea are fundamentally different to Bacteria in the components and architecture of the cell envelope: they contain isoprenyl di- and tetraether glycerol-based lipids (Boucher 2007); they lack muramic acid and a lipopolysaccharide-containing outer membrane (Kandler and Hippe 1977; Kandler and König 1978, 1993); and cells of *Halobacterium* (Houwink 1956) and of *Sulfolobus* (Weiss 1973, 1974) were found to be covered by a regular surface layer (S-layer). The surface layer glycoprotein of *Halobacterium salinarum* was the first glycoprotein discovered in prokaryotes (Mescher and Strominger 1976a; Mescher and Strominger 1976b). The phylogenetic diversity of all Archaea is reflected in a large diversity of cell envelope types, which have been summarized in various reviews, e.g. recently in König et al. 2007 (see also Baumeister et al 1989; Eichler 2003; Kandler and König 1993; Lechner and

Wieland 1989; Messner and Schäffer 2003). Initially, the novel cell wall structures were viewed as curiosities, and their taxonomic significance was not realized until the concept of the Archaea was published (Woese 1987). In fact, the results of cell wall studies correlate with our current view of the phylogeny of the prokaryotes. Within the Archaea, many species possess proteinaceous surface layers (Baumeister and Lembcke 1992; Beveridge and Graham 1991; Kandler and König 1993; Messner and Sleytr 1992; Sumper and Wieland 1995).

If the view is restricted, as for the purpose of this article, to the domain of the Crenarchaeota, it is evident that for almost all species known today – with two notable exceptions, *Thermosphaera aggregans* and all species of the genus *Ignicoccus* – S-layers are distinct and prominent parts of the cell envelope. S-layers, by definition, are surface layers: regular, two-dimensional crystalline arrays of proteins. They are directly associated with or anchored in the cytoplasmic membrane (Fig. 9.1) and composed of many identical copies of a single or of two protein species, which are often glycosylated. For the species of the Crenarchaeota described, the S-layers (König et al. 2007) were shown to contain morphological building blocks, each of which is composed of six, four, or three subunits of one type of a (glyco-)protein. Accordingly, the symmetry axis with the highest symmetry is p6, p4, or p3 (Fig. 9.2; Table 9.1). In most species, the S-layer is the sole cell wall polymer; it is anchored into the cytoplasmic membrane by an elongated, filamentous protrusion, spanning the quasi-periplasmic space between the S-layer and the membrane (Fig. 9.1).

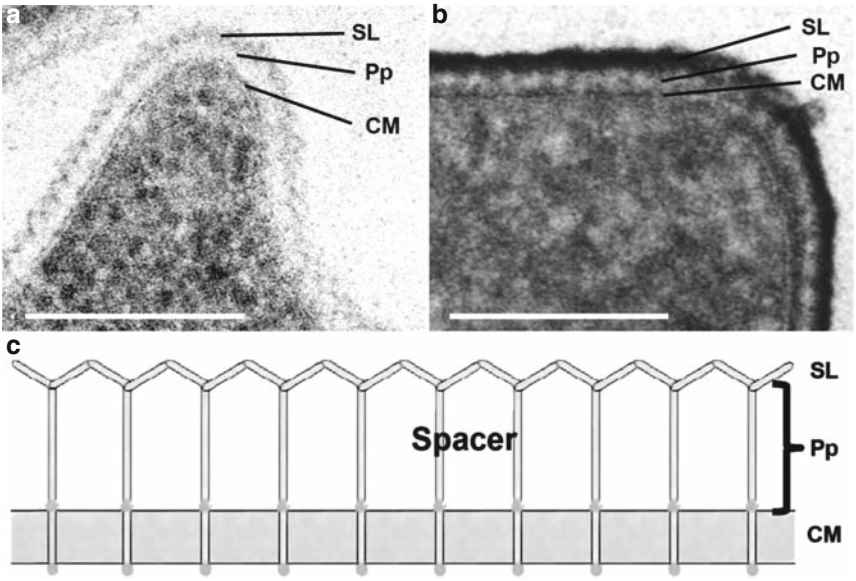

Fig. 9.1 Ultrathin sections of cells of (**a**) *Metallosphaera sedula*, (**b**) *Pyrobaculum aerophilum* and (**c**) Simplified model of the cell wall profile of the Crenarchaeota known today. SL, S-layer, Pp, periplasm, CM, cytoplasmic membrane. Bar: 200 nm

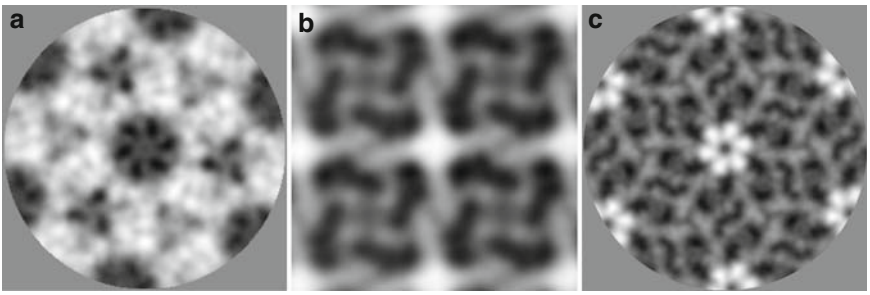

Fig. 9.2 "Correlation averages" deduced from electron micrographs of negatively stained S-layer sheets from (**a**) a *Sulfolobus* strain, (**b**) an *Aeropyrum* strain, and (**c**) a *Pyrobaculum* strain. *Dark*: negative stain; *white*: protein. The images show the representative arrangement of S-layer subunits. Type of symmetry: (**a**) p3; (**b**) p4; (**c**) p6

Table 9.1 Characteristic features of crenarchaeal S-layers

Genus/species	Symmetry	Center–center (nm)	Width of periplasm (nm)
Sulfolobales			
Sulfolobus sp.	p3	20–21	20–25
Metallosphaera sp.	p3	21	25
Acidianus brierleyi	p3	21	25
Thermoproteales			
Pyrobaculum organotrophum	p6	~30	25
Pyrobaculum islandicum	p6	~30	25
Thermoproteus tenax	p6	~30	25
Thermofilum sp.	p6	27	25
Desulfurococcales			
Desulfurococcus mobilis	p4	18	30
Staphylothermus marinus	p4	36	60–70
Aeropyrum pernix	p4	19	30
Pyrolobus fumarii	p4	18.5	45
Pyrodictium sp.	p6	21	35
Hyperthermus butylicus	p6	25	20 (?)
Ignicoccus sp.	–	–	20–400
Candidatus "Cenarchaeum symbiosum"	?	?	?
Candidatus "Nitrosopumilus marinus"	?	?	?
Candidatus "Nitrososphaera gargensis"	?	?	?
Nanoarchaeum equitans	p6	16	20
Candidatus "Korarchaeum cryptofilum"	p6?	~14	?

The center-to-center spacing varies among the known genera and species between about 18 nm (*Desulfurococcus mobilis*; Wildhaber et al. 1987), about 21 nm (species of the Sulfolobales), about 30 nm (most species of the order Thermoproteales, such as *Thermoproteus tenax* (Messner et al. 1986; Wildhaber and Baumeister 1987) or *Pyrobaculum islandicum* (Phipps et al. 1990)), and up to 36 nm (*Staphylothermus marinus*; Peters et al. 1995). The molecular masses of the surface proteins range from 40 to 325 kDa (Messner and Sleytr 1992).

For one crenarchaeal species, a strain of *Sulfolobus*, a first detailed characterization of the S-layer was published in the early 1980s (Taylor et al. 1982). Soon, the technique of protein electron crystallography became routinely applicable, and a number of structural studies were conducted on the cell wall of various crenarchaeal isolates, belonging to all three orders, the Sulfolobales, the Thermoproteales, and the Desulfurococcales. Today, the two- and three-dimensional structures of isolated S-layer sheets have been studied in numerous species by electron crystallography: *S. acidocaldarius* (Deatherage et al. 1983; Lembcke et al. 1990; Taylor et al. 1982), *S. solfataricus* (Prüschenk and Baumeister 1987), *Sulfolobus shibatae* (Lembcke et al. 1993), *Acidianus brierleyi* (Baumeister et al. 1991), *T. tenax* (Messner et al. 1986; Wildhaber and Baumeister 1987), *P. islandicum* (Phipps et al. 1990), *Pyrobaculum organotrophum* (Phipp et al. 1991), *Desulfurococcus mobilis* (Wildhaber et al. 1987), *Pyrodictium occultum* (Hegerl and Baumeister 1988), *Pyrodictium brockii* (Dürr et al. 1991), and *Hyperthermus butylicus* (Baumeister et al. 1990). Complementary structural information was obtained by studying freeze-fractured or freeze-etched cells, ultrathin sections, and freeze-dried and heavy-metal-shadowed isolated S-layer sheets. Structural information at lower resolution is available for some newer isolates such as *Pyrobaculum aerophilum* (Völkl et al. 1993), *Pyrodictium abyssi* (Rieger et al. 1995), and *Pyrolobus fumarii* (Blöchl et al. 1997). In some studies, the arrangement of the subunits is characteristic of the genus (e.g. *Pyrodictium* vs. *Pyrolobus* vs. *Hyperthermus*). In other cases, it is similar or identical in all species of the genera belonging to a family (*Thermoproteus* and *Pyrobaculum* within the Thermoproteaceae; *Sulfolobus*, *Acidianus* and *Metallosphaera* within the Sulfolobaceae). These results indicate that, at least for the phylogenetic group discussed here, the domain Crenarchaeota, the S-layer structure correlates with the organism's phylogeny (Baumeister and Lembcke 1992), as determined by sequencing the ribosomal DNA. As far as is known today, this similarity in the quarternary structure, as observed by TEM, is not reflected in a similarity on the gene or protein level of the major S-layer protein (Veith et al. 2009, under revision).

These electron microscopy studies revealed the structural data available today. Structural data are missing so far for *Candidatus* Cenarchaeum symbiosum, for *Candidatus* Nitrosopumilus marinus, and for *Candidatus* Nitrososphaera gargensis. Preliminary data are available for *Candidatus* Korarchaeum cryptofilum: these cells possess an S-layer with unknown symmetry (possibly p6), and a lattice constant of 16 nm (Elkins et al. 2008). The biochemical structure of a crenarchaeal S-layer glycoprotein has been determined in detail for *Staphylothermus marinus* (Peters et al. 1995), and for three Sulfolobales species (Veith et al 2009, under revision).

In the following sections, the S-layer structures of Crenarchaeota are described in a phylogenetic context.

9.3 The S-Layers of Crenarchaeota

9.3.1 Sulfolobales

The S-layer of *Sulfolobus acidocaldarius* (Weiss 1973, 1974) was first isolated by lysing the cells with SDS (0.15%), DNase digestion, and repeated treatment of the S-layer sheets with SDS. Following disintegration in phosphate buffer pH 9 at 60°C, the solubilized S-layer protein was purified by size-exclusion chromatography (Michel et al. 1980). Chemical analysis revealed the S-layer to be composed of a single glycoprotein occurring in two modifications of apparent molecular masses of about 140 and 170 kDa, respectively. The glycoproteins contained high levels of serine and threonine and low levels of basic amino acids and dicarboxylic amino acids (Michel et al. 1980). Evidence was provided for the presence of a second protein subunit which may anchor the S-layer into the cell membrane of *S. acidocaldarius* (Grogan 1996).

The first electron micrographs of purified S-layers of *S. acidocaldarius* were interpreted such that the subunits were arranged on a hexagonal lattice, with a two-sided plane group p6, and a 22-nm unit cell dimension. The three-dimensional structure of the S-layer, elucidated in one of the first electron crystallography studies on biological objects (Deatherage et al. 1983; Taylor et al. 1982), showed dimeric building blocks, arranged to form a series of hexagonal and triangular holes. This first 3D structure of an archaeal S-layer identified a feature that has subsequently been found to occur in many archaeal S-layers: the external surface is fairly smooth, while the surface facing to the interior of the cell appears "rough", exhibiting large dome-shaped cavities and protruding "pedestals", which are known to anchor the S-layer into the lipid bilayer of the cytoplasmic membrane (Baumeister and Lembcke 1992). The protein substructure consists of three types of globular domains, diad (D), triad (T), and ring region (R), connected by narrow bridges. These may act as "hinges," allowing the S-layer to form a curved surface (*Sulfolobus* cells are lobed) and to follow the movements of the cell surface during growth. Similar results were subsequently obtained for the S-layer of *Sulfolobus solfataricus* (Prüschenk and Baumeister 1987). It is noteworthy that the larger pores of the S-layer have the same diameter as pili (Weiss 1973). Pili have been observed that might attach cells to sulfur crystals (Weiss 1973), and, recently, that are involved in cell–cell contacts: *Sulfolobus* treated with UV radiation formed aggregates (Fröls et al. 2007), and, concomitantly, pili were observed on cell surfaces.

The interpretation that the S-layers of *Sulfolobus* species had sixfold symmetry was later shown to be preliminary (Baumeister and Lembcke 1992; Lembcke et al. 1990, 1993). By improving the technique of sample preparation, image recording

using cryo-electron microscopy, and refining the image processing methods of the electron micrographs (including image classification), it became clear that the S-layer of *S. acidocaldarius* had "only" threefold symmetry (Lembcke et al. 1990). The S-layer of *S. shibatae* was also found to have p3 symmetry and the same fine structure (distribution of protein mass) as determined for *S. acidocaldarius*. The protein complexes were found to be arranged as in a mosaic, with frequent occurrence of twin boundaries (neighboring unit cells are rotated by 120°) and distortions (unit cells on one lattice line are slightly displaced and rotated relative to each other) (Lembcke et al. 1993).

It has now been established that the S-layers of the phylogenetically related organisms of this order (Fig. 9.3), *S. acidocaldarius, S. solfataricus*, *S. shibatae*, *A. infernus* (see Figs. 9.2a and 9.4a; R. Rachel and H. Huber, unpublished results), *A. brierleyi* (Baumeister et al. 1991), *Metallosphaera sedula,* and *Metallosphaera prunae* (Fuchs et al. 1995), all have a distinctive structural similarity to each other. Their fine structure and mass distribution, the unique p3 symmetry, the center-to-center distance of about 21 nm, and the width of their periplasm (about 25 nm) are almost identical (Baumeister and Lembcke 1992). These structural characteristics appear to be common to many, if not all species belonging to the crenarchaeal order *Sulfolobales*.

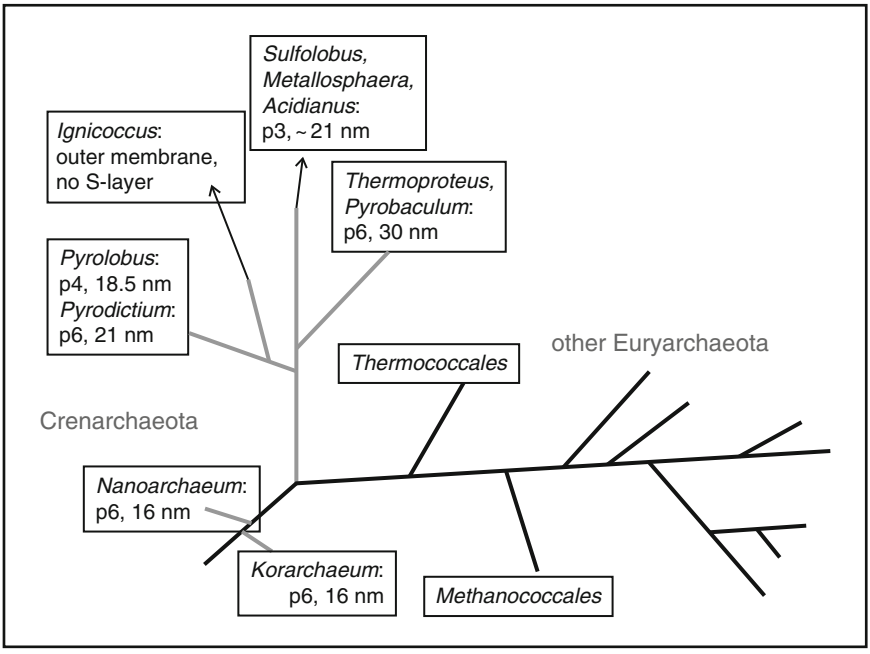

Fig. 9.3 Phylogenetic tree of those Archaea which have been cultivated and characterized, with a focus on the Crenarchaeota, based on 16S rDNA sequences. Data compilation by Harald Huber and Reinhard Rachel

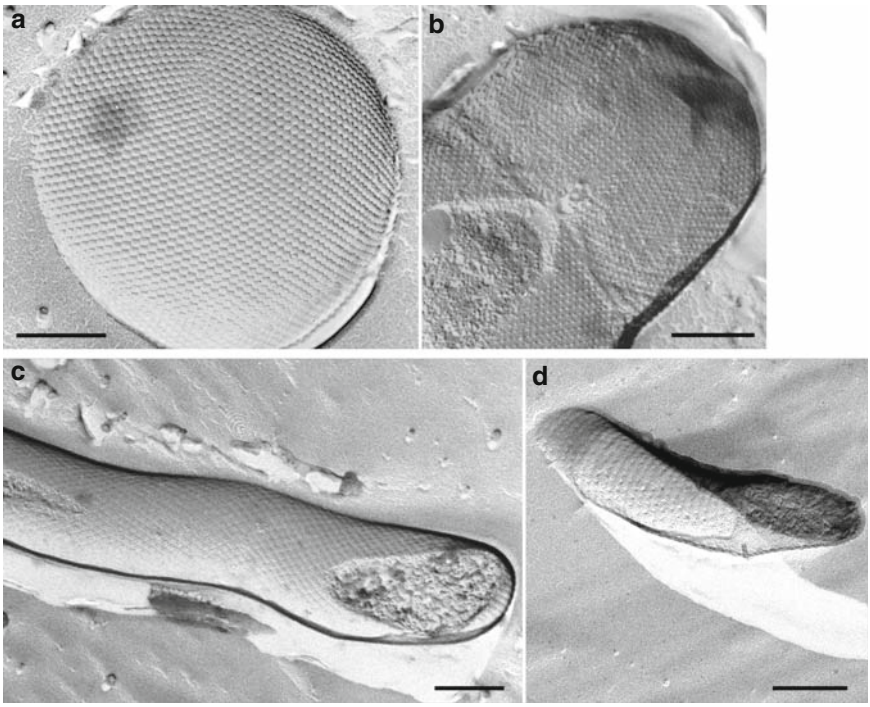

Fig. 9.4 Transmission electron micrographs of different Crenarchaeota, prepared by freeze-etching. (**a**) *Metallosphaera sedula* – p3 symmetry; (**b**) *Aeropyrum pernix* – p4 symmetry; (**c**) *Thermoproteus tenax* – p6 symmetry; (**d**) *Thermofilum pendens* – p6 symmetry. All bars: 0.2 μm

9.3.2 Desulfurococcales

For *Desulfurococcus mobilis*, the S-layer was shown to exhibit p4 symmetry and a rather open meshwork of protein, with a lattice of 18 nm (Wildhaber et al. 1987). Two other species in this order also have an S-layer with p4 symmetry, although with a different fine structure. *Pyrolobus fumarii*, an archaeon with a T_{max} of 113°C, has an S-layer with a lattice of 18.5 nm; it encloses a 40-nm-wide "quasi-periplasmic space" (Blöchl et al. 1997). The *Staphylothermus marinus* S-layer is composed of a unique glycoprotein, named "tetrabrachion", which was intensively studied by biochemical methods, gene sequencing, and electron microscopy (Peters et al. 1995, 1996). The morphological subunits appear as a branched filiform meshwork. They form a canopy with a distance of about 70 nm from the cell membrane, thus enclosing a fairly large "quasi-periplasmic space" (Mayr et al. 1996). The morphological subunit is a tetramer of polypeptides (M_r 92 kDa), forming a parallel, four-stranded α-helical rod, 70 nm long. It separates at one end into four strands, or straight arms; hence the name "tetra-brachion", "four arms". Each polypeptide arm has a mass of 85 kDa, and is 24 nm in length. It is composed of β-sheets and provides lateral connectivity to neighboring morphological subunits by end-to-end

contacts (Peters et al. 1996). Attached to the middle of each stalk, i.e. about in the middle of the periplasmic space, are two copies of a protease, that provide an exodigestive function related to the heterotrophic energy metabolism of the organism (Mayr et al. 1996). Both proteins, the tetrabrachion and the protease, exhibit an unusually high thermal stability (Peters et al. 1995, 1996).

Recently, freeze-etching was used for investigating the fine structure of cells of *Aeropyrum pernix*, a closely related *Aeropyrum* isolate, and three other isolates (CB9, HVE1, CH11), which were obtained from hot springs (R. Rachel, I. Wyschkony, H. Schmidt, and H. Huber; unpublished results). The physiological characterization of the three new isolates is incomplete. According to their 16S rRNA gene sequence, they all belong to the Desulfurococcales. Electron micrographs of freeze-etched cells (Fig. 9.4b) revealed that they have S-layers with p4 symmetry and a lattice of about 18–19 nm, with obvious differences in their surface relief and in the mass distribution of the protein complexes. An S-layer with p4 symmetry, an open network of protein, and a comparably large quasi-periplasmic space (30–70 nm) is a common feature of these organisms; in these features, they have a certain degree of similarity to three other species, *Desulfurococcus mobilis, Staphylothermus marinus*, and *Pyrolobus fumarii*.

In contrast to the *Aeropyrum*-related strains, cells of the genera *Hyperthermus* and *Pyrodictium* (Fig. 9.5) have S-layers with p6 symmetry. For all isolates of the genus *Pyrodictium*, *P. occultum*, *P. abyssi*, *P. abyssi* strain TAG11 and *P. brockii*, the center–center distance of the S-layer is about 21 nm. Their mass distribution, surface reliefs and 3D structures are almost indistinguishable (Dürr et al. 1991; Hegerl and Baumeister 1988; Rieger et al. 1995). However, distribution of protein mass and surface relief of the *H. butylicus* S-layer is clearly different to that of the *Pyrodictium* species (Baumeister et al. 1990); also its center–center distance of 25 nm is significantly larger than for cells of the genus *Pyrodictium*. This shows that the phylogeny in this family, as determined by the sequence of the 16S rDNA, correlates with S-layer ultrastructure.

9.3.3 Thermoproteales

T. tenax (Fig. 9.4c) and *Thermofilum pendens* (Fig. 9.4d) possess extraordinarily rigid S-layer sacculi of hexagonally arranged subunits that are resistant to detergent and protease treatment (Zillig et al. 1981, Zillig et al. 1982). The S-layer can easily be isolated by disrupting the cells using sonication followed by incubation with DNase and RNase, SDS treatment (2% SDS, 80°C or even 100°C, 30 min), and differential centrifugation (König and Stetter 1986; Wildhaber and Baumeister 1987). In *Termoproteus tenax*, the 25 nm wide interspace is due to long protrusions that extend from the relatively thin (3–4 nm) filamentous network of the outer surface of the S-layer toward the cytoplasmic membrane. The distal ends of these pillar-like protrusions most likely penetrate the membrane, thus serving as membrane anchors (Messner et al. 1986; Wildhaber and Baumeister 1987). The same

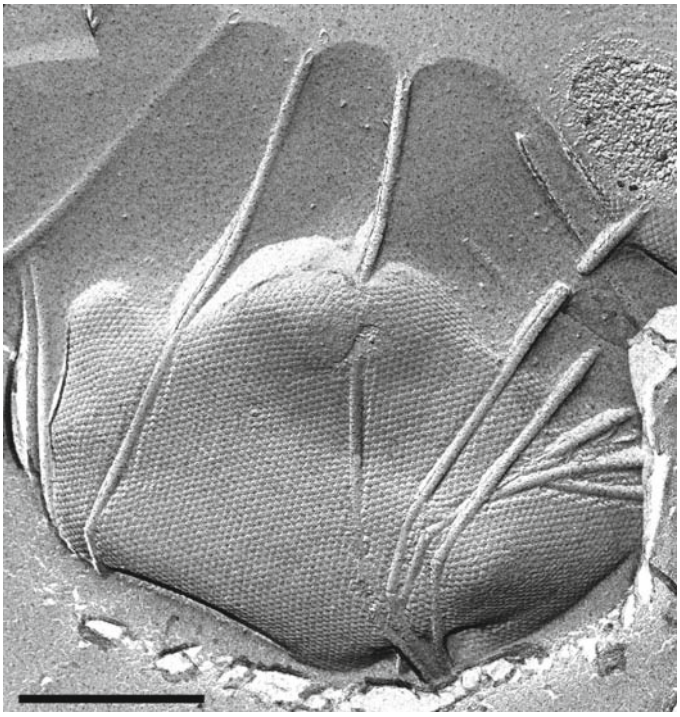

Fig. 9.5 Transmission electron micrograph of a cell of *P. occultum,* after freeze-etching, exhibiting an S-layer with p6 symmetry. Bar: 0.2 μm. The tubes are extracellular cannulae, which interconnect the cells and are made of glycoproteins; this is a specific feature of the genus *Pyrodictium*

S-layer structure as determined for *T. tenax* is also found in three species belonging to the genus *Pyrobaculum*, *P. islandicum* (Phipps et al. 1990), *P. organotrophum* and *P. aerophilum* (Völkl et al. 1993). Again, it consists of a delicate but thermostable protein meshwork with a center-to-center distance of about 30 nm; long protrusions serve as membrane anchors that enclose a 25-nm-wide periplasm. Preliminary investigations with cells of two *Thermofilum* species showed a similar S-layer fine structure, with p6 symmetry and a center–center distance of 27 nm (R. Rachel, unpublished results).

9.4 Gene and Protein Sequences

9.4.1 Sequences

Crenarchaeal S-layer protein sequences have so far only been analyzed in a few cases. The only one analyzed thoroughly is from the species *Staphylothermus marinus* (Peters et al. 1995, 1996). In this case, the single gene and the two highly

glycosylated proteins (both derived from the precursor protein) were both sequenced, and in addition the whole protein complex was investigated by electron microscopy and by analysis of its secondary structure. The results have been published in several articles and are, therefore, only briefly summarized here. The protein is composed of the S-layer as such, which builds a kind of canopy, and includes a long α-helical stalk. A protease is found to be associated with the stalk, which, therefore, is located in the periplasm of the cells. The sequence of the tetrabrachion protein of *S. marinus* suggests the presence of a characteristic N-terminal signal sequence (Bendtsen et al. 2004; Peters et al. 1996), with a non-hydrophobic (n)-region, a hydrophobic (h)-core, a charged (c)-region and an alanine residue at the peptide cleavage site. The signal peptide allows its secretion across the cytoplasmic membrane by the general secretory pathway (Boot and Pouwels 1996; Fernandez and Berenguer 2000; Sára and Sleytr 2000). The putative leader peptide of 26 amino acids was predicted on the basis of its sequence, but could not be identified directly, because the protein was found to be N-terminally blocked. The example of the *S.marinus* S-layer shows that extensive posttranslational modification by glycosylation and proteolytic processing can occur (and probably are a common feature) of (cren-)archaeal S-layers (see also: Eichler 2001, 2003).

In addition, the S-layer protein of *Sulfolobus acidocaldarius* was chemically analyzed (Michel et al. 1980; Grogan 1996), but, at that time, no gene sequencing was performed, nor were the genomes available for these studies. Later, the putative S-layer gene of *S. acidocaldarius* DSM 639 was identified in the published whole genome sequence (Claus et al. 2005). The main protein consists of 1,424 amino acids, corresponding to a molecular mass of 151 kDa. A putative leader peptide of 29 amino acids would be cleaved after membrane translocation to obtain the mature protein. Cysteine is present, as was found in S-layer (glyco)proteins from other hyperthermophiles. In a recent study, S-layer proteins and genes of three Sulfolobales species, *Acidianus ambivalens*, *Sulfolobus solfataricus*, and *Metallosphaera sedula*, were compared in detail. The S-layers are built of two proteins, a large one and a smaller one. SlaA builds the outer S-layer sheet, and SlaB, the stalk protein, anchors SlaA into the cytoplasmic membrane (Veith et al 2009, under revision). The *slaAB* genes, coding for the two proteins, are arranged in one operon in the genome: they are co-transcribed in distinct amounts, as shown by PCR analysis. The arrangement of the genes is conserved in all Sulfolobales species. SlaB proteins of Sulfolobales show a high degree of similarity in their primary structure, while the SlaA proteins exhibit a low degree of similarity, if any.

9.4.2 Glycosylation

Glycosylation of S-layer proteins is generally well characterized (Messner and Sleytr 1992) among Euryarchaeotes, e.g. for the hyperthermophilic methanogenic species *M. fervidus* and *Methanothermus sociabilis* (Bröckl et al. 1991; Kärcher

et al. 1993; Nußer et al. 1988). The S-layers of *S. marinus* and *Sulfolobus* species are also glycosylated. In addition, *Sulfolobus acidocaldarius* is sensitive to tunicamycin (Eichler 2001). Experiments with exogenously added peptides with an *N*-glycosylation site, which cannot penetrate the cytoplasmic membrane, indicated an oligosaccharide transfer outside of the cytoplasmic membrane (Lechner and Wieland 1989), because oligosaccharides were linked to these peptides by the cells. The proteins can also undergo further posttranslational modifications, which may include isoprenylation (Konrad and Eichler 2002) or linkage of diphytanylglyceryl phosphate residues (Kikuchi et al. 1999).

9.4.3 Secondary Structures

The stalk of the *S. marinus* S-layer was shown to be composed of a tetrameric bundle of α-helices (Peters et al. 1996). In fact, the ultrastructure of the cells, as visualized in ultrathin sections, shows the presence of a periplasmic space in almost all species, strongly suggesting that such stalks are a common structural part of crenarchaeal S-layers. It is tempting to speculate that they are all arranged in a similar way, as a bundle of intertwined α-helices. On the basis of structural predictions using a variety of programs, this was recently suggested for the stalks, i.e. SlaB proteins, of three different Sulfolobales species (Veith et al. 2009, under revision). Nevertheless, a thorough genome and protein analysis needs to be done in order to be verify whether this is a general feature of many or most species of the Crenarchaeota.

9.4.4 Sequence Comparison

In this respect, analysis for crenarchaeal S-layers and in particular comparison of proteins from different organisms is scarce. While a comparison of methanococcal S-layer genes was carried out some time ago (Akca et al. 2002), no comparative analyses of crenarchaeal S-layers has yet been performed. Beside the S-layers of *Sulfolobus* and *Staphylothermus*, no other primary structures of crenarchaeal S-layer proteins have yet been directly identified on the protein level and analyzed. From the data available, it is deduced that the S-layer proteins of halobacteria and of the hyperthermophilic crenarchaeon *Staphylothermus marinus* share no homologies with the S-layers from Thermococcales or methanogens (Claus et al. 2001, 2002).

9.5 Extracellular Cannulae of the Genus *Pyrodictium*

Pyrodictium species typically grow at temperatures between 75 and 110°C under anoxic conditions. The cells are covered by an S-layer with hexagonal symmetry and a lattice constant of ~21 nm (Fig. 9.5). The dome-shaped complexes are

anchored to the cytoplasmic membrane by filiform stalks and span the periplasmic space with a constant width of approximately 35 nm (Rieger et al. 1995; Dürr et al. 1991; Hegerl and Baumeister 1988). During growth on elemental sulfur, *Pyrodictium* cells form an extracellular matrix, in which the cells are entrapped. This matrix is an extended network of hollow cylinders consisting of helically arranged subunits (König et al. 1988; Rieger et al. 1995). Each of the cannulae – outer diameter 25 nm (Fig. 9.5) – is made up of (at least) three homologous glycoproteins. In vivo observations of growing *Pyrodictium* cells at 90°C under anoxic conditions demonstrated that this network is dynamic; cell division and synthesis of the cannulae are directly linked (Horn et al. 1999). After cell division, the daughter cells remain interconnected by cannulae loops. Multiple generations result in the formation of a colony of cells entrapped in a dense cannulae network, in which each cell has connections with its neighbors. Analysis of dual-axis tilt series in cryo-electron tomography helped to reveal that the cannulae interconnect individual cells with each other, but only on the level of the periplasmic spaces of the cells; the cannulae do not enter the cytoplasm (Nickell et al. 2003).

9.6 The Cell Envelopes of *Ignicoccus* and of *Nanoarchaeum*

From a sample taken at a hydrothermal vent of the Kolbeinsey Ridge, north of Iceland, a co-culture of two coccoid, hyperthermophilic Archaea was obtained (Huber et al. 2002). Based on 16S rRNA gene sequences, one belongs to the genus *Ignicoccus*, while the other was so different that it was attributed to a new phylum, Nanoarchaeota. Cells of *Ignicoccus* can be cultivated independently under strictly anaerobic conditions, and thrive by sulfur-hydrogen autotrophy. However, *Nanoarchaeum equitans* can only be grown in co-culture with, and in close contact to, cells of *Ignicoccus* sp. strain KIN4I. *Ignicoccus* cells are unique among the Archaea in two important ways (Rachel et al. 2002): (1) they have a huge periplasm with variable width (20–500 nm), which contains vesicles; (2) cells do not possess an S-layer or any other cell wall polymer but have a unique outer membrane (Burghardt et al. 2007). Freeze-etching experiments revealed that the *Ignicoccus* outer membrane fractures into two halves (Fig. 9.6). This type of behavior is similar to the way in which many biological lipid membranes respond during freeze-etching. The outer membrane is a highly dynamic structure: periplasmic vesicles can be observed in various stages of a fusion process, and vesicles are also released into the culture medium (Näther and Rachel 2004). The outer membrane is tightly packed with protein complexes, although there is no indication for crystallinity and the proteins are currently being investigated (Burghardt et al. 2008; Junglas et al. 2008).

Ignicoccus sp. strain KIN4I and *N. equitans* both contain qualitatively identical amounts of glycerol ether lipids, archaeol and, to a minor degree, caldarchaeol (Jahn et al. 2004). Cells of *N. equitans* are the smallest archaeal cocci presently known and their ability to reproduce relies on the direct interaction with *Ignicoccus* cells. The reliance on a host is reflected in its genome sequence, which lacks genes

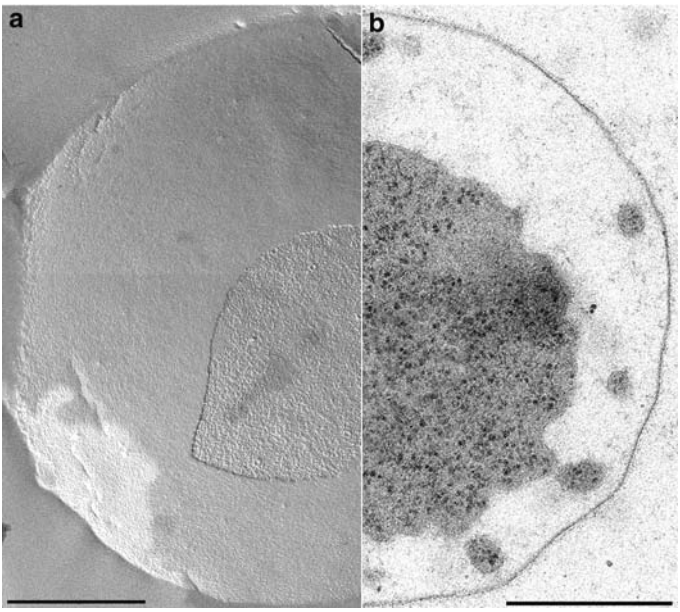

Fig. 9.6 Transmission electron micrographs of cells of the genus *Ignicoccus*. (**a**) Half of a freeze-etched cell; (**b**) ultrathin section of half a cell. Bar: 0.5 μm

for many important metabolic pathways (Waters et al. 2003). The ultrastructure of *N. equitans* is similar to many Archaea. The cytoplasmic membrane is surrounded by a quasi-periplasmic space of constant width (20 nm), and covered by an S-layer with sixfold symmetry and a lattice constant of about 15 nm (Huber et al. 2003). Future investigations of the unusual symbiosis of these two hyperthermophilic Archaea aim at elucidating which proteins of both cell envelopes are directly involved in the physical interaction and in the exchange of metabolites from one cell to the other (Burghardt et al. 2008).

9.7 Conclusions

The cell envelopes of the Crenarchaeota are often directly exposed to extreme environmental conditions and they cannot be stabilized by cellular factors, like chaperonins. Adaptation to the same type of extreme environment has not led to the evolution of similar cell surface structures; see, for example, the different cell surface architecture of *Pyrolobus fumarii* and *Ignicoccus pacificus* (both from the Pacific), or *Aquifex pyrophilus* (a hyperthermophilic bacterium) and *Ignicoccus hospitalis* (both from the Kolbeinsey Ridge north of Iceland), which all thrive in similarly hot, deep marine biotopes. On the contrary, an almost identical S-layer

architecture, as found for the various members of the Thermoproteales or of the Sulfolobales, does not reflect a similar biotope or lifestyle.

Differences in cell surface structures also exist for other Crenarchaeota. Therefore, they provide a range of models for elucidating survival strategies of extracellular biopolymers, and may give clues about molecular mechanisms of resistance against high temperature, high pressure, or extreme pH (Evrard et al. 1999; Eichler 2003).

In future studies, the analysis of the primary and secondary structure of the proteins will be greatly facilitated by the increased knowledge of full genomes which have now become publicly available. The genes of some archaeal S-layer proteins have been characterized (Akca et al. 2002; Bröckl et al. 1991; Claus et al. 2001, 2002, 2005; Lechner and Sumper 1987; Sumper et al. 1990; Yao et al. 1994), and complete genome sequences have been published for many of the species described in this chapter (Chen et al. 2005; Fitz-Gibbon et al. 2002; Kawarabayasi et al. 1999, 2001; She et al. 2001; Waters et al. 2003). To date, most genes involved in cell wall biosynthesis have not been unambigously defined. Knowledge of the complete genome may be helpful for identifying special enzymes involved in the biosynthesis and degradation of cell wall polymers.

For further analyses, improved tools for secondary structure predictions are now available (among many other sites, see e.g. http://bp.nuap.nagoya-u.ac.jp/sosui; http://www.ebi.ac.uk/Tools/; http://www.expasy.ch; see also Veith et al. 2009, under revision). The high-resolution structural analysis of the tertiary and quarternary structure is far more challenging. Considerable work needs to be done to overcome technical limitations and to reach atomic resolution, which has, for archaeal S-layers, not yet been achieved. Two routes can be envisaged, either electron crystallography (Glaeser et al. 2007) or X-ray crystallography (Drenth 2007). For bacterial S-layers, one exceptional study has reached this goal, although not with the native protein complex; a high resolution map of a truncated version of the *Geobacillus* S-layer protein SbsC was published recently (Pavkov et al. 2008).

Through studies of their cell surfaces, these investigations may lead to applications of new biomaterials. S-layers represent the most common cell surface structure of Crenarchaeota, can easily be isolated, and are the simplest biological membranes found in nature. A wide spectrum of applications for S-layers has emerged. Isolated S-layer subunits assemble into monomolecular crystalline arrays in suspension, on surfaces and interfaces. These lattices have functional groups on the surface in an identical position and orientation in the nanometer range. These characteristics have led to their application as ultrafiltration membranes, immobilization matrices for functional molecules, affinity microcarriers and biosensors, conjugate vaccines, carriers for Langmuir–Blodgett films, and reconstituted biological membranes and patterning elements in molecular nanotechnology (Sleytr et al. 1994).

In the past, applied studies have been performed almost exclusively with bacterial S-layers. Since crenarchaeal S-layer (glyco-)proteins are resistant to extreme conditions, a broad spectrum of future developments can be envisaged.

The resolution of the 3D structure will be necessary to obtain a better knowledge of the molecular stabilization mechanisms of hyperthermophilic S-layer proteins.

The first successful crystallizations indicate that this goal is indeed achievable (Claus et al. 2001; Debaerdemaeker et al. 2002; Evrard et al. 1999; Pavkov et al. 2008).

Acknowledgements Financial support by the Deutsche Forschungsgemeinschaft (Bonn) is gratefully acknowledged.

References

Akca E, Claus H, Schultz N, Karbach G, Schlott B, Debaerdemaeker T, Declercq JP, König H (2002) Genes and derived amino acid sequences of S-layer proteins from mesophilic, thermophilic and extremely thermophilic methanococci. Extremophiles 6:351–358

Baumeister W, Lembcke G (1992) Structural features of archaebacterial cell envelopes. J Bioenerg Biomembr 24:567–575

Baumeister W, Wildhaber I, Phipps BM (1989) Principles and organization in eubacterial and archaebacterial surface proteins. Can J Microbiol 35:215–227

Baumeister W, Santarius U, Volker S, Dürr R, Lemcke G, Engelhardt H (1990) The surface protein of *Hyperthermus butylicus*: three dimensional structure and comparison with other archaebacterial surface proteins. Syst Appl Microbiol 13:105–111

Baumeister W, Volker S, Santarius U (1991) The three-dimensional structure of the surface layer protein of *Acidianus brierleyi* determined by electron crystallography. Syst Appl Microbiol 14:103–110

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: signalP 3.0. J Mol Biol 16:783–795

Beveridge TJ, Graham LL (1991) Surface layers of bacteria. Microbiol Rev 55:684–705

Blöchl E, Rachel R, Burggraf S, Hafenbradl D, Jannasch HW, Stetter KO (1997) *Pyrolobus fumarii*, gen. and sp. nov. represents a novel group of *Archaea*, extending the upper temperature limit for life to 113°C. Extremophiles 1:14–21

Boot HJ, Pouwels PH (1996) Expression, secretion and antigenic variation of bacterial S-layer proteins. Mol Microbiol 21:1117–1123

Boucher Y (2007) Lipids: biosynthesis, function, and evolution. In: Cavicchioli R (ed) Archaea: molecular and cellular biology. ASM Press, Washington, USA, pp 341–353

Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P (2008) Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. Nature Rev Microbiol 6:245–252

Bröckl G, Behr M, Fabry S, Hensel R, Kaudewitz H, Biendl E, König H (1991) Analysis and nucleotide sequence of the genes encoding the surface-layer glycoproteins of the hyperthermophilic methanogens *Methanothermus fervidus* and *Methanothermus sociabilis*. Eur J Biochem 199:147–152

Burggraf S, Huber H, Stetter KO (1997) Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. Int J Syst Bacteriol 47:657–660

Burghardt T, Näther DJ, Junglas B, Huber H, Rachel R (2007) The dominating outer membrane protein of the hyperthermopilic Archaeum *Ignicoccus hospitalis*: a novel pore-forming complex. Mol Microbiol 63:166–176

Burghardt T, Saller M, Gürster S, Müller D, Meyer C, Jahn U, Hochmuth E, Deutzmann R, Siedler F, Babinger P, Wirth R, Huber H, Rachel R (2008) Insight into the proteome of the hyperthermophilic Crenarchaeon *Ignicoccus hospitalis*: the major cytosolic and membrane proteins. Arch Microbiol 190:379–394

Chen L, Brugger K, Skovgaard M, Redder P, She Q, Torarinsson E, Greve B, Awayez M, Zibat A, Klenk H-P, Garrett RA (2005) The genome of *Sulfolobus acidocaldarius*, a model organism of the Crenarchaeota. J Bacteriol 187:4992–4999

Claus H, Akca E, Schultz N, Karbach G, Schlott B, Debaerdemaeker T, Declercq JP, König H (2001) Surface (glyco-)proteins: primary structure and crystallization under microgravity conditions. In: Proccedings of the first European workshop on exo-/astro-biology, Frascati, 21–23 May 2001, ESA SP-496, August 2001; ISBN No 92-9092-806-9, pp 806–809

Claus H, Akca E, Debaerdemaeker T, Evrard C, Declercq JP, König H (2002) Primary structure of selected archaeal mesophilic and extremely thermophilic outer surface layer proteins. Syst Appl Microbiol 25:3–12

Claus H, Akca E, Debaerdemaeker T, Evrard C, Declercq JP, Harris JR, Schlott B, König H (2005) Molecular organization of selected prokaryotic S-layer proteins. Can J Microbiol 51:731–743

Deatherage JF, Taylor KA, Amos LA (1983) Three-dimensional arrangement of the cell wall protein of *Sulfolobus acidocaldarius*. J Mol Biol 167:823–852

Debaerdemaeker T, Evrard C, Declercq JP, Claus H, Akca E, König H (2002) The first crystallization of the outer surface (S-layer) glycoprotein of the mesophilic bacterium *Bacillus sphaericus* and the hyperthermophilic archaeon *Methanothermus fervidus*. In: Proccedings of the 2nd European workshop on exo/astro-biology, Graz, Austria, 16–19 Sept 2002, ESA SP-518, November 2002; ISBN No 92-9092-828-X, pp 411–412

Drenth J (2007) Principles of protein X-ray crystallography, 3rd edn. Springer, New York

Dürr R, Hegerl R, Volker S, Santarius U, Baumeister W (1991) Three-dimensional reconstruction of the surface protein of *Pyrodictium brockii*: comparing two image processing strategies. J Struct Biol 106:181–190

Eichler J (2001) Post-translational modification of the S-layer glycoprotein occurs following translocation across the plasma membrane of the haloarchaeon *Haloferax volcanii*. Eur J Biochem 268:4366–4373

Eichler J (2003) Facing extremes: archaeal surface-layer (glyco)proteins. Microbiology 149:3347–3351

Elkins JG, Podar M, Graham DE, Makarova KS et al (2008) A korarchaeal genome reveals insights into the evolution of the Archaea. PNAS 105:8102–8107

Evrard C, Declercq JP, Debaerdemaeker T, König H (1999) The first successful crystallization of a prokaryotic extremely thermophilic outer surface layer glycoprotein. Z Kristallogr 214:427–429

Fernandez LA, Berenguer J (2000) Secretion and assembly of regular surface structures in gram-negative bacteria. FEMS Microbiol Rev 24:21–44

Fitz-Gibbon ST, Ladner H, Kim U-J, Stetter KO, Simon MI, Miller JH (2002) Genome sequence of the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. Proc Natl Acad Sci USA 99:984–989

Fröls S, Gordon PMK, Panlilio MA, Duggin IG, Bell SD, Sensen CW, Schleper C (2007) Response of the hyperthermophilic archaeon Sulfolobus solfataricus to UV damage. J Bacteriol 189:8708–8718

Fuchs T, Huber H, Teiner K, Burggraf S, Stetter KO (1995) *Metallosphaera prunae*, sp.nov., a novel metal-moblizing, thermoacidophilic archaeum, isolated from a uranium mine in Germany. Syst Appl Microbiol 18:560–566

Glaeser RM, Downing K, DeRosier D (2007) Electron crystallography of biological macromolecules. Oxford University Press, New York

Gogarten JP, Kibak H, Dittrich P, Taiz L, Bowman EJ, Bowman BJ, Manoson MF, Poole RJ, Date T, Oshima T, Konishi J, Denda K, Yoshida M (1989) Evolution of the vacuolar H^+-ATPase: implications for the origin of eukaryotes. Proc Natl Acad Sci USA 86:6661–6665

Grogan DW (1996) Organization and interactions of cell envelope proteins of the extreme thermoacidophile *Sulfolobus acidocaldarius*. Can J Microbiol 42:1163–1171

Hallam SJ, Konstantinidis KT, Putnam N, Schleper C, Watanabe Y, Sugahara J, Preston C, de la Torre J, Richardson PM, DeLong EF (2006) Genomic analysis of the uncultivated marine crenarchaeote *Cenarchaeum symbiosum*. Proc Natl Acad Sci USA 103:18296–18301

Hatzenpichler R, Lebedeva EV, Spieck E, Stoecker K, Richter A, Daims H, Wagner M (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proc Natl Acad Sci USA 105:2134–2139

Hegerl R, Baumeister W (1988) Correlation averaging of a badly distorted lattice: the surface protein of *Pyrodictium occultum*. J Electron Microsc Tech 9:413–419

Horn C, Paulmann B, Kerlen B, Junker N, Huber H (1999) In vivo observation of cell division of anaerobic hyperthermophiles by using a high-intensity dark-field microscope. J Bacteriol 181:5114–5118

Houwink AL (1956) Flagella, gas vacuoles and cell-wall structure in *Halobacterium halobium*; an electron microscope study. J Gen Microbiol 15:146–150

Huber H (2006) Crenarchaeota. Encyclopedia of life sciences. Wiley, New York, p 1–11. doi: 10.1038/npg.els.0004242

Huber H, Hohn MJ, Rachel R, Fuchs T, Wimmer VC, Stetter KO (2002) A new phylum of *Archaea* represented by a nanosized hyperthermophilic symbiont. Nature 417:63–67

Huber H, Hohn MJ, Stetter KO, Rachel R (2003) The phylum *Nanoarchaeota*: present knowledge and future perspectives of a unique form of life. Res Microbiol 154:165–171

Iwabe N, Kuma K-I, Hasegawa M, Osawa S, Miyata T (1989) Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. Proc Natl Acad Sci USA 86:9355–9359

Jahn U, Summons R, Sturt H, Grossjean E, Huber H (2004) Composition and source of the lipids of *Nanoarchaeum equitans* and their origin in the cytoplasmic membrane of its host *Ignicoccus* sp. KIN4I. Arch Microbiol 182:404–413

Junglas B, Briegel A, Burghardt T, Walther P, Huber H, Rachel R (2008) *Ignicoccus hospitalis* and *Nanoarchaeum equitans*: Ultrastructure, cell–cell interaction, and 3D reconstruction from serial sections of freeze-substituted cells and by electron cryotomography. Arch Microbiol 190:395–408

Kandler O, Hippe H (1977) Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Arch Microbiol 113:57–60

Kandler O, König H (1978) Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch Microbiol 118:141–152

Kandler O, König H (1993) Cell envelopes of archaea: structure and chemistry. In: Kates M et al (eds) The biochemistry of *Archaea* (Archaebacteria). Elsevier, Amsterdam, pp 223–259

Kärcher U, Schröder H, Haslinger E, Allmeier G, Schreiner R, Wieland F, Haselbeck A, König H (1993) Primary structure of the heterosaccharide of the surface glycoprotein of *Methanothermus fervidus*. J Biol Chem 268:26821–26826

Kawarabayasi Y, Hino Y, Horikawa H, Yamazaki S, Haikawa Y, Jin-no K, Takahashi M, Sekine M, Baba S, Ankai A et al (1999) Complete genome sequence of an aerobic hyperthermophilic crenarchaeon, *Aeropyrum pernix* K1. DNA Res 6:83–101

Kawarabayasi Y, Hino Y, Horikawa H, Jin-no K, Takahashi M, Sekine M, Baba S, Ankai A, Kosugi H, Hosoyama A, Fukui S, Nagai Y, Nishijima K, Otsuka R, Nakazawa H, Takamiya M, Kato Y, Yoshizawa T, Tanaka T, Kudoh Y, Yamazaki J, Kushida N, Oguchi A, Aoki K, Masuda S, Yanagii M, Nishimura M, Yamagishi A, Oshima T, Kikuchi H (2001) Complete genome sequence of an aerobic thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain7. DNA Res 8:123–140

Kikuchi A, Sagami H, Ogura K (1999) Evidence for the covalent attachment of diphytanylglycerol phosphate to the cell-surface glycoprotein of *Halobacterium halobium*. J Biol Chem 274:18011–18016

König H, Stetter KO (1986) Studies on archaebacterial S-layers. Syst Appl Microbiol 7:300–309

König H, Messner P, Stetter KO (1988) The fine structure of the fibers of *Pyrodictium occultum*. FEMS Microbiol Lett 49:207–212

König H, Rachel R, Claus H (2007) Proteinaceous surface layers of archaea: ultrastructure and biochemistry. In: Cavicchioli R (ed) Archaea: molecular and cellular biology. ASM Press, Washington, USA, pp 315–340

Könnecke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437:543–546

Konrad Z, Eichler J (2002) Lipid modification of proteins in *Archaea*: attachment of a mevalonic acid-based lipid moiety to the S-layer glycoprotein of *Haloferax volcanii* follows protein translocation. Biochem J 366:959–964

Lechner J, Sumper M (1987) The primary structure of a procaryotic glycoprotein. Cloning and sequencing of the cell surface glycoprotein gene of halobacteria. J Biol Chem 262:9724–9729

Lechner J, Wieland F (1989) Structure and biosynthesis of prokaryotic glycoproteins. Annu Rev Biochem 58:173–194

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442: 806–809

Lembcke G, Dürr R, Hegerl R, Baumeister W (1990) Image analysis and processing of an imperfect two-dimensional crystal. The surface layer of *Sulfolobus acidocaldarius* reinvestigated. J Microsc 161:263–278

Lembcke G, Baumeister W, Beckmann E, Zemlin F (1993) Cryo-electron microscopy of the surface protein of *Sulfolobus shibatae*. Ultramicroscopy 49:397–406

Ludwig W, Strunk O, Westram R, Richter L et al (2004) ARB: a software environment for sequence data. Nucleic Acid Res 32:1363–1371

Mayr J, Lupas A, Kellermann J, Eckerskorn C, Baumeister W, Peters J (1996) A hyperthermostable protease of the subtilisin family bound to the surface layer of the archaeon *Staphylothermus marinus*. Curr Biol 6:739–749

Mescher MF, Strominger JL (1976a) Structural (shape-maintaining) role of the cell surface glycoprotein of *Halobacterium salinarium*. Proc Natl Acad Sci USA 73:2687–2691

Mescher MF, Strominger JL (1976b) Purification characterisation of a prokaryotic glycoprotein from the cell envelope of *Halobacterium salinarium*. J Biol Chem 251:2005–2014

Messner P, Schäffer C (2003) Prokaryotic glycoproteins. In: Herz H, Falk H, Kirby GW (eds) Progress in the chemistry of organic natural products. Springer, Heidelberg, pp 51–124

Messner P, Sleytr UB (1992) Crystalline bacterial cell-surface layers. Adv Microbial Physiol 33:213–274

Messner P, Pum D, Sara M, Stetter KO, Sleytr UB (1986) Ultrastructure of the cell envelope of the Archaebacteria *Thermoproteus tenax* und *Thermoproteus neutrophilus*. J Bacteriol 166: 1046–1054

Michel H, Neugebauer D-C, Oesterhelt D (1980) The 2-d crystalline cell wall of *Sulfolobus acidocaldarius*: Structure, solubilization, and reassembly. In: Baumeister W, VogelI W (eds) Electron microscopy at molecular dimensions. Springer, Berlin, pp 27–35

Näther DJ, Rachel R (2004) The outer membrane of the hyperthermophilic archaeon *Ignicoccus*: dynamics, ultrastructure and composition. Biochem Soc Trans 32:199–203

Nickell S, Hegerl R, Baumeister W, Rachel R (2003) *Pyrodictium* cannulae enter the periplasmic space but do not enter the cytoplasm, as revealed by cryo-electron tomography. J Struct Biol 141:34–42

Niederberger TD, Götz DK, McDonald IR, Ronimus RS, Morgan HW (2006) *Ignisphaera aggregans* gen. nov., sp. nov., a novel hyperthermophilic crenarchaeote isolated from hot springs in Rotorua and Tokaanu, New Zealand. Int J Syst Evol Microbiol 56:965–971

Nußer E, Hartmann E, Allmeier H, König H, Paul G, Stetter KO (1988) A glycoprotein surface layer covers the pseudomurein sacculus of the extreme thermophile *Methanothermus fervidus*. In: Sleytr UB, Messner P, Pum D, Sàra M (eds) Crystalline bacterial cell surface layers. Springer, Berlin, pp 21–25

Pavkov T, Egelseer EM, Tesarz M, Svergun DI, Sleytr UB, Keller W (2008) The structure and binding behavior of the bacterial cell surface layer protein SbsC. Structure 16:1226–1237

Peters J, Nitsch M, Kühlmorgen B, Golbik R, Lupas A, Kellermann J, Engelhardt H, Pfander JP, Müller S, Goldie K, Engel A, Stetter KO, Baumeister W (1995) Tetrabrachion: a filamentous archaebacterial surface protein assembly of unusual structure and extreme stability. J Mol Biol 245:385–401

Peters J, Baumeister W, Lupas A (1996) Hyperthermostable surface layer protein tetrabrachion from the archaebacterium *Staphylothermus marinus*: evidence for the presence of a right-handed coiled coil derived from the primary structure. J Mol Biol 257:1031–1041

Phipp BM, Huber R, Baumeister W (1991) The cell envelope of the hyperthermophilic archaebacterium *Pyrobaculum organotrophum* consists of two regularly arrayed protein layers: three-dimensional structure of the outer layer. Mol Microbiol 5:253–265

Phipps BM, Engelhardt H, Huber R, Baumeister W (1990) Three-dimensional structure of the crystalline protein envelope layer of the hyperthermophilic archaebacterium *Pyrobaculum islandicum*. J Struct Biol 103:152–163

Preston CM, Wu KY, Molinski TF, DeLong EF (1996) A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. Proc Natl Acad Sci USA 93:6241–6246

Prüschenk R, Baumeister W (1987) Three-dimensional structure of the surface protein of *Sulfolobus solfataricus*. Eur J Cell Biol 45:185–191

Rachel R, Wyschkony I, Riehl S, Huber H (2002) The ultrastructure of *Ignicoccus*: evidence for a novel outer membrane and for intracellular vesicle budding in an archaeon. Archaea 1:9–18

Rieger G, Rachel R, Hermann R, Stetter KO (1995) Ultrastructure of the hyperthermophilic archaeon *Pyrodictium abyssi*. J Struct Biol 115:78–87

Sára M, Sleytr UB (2000) S-layer proteins. J Bacteriol 182:859–868

Schleper C, Jurgens G, Januscheit M (2005) Genomic studies of uncultivated Archaea. Nat Rev Microbiol 3:479–488

She Q, Singh RK, Confalonieri F, Zivanovic Y, Allard G, Awayez MJ, Chan-Weiher CC, Clausen IG, Curtis BA, De Moors A, Erauso G, Fletcher C, Gordon PM, Heikamp-de Jong I, Jeffries AC, Kozera CJ, Medina N, Peng X, Thi-Ngoc HP, Redder P, Schenk ME, Theriault C, Tolstrup N, Charlebois RL, Doolittle WF, Duguet M, Gaasterland T, Garrett RA, Ragan MA, Sensen CW, Van der Oost J (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. Proc Natl Acad Sci USA 98:7835–7840

Sleytr UB, Sára M, Messner P, Pum D (1994) Two-dimensional protein crystals (S-layers): fundamentals and applications. J Cell Biochem 56:171–176

Snel B, Huynen MA, Dutilh BE (2005) Genome trees and the nature of genome evolution. Annu Rev Microbiol 59:191–209

Sumper M, Wieland FT (1995) Bacterial glycoproteins. In: Montreuil J, Vliegenthart JFG, Schachter H (eds) Glycoproteins. Elsevier, Amsterdam, pp 455–473

Sumper M, Berg E, Mengele R, Strobel I (1990) Primary structure and glycosylation of the S-layer protein of *Haloferax volcanii*. J Bacteriol 172:7111–7118

Taylor KA, Deatherage JF, Amos LA (1982) Structure of the S-layer of *Sulfolobus acidocaldarius*. Nature 299:840–842

Veith A, Klingl A, Zolghadr B, Lauber K, Mentele R, Lottspeich F, Rachel R, Albers S-V, Kletzin A (2009) *Acidianus*, *Sulfolobus* and *Metallosphaera* surface layers: structure, composition and gene expression. Mol Microbiol 73(1):58–72

Völkl P, Huber R, Drobner E, Rachel R, Burggraf S, Trincone A, Stetter KO (1993) *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic Archaeum. Appl Environ Microbiol 59:2918–2926

Waters E, Hohn MJ, Ahel I, Graham DE, Adams MB, Barnstead M, Beeson KY, Bibbs L, Bolanos R, Keller M, Kretz K, Lin X, Mathur E, Ni J, Podar M, Richardson TH, Sutton S, Simon M, Soll D, Stetter KO, Short JM, Noorderwier M (2003) The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. Proc Natl Acad Sci USA 100:12984–12988

Weiss LR (1973) Attachment of bacteria to sulphur in extreme environments. J Gen Microbiol 77:501–507

Weiss LR (1974) Subunit cell wall of *Sulfolobus acidocaldarius*. J Bacteriol 118:275–284

Wildhaber I, Baumeister W (1987) The cell envelope of *Thermoproteus tenax*: three-dimensional structure of the surface layer and its role in shape maintenance. EMBO J 6:1475–1480

Wildhaber I, Santarius U, Baumeister W (1987) Three-dimensional structure of the surface protein of *Desulfurococcus mobilis*. J Bacteriol 169:5563–5568

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Yao R, Macario AJ, Conway de Macario E (1994) An archaeal S-layer gene homolog with repetitive units. Biochim Biophys Acta 1219:697–700

Zillig W, Stetter KO, Schäfer W, Janékovic D, Wunderl S, Holz I, Palm P (1981) Thermoproteales: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras. Zentralbl Bakteriol Mikrobiol Hyg C 2:205–227

Zillig W, Stetter KO, Prangishvilli D, Schäfer W, Wunderl S, Janékovic D, Holz I, Palm P (1982) *Desulfurococcaceae*, the second family of the extremely thermophilic, anaerobic, sulfur respiring Thermoproteales. Zentralbl Bakteriol Mikrobiol Hyg C 3:304–317

Part III
Biological Activities

Chapter 10
Immunochemistry of the Cell Walls of Methanogenic Archaea: A View from the Past into the Future

Everly Conway de Macario and Alberto J.L. Macario

10.1 Introduction

Cells walls occur in archaea (Kandler and König 1978; Kandler 1981, 1982), bacteria (Buynak 2007; Lange et al. 2007), fungi (Coronado et al. 2007; Funkhouser and Aronson 2007), algae (Domozych et al. 2007; Kodama and Fujishima 2007), plants (Dvorakova et al. 2007; Pauly et al 1999), and some protists (Harold 2002; Milligan and Morel 2002). The composition and structure of the archaeal cell walls varies, depending on taxonomic group (Claus et al. 2002, 2005; Firtel et al. 1993; Hartmann and König 1990; Kandler 1981, 1982; Kandler and König 1978, 1985, 1998; König et al. 1982, 1983; König 1986). The major common characteristic of these cell walls is that they are made of polymers such as pseudopeptidoglycan, polysaccharides, or S-layers (Claus et al. 2005; Kandler 1982; Kandler and König 1985, 1998; König et al. 1982; König 1986; Messner et al. 1997). The latter are composed of proteins with sugars, i.e., they are mostly built of glycoproteins, in which the sugars are in various proportions, depending on the organism (Claus et al. 2002; Firtel et al. 1993; Messner et al. 1997).

Cell walls are studied by numerous means, including immunological methods (Bryniok and Trösch 1989; Conway de Macario and Macario 1986; Macario and Conway de Macario 1985a; Southam and Beveridge 1991). These methods allow identification of immunotypes, classification of phenotypes, manipulation of microbial populations, and many other studies in basic and applied fields (Conway de Macario and Macario 1986; Macario and Conway de Macario 1985a, b, c). The advent of monoclonal antibodies provided powerful tools for immunochemical dissection of the cell walls of a variety of organisms belonging to the three evolutionary domains, Bacteria, Archaea, and Eukaryota. In turn, cell wall immunochemistry

E. Conway de Macario (✉)
Center of Marine Biotechnology, University of Maryland Biotechnology Institute (UMBI), Columbus Center, 701 E, Pratt Street, Baltimore, MD 21202, USA
e-mail: macarioe@umbi.umd.edu

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_10,

provides basic information on the antigenic composition of any given organism, and on the immunochemical structure of any given antigenic determinant (also named epitope). The information obtained completes and expands data from chemical and biochemical analyses, and thus provides a solid basis to develop strategies and methods for identification, classification, modification, and manipulation of microbes. For example, identification of archaeal immunotypes will allow a correlation between them and other parameters of biotechnological or clinical importance, such as functional performance in a bioreactor or, pertinent to clinical sciences, participation in pathogenicity (see Conclusion). For these and other applications, antibodies have no equal and are not only indispensable but also irreplaceable. An antibody is most useful and efficient when it is well characterized, i.e., its specificity spectrum, molecular specificity, and binding affinity are defined. Specificity spectrum refers to the range of different species/strains with which the antibody reacts after proper calibration. The molecular specificity is defined by the molecules of known structure that are recognized by the antibody and that tell the composition/structure of the epitope recognized by the antigen-binding site of the antibody. The strength of the interaction between the antibody and the epitope(s) it recognizes defines its binding affinity.

In this Chapter, we shall discuss our studies on the cell wall immunochemistry of various methanogens performed with panels of calibrated monoclonal antibodies, correlate the findings with what is known about the chemical make-up of the archaeal cell walls, and draw conclusions pertaining to future developments within basic and applied fields.

10.2 Antigenic Mosaic of Methanogens Elucidated by Monoclonal Antibodies

The following is an example of how the use of antibodies contributes to the elucidation of antigenic mosaics. A panel of monoclonal antibodies was generated against methanogens of four genera from the families Methanobacteriaceae, Methanococcaceae, and Methanomicrobiaceae (Conway de Macario et al. 1982). Their specificity spectra were determined and those recognizing only the immunizing organisms were identified. In addition, the immunochemical specificities of four of these monospecific antibodies were determined, each made against a representative species of the four genera, *Methanobacterium thermoautotrophicum* Delta H (now designated *Methanothermobacter thermautotrophicum* Delta H), *Methanobrevibacter arboriphilus* DH1, *Methanococcus vannielii* SB, and *Methanospirillum hungatei* JF1 (for current information on the nomenclature and classification of methanogens visit http://www.the-icsp.org/subcoms/Methanogens.htm#taxa). Immunochemical specificities were determined by inhibition-blocking experiments with compounds of known structure. The antibodies against the two species from the Methanobacteriaceae, which have pseudomurein in their cell walls (Kandler 1982;

Kandler and König 1978, 1985, 1998; König et al. 1982, 1983), were inhibited by compounds representing pseudomurein components. In contrast, these compounds were not recognized by the other antibodies made against organisms from the Methanococaceae and Methanospirillaceae, which do not have pseudomurein (Claus et al. 2002; Firtel et al. 1993; Kandler 1982; Kandler and König 1978, 1985, 1998; Messner et al. 1997; Southam and Beveridge 1991). Detailed descriptions of the antigenic mosaics of methanogens follows.

10.3 Antigenic Mosaic of *Methanothermobacter thermoautotrophicus*

The antigenic mosaic of *Methanothermobacter thermautotrophicum* Delta H (previously known as *Methanobacterium thermoautotrophicum* Delta H; http://www.the-icsp.org/subcoms/Methanogens.htm#taxa) was analyzed with a panel of six monoclonal antibodies of which three (4A, 4B, and 4C) reacted with the immunizing organism, Delta H, and with the crossreactive strain GC1; one (4D) reacted only with GC1, and two (4E and 4F) reacted only with Delta H, i.e., the immunizing organism (Conway de Macario et al. 1983). They were calibrated as shown in Fig. 10.1, using as antigen the strain Delta H, or the strain GC1 in the case of

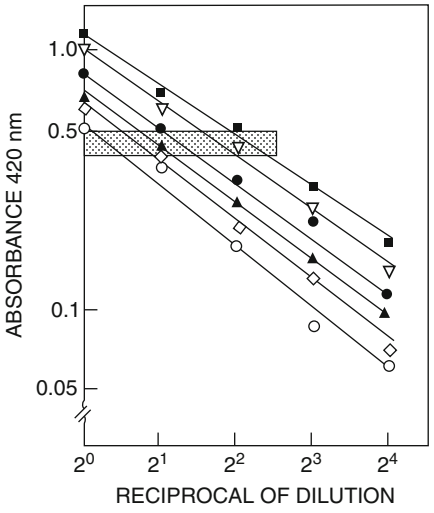

Fig. 10.1 Monoclonal antibody calibration. A series of twofold dilutions of each antibody was assayed against the immunizing strain Delta H, or the crossreactive strain GC1 in the case of antibody 4D. The *shaded box* indicates the range of calibration within which all antibodies were used for the comparative analyses of their specificity spectra and molecular specificities. Anbiody 4A, *closed circles*; 4B, *closed triangles*; 4C, *open circles*; 4E, *squares*; 4F, *open triangles*. Details and methods are provided in the text and in the original reference (Proc Natl Acad Sci USA 80: 6346–6350, 1983) from which the figure was reproduced with permission from the copyright owner

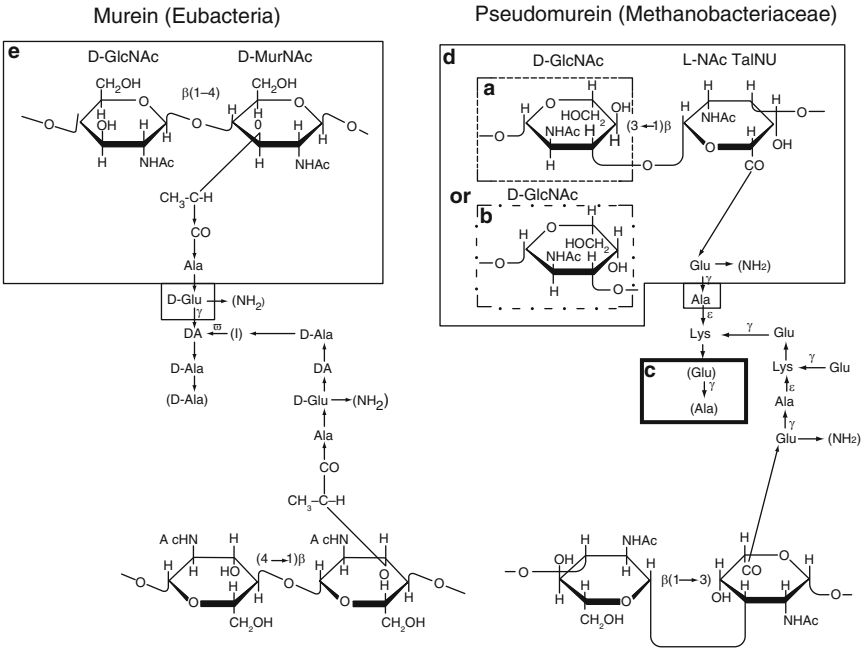

Fig. 10.2 Primary structure of murein (*left*) and pseudomurein (*right*). The boxed structures were used in the inhibition-blocking experiments. (**a**) GlcNAc; (**b**) GalNAc; (**c**) gamma-Glu–Ala; (**d**) pseudomurein glycan; and (**e**) murein glycan. Details and methods are provided in the text and in the original reference (Proc Nac Acad Sci USA 80:6346–6350, 1983) from which the figure was reproduced with permission from the copyright owner

antibody 4D. The immunochemical specificity of these six antibodies was determined by inhibition-blocking assays, using a series of compounds of known structure, some of which are shown in Fig. 10.2. An example of inhibition-blocking results is presented in Fig. 10.3, and the results for all antibodies and compounds are shown in Fig. 10.4. Five distinct epitopes could be identified on the Delta H cell wall. GlcNAc, GalNAc, and γ-Glu–Ala are involved in the make-up of three of these five determinants. Since the acetylated and non-acetylated forms of the analogs were inhibitory, it is likely that the hydroxyl groups of carbons 3 and 4 of the amino sugar are part of two of these three determinants while the carboxyl terminus of the peptide is involved in the make-up of the third epitope. A fourth determinant includes TalNu and the fifth epitope does not include any of the amino sugars tested or γ-Glu–Ala.

Antibody 4A recognized on strain Delta H a determinant containing GlcNAc, which is present also on strain GC1. However, the determinant is not exactly the same on Delta H and GC1 in terms of antigenicity with regard to the assays performed, although structurally it could be the same. The different antigenicity of the Delta H epitope as compared with the GC1 counterpart could be due to

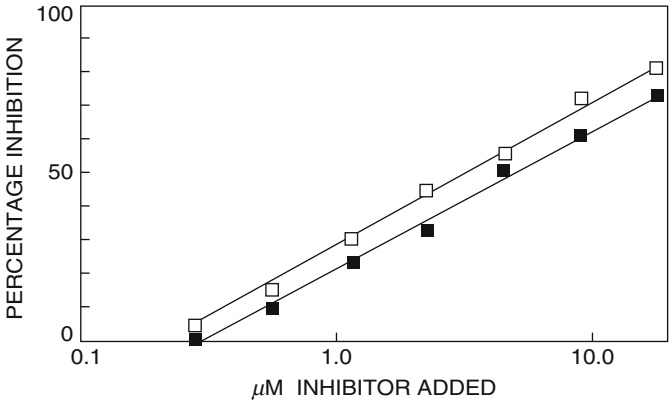

Fig. 10.3 Example of quantitative inhibition–blocking experiment. A series of samples from a calibrated aliquot of antibody 4B was incubated with an array of doses of NAc–γ-Glu–Ala and centrifuged. The supernatants were assayed with the immunizing organism, Delta H (*open squares*) or the crossreactive strain GC1 (*closed squares*). Details and methods are provided in the text and in the original reference (Proc Natl Acad Sci USA 80:6346–6350, 1983) from which the figure was reproduced with permission from the copyright owner

differences in their topological availability on the cell surface rather than to structural differences.

Antibodies 4B and 4E identified a determinant containing NAc–γ-Glu–Ala. The data do not elucidate whether the antibodies recognize different portions of one determinant or different determinants altogether. Antibody 4B reacted with Delta H and GC1, while antibody 4E reacted only with Delta H, but it was not determined whether both antibodies recognize the same determinant on Delta H.

Antibody 4C identified a determinant that contains GalNAc, on Delta H and GC1, and also in *Methanobacterium formicicum* MF, which is known to have pseudomurein in its cell wall like *Methanothermobacter thermoautrophicum* (Kandler 1982; Kandler and König 1978, 1985, 1998; König et al. 1983). However, the determinant on each of these organisms is likely not the same. The data indicate a greater similarity between the determinants on strains GC1 and MF than between the determinants on these two strains and that on Delta H. The determinant recognized by antibody 4C is probably strongly immunogenic as suggested by the chemical composition of the Delta H glycan strands and the inhibition-blocking results. The strong immunogenicity of the epitope seems to occur in spite of its relative low concentration in the cell wall of the immunizing organism, i.e., Delta H that was used to produce antibody 4C. This observation, and its occurrence in all three methanogens investigated, indicate that this epitope is widely distributed in nature and may have key biologic-evolutionary roles.

Antibody 4D identified a determinant that, although exposed on GC1, is masked on the immunizing strain, Delta H. This determinant does not include any of the compounds tested, at least not as immunodominant component.

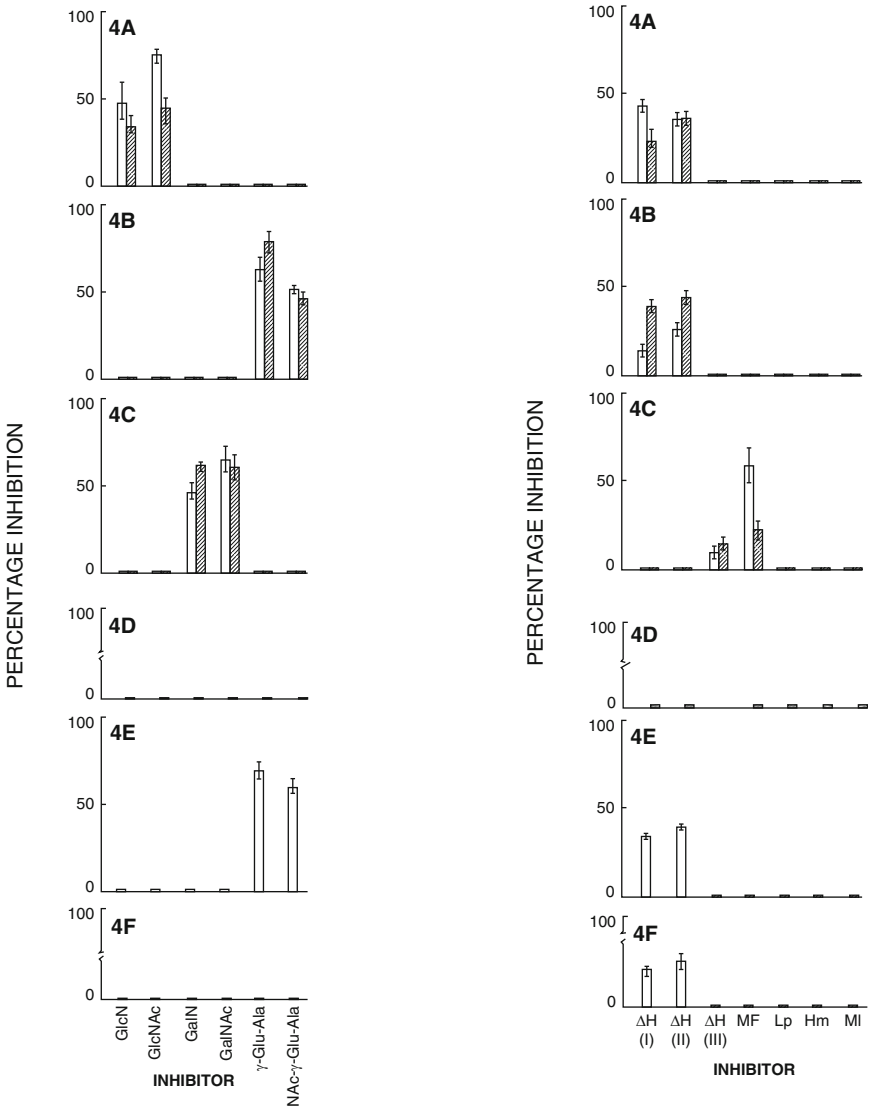

Fig. 10.4 Molecular specificity patterns of monoclonal antibodies 4A–4F. *Left panel*: Six inhibitors were used, and each bar represents the percentage of inhibition of the binding of the antibody to the immunizing strain Delta H (*open bars*) or to the crossreactive strain GC1 (*shaded bars*). Mean ± range; $n = 3$–6. *Right panel*: The inhibitors were cell wall glycan strand preparations from Delta H (Δ*H*), *Methanobacterium formicicum* MF (MF), *Lactobacillus plantarum* (Lp), *Micrococcus luteus* (Ml), and *Halococcus morrhuae* (Hm). *Open and shaded bars* are as in the left panel. Mean ± range; $n = 2$–3. Details and methods are provided in the text and in the original reference (Proc Natl Acad Sci USA 80:6346–6350, 1983) from which the figure was reproduced with permission from the copyright owner

Antibody 4F recognized a determinant including TalNU, a structure that occurs only in the peptidoglycan of the Methanobacteriaceae (Kandler 1982; Kandler and König 1978, 1985, 1998; König et al. 1982, 1983) and which, according to the results provided by antibody 4f, is immunogenic.

The data suggest that the epitopes identified by antibodies 4E and 4F are strain Delta H specific; the epitopes identified by antibodies 4A, 4B, and 4D are species (*M. thermautotrophicum*) specific; and the determinant identified by antibody 4C is a common antigen.

10.4 Antigenic Mosaic of *Methanococcus vannielii*

A panel of six monoclonal antibodies was calibrated and used to study the antigenic mosaic of *Methanococcus vannielii* strain SB (Conway de Macario et al. 1984). *M. vannielii* is a representative of the family Methanococcaceae (http://www.the-icsp.org/subcoms/Methanogens.htm#taxa), whose members do not have pseudomurein in their cell walls (Claus et al. 2002; Kandler 1982; Kandler and König 1978, 1985, 1998; König 1986; Messner et al. 1997). These antibodies were made against *M. vannielii* SB, the immunizing strain, and four (5C–5F) were found to react only with SB, while the other two (5A and 5B) reacted with strain SB and the crossreactive *M. voltae* PSv. Inhibition-blocking experiments with a set of compounds of known structure or composition revealed six distinct epitopes, each identified by one antibody in the panel. No sugars could be detected in the make-up of these determinants, which confirmed pre-existing biochemical data showing that *M. vannielii* does not have sugars in its envelope, or if it has them, they are present in very minimal amounts (Claus et al. 2002; Kandler 1982; Kandler and König 1978, 1985, 1998; Messner et al. 1997).

To elucidate further the antigenic mosaic of *M. vannielii* SB, proteins from the S-layer isolated from this organism and separated by electrophoresis were tested with two of the antibodies, 5D and 5E. Six distinct protein bands were resolved by SDS-PAGE. While the epitope recognized by antibody 5E was present in all the protein bands, the epitope recognized by 5D was present only in five of the six proteins. In conclusion, the epitopes recognized by the antibodies are in the S-layer, are proteinic in nature, and are exposed to the outside of the cells as demonstrated by direct binding assays with whole cells fixed and unfixed, and by immunoelectron microscopy.

10.5 Antigenic Mosaic of *Methanogenium* Species

The antigenic mosaic of *Methanogenium* species was studied with a panel of calibrated polyclonal antibodies and with two monoclonal antibodies made against *M. cariaci* JR1(Macario et al. 1987). Two determinants were identified with the monoclonal antibodies (Fig. 10.5). Antibody 7A was consistently inhibited–

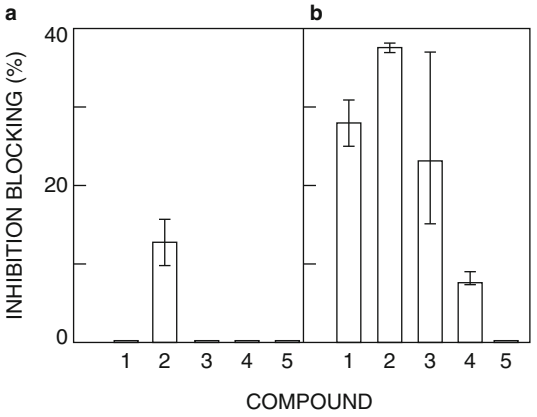

Fig. 10.5 Molecular specificity of monoclonal antibodies 7A (**a**) and 7B (**b**) made against *Methanogenium cariaci* JR1. Inhibition-blocking assays were carried out with the following compounds: (1) glycyl-L-glutamic acid; (2) L-alanyl-glycyl-L-seryl-L-glutamic acid; (3) L-glutamyl-L-alanyl-L-glutamyl-L-asparagine; (4) L-alanyl-L-glutamic acid; and (5) others (all were non-inhibitory when tested separately in independent assays) listed in the original reference. Bars represent arithmetic mean ± range ($n = 3$–5). Details and methods are provided in the text and in the original reference (J Bacteriol 169:666–669, 1987) from which the figure was reproduced with permission from the copyright owner

blocked only by L-allanyl-glycyl-L-seryl-L-glutamic acid. In contrast, antibody 7B was inhibited–blocked by this compound and also by others, but none were sugars (Fig. 10.5). These results also corroborate that the S-layer of *Methanogenium* species (Claus et al. 2002; Kandler 1982; Kandler and König 1978, 1985, 1998; Messner et al. 1997) does not contain carbohydrates, or if it does contain sugars they must be present in very minimal quantities and/or they are very poor immunogens. The data also demonstrate that the epitopes recognized by antibodies 7A and 7B are proteinic in nature. The two determinants were found expressed on the surface of *M. cariaci* JR1 as demonstrated by indirect immunofluorescence with the monoclonal antibodies. The whole array of determinants identified on *Methanogenium* species with the poly- and monoclonal antibody probes is displayed in Table 10.1.

The molecular specificity of antibody 7A was further investigated to identify the protein components in the S-layer of *M. cariaci* JR1 that reacted with it (Conway de Macario et al. 1986). The results are shown in Fig. 10.6 and Table 10.2. The epitope recognized by antibody 7A was present in all five protein components resolved by SDS-PAGE of S-layer from *M. cariaci* JR1. Nevertheless, 7A did not react with any other organism, even those known to be antigenically related as shown by calibrated polyclonal antibody probes, emphasizing the remarkable monospecificity of the antibody as regards a particular epitope. The data also dramatically illustrate the fact that a monoclonal antibody can recognize more than one protein even if it is very specific for a single epitope; all proteins, no matter how different overall from one another, will react with the antibody as long as they all share the epitope

Table 10.1 Antigenic mosaic of *Methanogenium* species

Organism	Determinant[a]					
	7a	7b	I	II	III	IV
M. cariaci JR1	+	+	+	−	+	+
M. marisnigri JR1	−	−	+	+	+	+
M. thermophilicum CR-1	−	−	+	+	−	+
M. thermophilicum Ratisbona	−	−	−	−	−	+
M. tatti DSM 2702	−	−	−	−	−	+
M. bourgense MS2	−	−	−	−	−	+
M. aggregans MSt	−	−	−	−	−	+
H2-oxidizing	−	−	−	−	−	− (+)[b]
Methanospirillum hungatei JF1	−	−	−	−	+	−
Methanomicrobium mobile BP	−	−	+	−	−	+

[a]7a and 7b were identified by monoclonal antibodies 7A and 7B, respectively (see text and original reference). I, II, III, and IV, were identified by polyclonal antibody probes for *M. cariaci* JR1, *M. marisnigri* JR1, *Methanospirillum hungatei* JF1, and *Methanomicrobium mobile* BP, respectively. +, positive reaction with the probe identifying the determinant; –, no reaction. *M. tatti* was *Methanofollis tationis*. Details and methods are provided in the text and in the original reference (Macario et al. 1987) from which the data were reproduced with permission from the copyright owner

[b]Positive reaction with the R probe for *M. mobile* BP

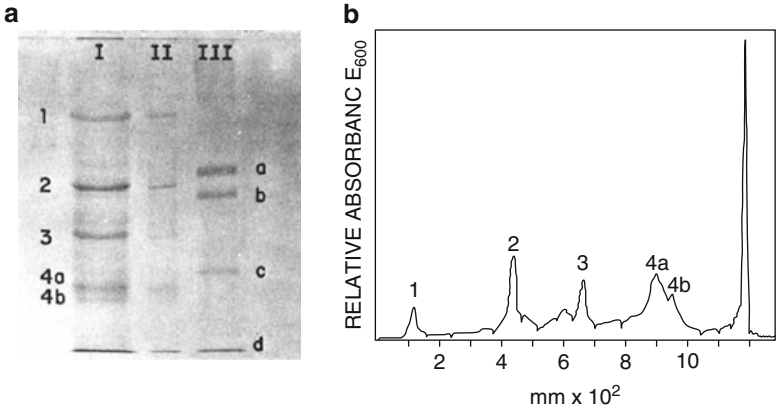

Fig. 10.6 Separation of S-layer proteins from *Methanogenium cariaci* JR1. *Left panel*, **a**. SDS-PAGE, lane I, crude S-layer preparation; lane II, same as lane I but diluted 10 fold; lane III, MW standards: albumin, 67 kDa (**a**); catalase, 60 kDa (**b**); lactate dehydrogenase, 36 kDa (**c**); and ferritin subunit, 18.5 kDa (**d**). *Left panel*, **b**. Densitometric scan of lane I showing the mayor protein components tested for antigenicity with antibody 7A. Details and methods are provided in the text and in the original reference (Arch Microbiol 144:20–24, 1986) from which the figure was reproduced with permission from the copyright owner

recognized by the antibody (Macario and Conway de Macario 1985a). It remains to be established whether the five proteins bearing the epitope recognized by antibody 7A are different proteins altogether, or are fragments or processing stages of one (or fewer) protein precursors.

Table 10.2 Molecular specificity of antibody 7A determined by direct binding assay with various antigens

Antigen preparation	Reaction with antibody 7A (SIA; OD_{450} nm)[a]
Methanogenium marisnigri JR1[b]	
Whole cells	0
S-layer	0
Methanogenium cariaci JR1[c]	
Whole cells	>1
S-layer	0.27
SDS-PAGE protein[d]	
1	0.18
2	0.26
3	0.12
4a	0.19
4b	0.37

[a]SIA: slide immunoenzymatic assay (Macario and Conway de Macario 1985b)
[b]*M. marisnigri* JR1 crossreacts with *M. cariaci* JR1 with rabbit and mouse polyclonal antibody probes
[c]The immunizing organism used to produce antibody 7A
[d]See Fig. 10.6. Details and methods are provided in the text and in the original reference (Arch Microbiol 144:20–24, 1986) from which the data were reproduced with permission from the copyright owner

10.6 Unique Antigen of *Methanospirillum hungatei*

An epitope distinctive of, perhaps unique to *Methanospirillum hungatei* JF1 was identified by a monoclonal antibody, 1A, produced using this methanogen as immunogen (Conway de Macario et al. 1986). Antibody 1A reacted only with the immunizing organism and this reaction was not inhibited–blocked by amino acids or sugars (Table 10.3); it was evident that the antibody was monospecific for JF1 but its molecular specificity was still unknown. It is known that JF1 and the closely related strain GP1 have a protein sheath and S-layer (Claus et al. 2002; Firtel et al. 1993; Kandler and König 1998; Messner et al. 1997; Southam and Beveridge 1991). Thus both strains were used to extract sheath and S-layer to assay them and their fractions with antibody 1A. The determinant recognized by 1A is in the sheath of JF1, in a protein of 12 kDa, as demonstrated by immunochemical analyses of sheath preparations resolved by SDS-PAGE (Table 10.3) (Conway de Macario et al. 1986).

10.7 Antigenic Mosaic of *Methanobrevibacter smithii*

The antigenic mosaic of *Methanobrevibacter smithii* is made up of several epitopes (Conway de Macario et al. 1985), at least seven, as demonstrated by a panel of six monoclonal antibodies made using strain PS as immunogen (Table 10.4) (Conway de Macario and Macario 1997). The determinants were revealed by testing a panel

Table 10.3 Molecular specificity of monoclonal antibody 1A determined by direct antigen binding and inhibition-blocking assays

Antigen[a]	Direct binding (SIA)[a]		Inhibition–blocking (%)
	Reaction intensity ($OD_{450\ nm}$)	(antigen) μg/circle	
Whole JF1 cells	>1.0	n.a.[b]	100
Whole GP1 cells[c]	0	n.a.	0
JF1 sheath prep 1	0.35	20	48
JF1 sheath prep 2	0.28	20	n.d.[d]
JF1 sheath prep 3	0.11	10	54
GP1 sheath[c]	0	10	0
GP1 cytoplasmic membrane[c]	0	10	0

[a]SIA, slide immunoenzymatic assay. Antigen preparations and binding and inhibition–blocking assays were done as described in the original publication (Arch Microbiol 144:20–24, 1986) from which the data were reproduced with permission from the copyright owner

[b]n.a., not applicable

[c]GP1 whole cells, sheath and cytoplasmic membrane crossreacted with the anti-*Methanospirillum hungatei* JF1 immune serum obtained from the mouse that produced the monoclonal antibody 1A

[d]n.d., not done

Table 10.4 Antigenic mosaic of *Methanobrevibacter smithii* isolates determined with a panel of six monoclonal antibodies

Isolate[a]		Determinant[b]						Total/cell[d]
Group[c]	Number (% of 46 isolates)	c	e	d	a	b	f	
I	5 (12)	+	+	+	+	+	+	6
II	6 (13)	−	+	+	+	+	+	5
III	8 (17)	−	−	+	+	+	+	4
IV	1 (2)	−	+	−	−	+	+	3
V	1 (2)	−	−	+	−	+	+	3
VI	2 (4)	−	−	−	+	+	+	3
VII	6 (13)	−	−	−	−	+	+	2
VIII	1 (2)	−	−	−	+	−	−	1
IX	16 (35)	−	−	−	−	−	−	0
Total	46	5[e]	12	20	22	29	29	
Percent	100	11	26	43	48	63	63	

[a]The isolates were determined to be *Methanobrevibacter smithii* by various complementary procedures

[b]Each antigenic determinant, a–f, was recognized by a single antibody, A–F, respectively

[c]Isolates in any given group possess and lack the same determinants (e.g., isolates in group II possess determinants a, b, d, e, and f, and lack determinant c)

[d]The figures indicate the number of different determinants recognized by the antibodies A–F that were present on the cell of each group (I–IX); e.g., while cells in group I had all six determinants, those in group II lacked one of them (determinant c)

[e]Figures in this row show how many times (i.e., the frequency, which as percentage is shown in the row underneath) the determinants occurred in the 46 isolates; e.g., determinant c occurred in 5 isolates out of 46, namely it occurred with a frequency of 11%. Details and methods are provided in the text and in the original reference (Conway de Macario and Macario 1997) from which the data were reproduced modified with permission from the copyright owner

of 46 *M. smithii* isolates. In this way it was found that the isolates could be sorted into nine groups according to their pattern of reaction/no-reaction with the antibodies. While isolates in groups I to VIII reacted with one or more antibodies, isolates in group IX, consisting of 16 isolates (i.e., 35% of the isolates tested), did not react with any of the antibodies. Thus, excluding group IX, it is apparent that determinant 6c (identified by antibody 6C) is present only in group I; 6e occurs in groups I, II, and IV; 6d is present in group I, II, III, and V; 6a occurs in groups I, II, III, VI, and VIII; and 6b and 6f are present in all isolates of groups I–VII. Despite this similar pattern of reaction with antibodies 6B and 6F, the corresponding determinants, 6b and 6f, are not the same. This was shown by measuring the intensity of the reaction of the two antibodies with a panel of isolates, which demonstrated distinctive patterns of reactivity. In conclusion, six determinants were identified with the antibodies, and in addition it can be inferred that there is at least another determinant in the mosaic of *M. smithii* that is different from the other six, and therefore it was not recognized by any of the six antibodies in the panel. Inhibition–blocking experiments with an array of sugars and amino acids were negative, except in the case of antibody 6A which was inhibited–blocked by α-Glu–ε-Lys, γ-Glu–ε-Lys, Glu–Lys, and Glu–Lys + Orn. These results show that antibody 6A is different from all the others in the panel, and suggest that determinant 6a contains a structure resembling the inhibitors. The three dipeptides that inhibited–blocked antibody 6A were the closest analogues that could be obtained to match those in the peptide bridge between the glycan strands of pseudomurein (Fig. 10.2). It is also interesting to note that Orn is distinctive of the pseudomurein of strain PS (Kandler 1982; König et al. 1982). Orn alone did not inhibit–block antibody 6A, but when tested together with Glu–Lys it did. Thus, Orn increased the level of inhibition–blocking effect of Gly–Lys above the levels caused by this dipeptide alone. The data indicate that determinant 6a is located in the peptide moiety of pseudomurein, in the region which is typical of strain PS, involving Glu, Lys, and Orn.

10.8 Antigenic Mosaic of *Methanosarcina barkeri*

The antigenic mosaic of *Methanosarcina* was studied with antibodies made against *Methanosarcina barkeri* 227 (Garberi et al. 1985). Two monoclonal antibodies, 8A and 8C, were characterized and calibrated for this study, using strain 227 and the antigenically related strain R1M3. Inhibition–blocking experiments with various cell and cell extract preparations showed that the antibodies 8A and 8C recognize two different antigenic structures (Fig. 10.7). Interestingly, the determinant recognized by 8C is not exposed on the surface of 227 but it is exposed on the surface of R1M3. Inhibition–blocking assays with compounds of known structures showed reactivity only for 8A and only with glucose, suggesting that, as expected, sugars are the main components of the cell wall of *Methanosarcina* (Kandler 1982; Kandler and König 1985, 1998). Chemical analysis of the antigen recognized by

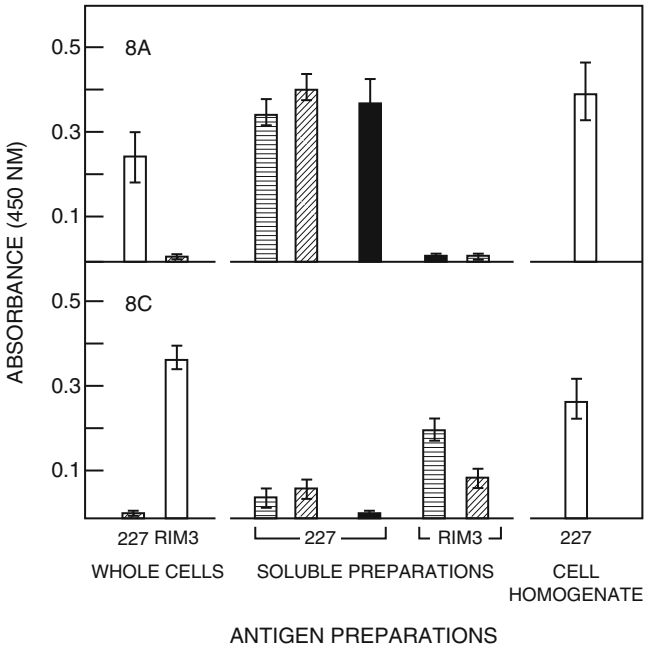

Fig. 10.7 Reaction of antibodies 8A and 8C with various antigen preparations. Bars represent mean ± SD; $n = 6$. Soluble preparations: *bars with horizontal lines*, PBS (phosphate-buffered saline) preparations stored at 4°C for 2 weeks; *bars with oblique lines*, used culture medium; *closed bars*, methanol extract. Details and methods are provided in the text and in the original reference (J Bacteriol 164:1–6, 1985) from which the figure was reproduced with permission from the copyright owner

8A showed carbohydrates to be abundant in comparison with amino acids (8.02 molar ratio in favor of sugars). However, many amino acids could be identified in this purified antigen, suggesting that they do form part of the cell wall, although in much lower proportions than the carbohydrates.

10.9 Conclusions

Recent advances in the understanding of cell walls open a variety of research alternatives with promising practical applications in the near future. The molecular details of wall synthesis and evolution, and the participation and role of the cell wall in cell division, growth and morphogenesis, sensing and adapting to environmental changes, invasion of hosts (if the cell does have this alternative in its life cycle), and in defense mechanisms against external aggression are being actively investigated in a variety of taxa (Buynak 2007; Cabeen and Jacobs-Wagner 2007; Coronado et al. 2007; Domozych et al. 2007; Dvorakova et al. 2007; Funkhouser and Aronson

2007; Harold 2002; 2007; Kodama and Fujishima 2007; Lange et al. 2007; Milligan and Morel 2002; Nakamura et al. 2006; Steenbakkers et al. 2006). Furthermore, the steady increase in environmental pollution with accumulation of all kinds of residues and the rise in oil prices have boosted interest in the development of alternative technologies for waste elimination–reutilization and for fuel production (Macario and Conway de Macario 1985a, b, c), including the use of methanogens for these purposes (Connaughton et al. 2006; de Bok et al. 2006; Li et al. 2006; Riffat and Krongthamchat 2007). Also, new developments in the field of the methanogenic microflora pertaining to veterinary sciences and to human medicine (Belay et al. 1988, 1990; Conway de Macario and Macario 2009; Conway de Macario et al. 1985, 1987; Mukhopadhyay et al. 1991) have re-emphasized the need to study methanogens, including their cell wall biochemistry, immunochemistry, and biology, particularly with regard to intermicrobial interactions and to microbe–host relationships (Eckburg et al. 2003; Samuel and Gordon 2006; Vianna et al. 2006; Vickerman et al. 2007; Conway de Macario and Macario 2009).Within this current scenario the utilization of immunochemical methods for analysis, modification, manipulation, and use of methanogens in animal and human medicines and in industry becomes a major instrument (Macario and Conway de Macario 1985a, b, c; Conway de Macario and Macario 2009). The data presented in this Chapter pertaining to the immunochemical and molecular characteristics of the cell walls of various methanogens, representing a range of evolutionary branches, should be useful to begin (or continue) the development of tools with direct practical applications. For example, the recognition of several distinct immunotypes in *M. smithii*, which is by far the most frequent methanogen inhabiting the human body, is the start for any investigation aiming at determining the organisms (immunotypes) most relevant for enhancing the pathogenesis of associated microbes. The same can be said for research directed toward the elucidation of which methanogenic immunotype is the most efficient for bioconversion of wastes in a waste-treatment plant.

References

Belay N, Johnson R, Rajagopal BS, Conway de Macario E, Daniels L (1988) Methanogenic bacteria from human dental plaque. Appl Environ Microbiol 54:600–603

Belay N, Mukhopadhyay B, Conway de Conway Macario E, Galask R, Daniels L (1990) Methanogenic bacteria in human vaginal samples. J Clin Microbiol 28:1666–1668

Bryniok D, Trösch W (1989) Taxonomy of methanogens by ELISA techniques. Appl Microbiol Biotechnol 32:243–247

Buynak JD (2007) Cutting and stitching: the cross-linking of peptidoglycan in the assembly of the bacterial cell wall. ACS Chem Biol 21:602–605

Cabeen MT, Jacobs-Wagner C (2007) Skin and bones: the bacterial cytoskeleton, cell wall, and cell morphogenesis. J Cell Biol 179:381–387

Claus H, Akça E, Debaerdemaeker T, Evrard C, Declercq JP, Harris JR, Schlott B, König H (2005) Molecular organization of selected prokaryotic S-layer proteins. Can J Microbiol 51:731–743

Claus H, Akça E, Debaerdemaeker T, Evrard C, Declercq JP, König H (2002) Primary structure of selected archaeal mesophilic and extremely thermophilic outer surface layer proteins. Syst Appl Microbiol 25:3–12

Connaughton S, Collins G, O'Flaherty V (2006) Psychrophilic and mesophilic anaerobic digestion of brewery effluent: a comparative study. Water Res 40:2503–2510

Conway de Macario E, Macario AJL (1997) S-layer and ABC-transporter genes in methanogenic archaea. FEMS Microbiol Rev 20:59–98

Conway de Macario E, Macario AJL (1986) Molecular structures of bacteria elucidated by monoclonal antibodies with special reference to antigenic determinants of the methanogens' envelopes. In: Macario AJL, Conway de Macario E (eds) Monoclonal antibodies against bacteria, vol III. Academic Press, Orlando, FL, pp. 181–202

Conway de Macario E, Macario AJL (2009) Methanogenic archaea in health and disease: a novel paradigm of microbial pathogenesis. Intl J Med Microbiol 299:99–108

Conway de Macario E, Macario AJL, Kandler O (1982) Monoclonal antibodies for immunochemical analysis of methanogenic bacteria. J Immunol 129:1670–1674

Conway de Macario E, Macario AJL, Magariños MC, König H, Kandler O (1983) Dissecting the antigenic mosaic of the archaebacterium *Methanobacterium thermoautotrophicum* by monoclonal antibodies of defined molecular specificity. Proc Natl Acad Sci USA 80:6346–6350

Conway de Macario E, König H, Macario AJL, Kandler O (1984) Six antigenic determinants in the surface layer of the archaebacterium *Methanococcus vannielii* revealed by monoclonal antibodies. J Immunol 132:883–887

Conway de Macario E, Macario AJL, Pastini A (1985) The superficial antigenic mosaic of *Methanobrevibacter smithii*. Identification of determinants and isolates by monoclonal antibodies. Arch Microbiol 142:311–316

Conway de Macario E, König H, Macario AJL (1986) Antigenic determinants distinctive of *Methanospirillum hungatei* and *Methanogenium cariaci* identified by monoclonal antibodies. Arch Microbiol 144:20–24

Conway de Macario E, Macario AJL, Miller T, Wolin MJ (1987) Antigenic diversity of methanogenic bacteria from intestinal tracts of animals. Syst Appl Microbiol 9:210–213

Coronado JE, Mniemneh S, Epstein SL, Qiu W, Lipke PN (2007) Conserved processes and lineage-specific proteins in fungal cell wall evolution. Eukaryot Cell 6:2269–2277

de Bok FA, van Leerdam RC, Lomans BP, Smidt H, Lens PN, Janssen AJ, Stams AJ (2006) Degradation of methanethiol by methylotrophic methanogenic archaea in a lab-scale upflow anaerobic sludge blanket reactor. Appl Environ Microbiol 72:7540–7547

Domozych DS, Serfis A, Kiemle SN, Gretz MR (2007) The structure and biochemistry of charophycean cell walls: I. Pectins of *Penium margaritaceum*. Protoplasma 230:99–115

Dvorakova L, Cvrckova F, Fischer L (2007) Analysis of the hybrid proline-rich protein families from seven plant species suggests rapid diversification of their sequences and expression patterns. BMC Genomics 128(1):412

Eckburg PB, Lepp PW, Relman DA (2003) Archaea and their potential role in human disease. Infect Immun 71:591–596

Firtel M, Southam G, Harauz G, Beveridge TJ (1993) Characterization of the cell wall of the sheathed methanogen *Methanospirillum hungatei* GP1 as an S layer. J Bacteriol 175:7550–7560

Funkhouser JD, Aronson NN Jr (2007) Chitinase family GH18: evolutionary insights from the genomic history of a diverse protein family. BMC Evol Biol 26(7):96

Garberi JC, Macario AJL, Conway de Macario E (1985) Antigenic mosaic of *Methanosarcinae*: partial characterization of *Methanosarcina barkeri* 227 surface antigens by monoclonal antibodies. J Bacteriol 164:1–6

Harold FM (2002) Force and compliance: rethinking morphogenesis in walled cells. Fungal Genet Biol 37:271–282

Harold FM (2007) Bacterial morphogenesis: learning how cells make cells. Curr Opin Microbiol 10:591–595

Hartmann E, König H (1990) Comparison of the biosynthesis of the methanobacterial pseudomurein and the eubacterial murein. Naturwissenschaften 77:472–475

Kandler O (1981) Archaebacteria and phylogeny of organisms. Naturwissenschaften 68:183–192

Kandler O (1982) Cell wall structure and their phylogenetic implications. Zentralbl Bakteriol Mikrobiol Hyg Abt 1 Orig Reihe C 3:149–160

Kandler O, König H (1978) Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch Microbiol 118:141–152

Kandler O, König H (1985) Cell envelopes of Archaebacteria. In: Woese K, Wolfe RS (eds) The bacteria: a treatise on structure and function. Academic Press, Orlando, FL, pp 413–457

Kandler O, König H (1998) Cell wall polymers in Archaea (Archaebacteria). Cell Mol Life Sci 54:305–308

Kodama Y, Fujishima M (2007) Infectivity of *Chlorella* species for the ciliate *Paramecium bursaria* is not based on sugar residues of their cell wall components, but on their ability to localize beneath the host cell membrane after escaping from the host digestive vacuole in the early infection process. Protoplasma 231:55–63

König H (1986) Chemical composition of cell envelopes of methanogenic bacteria isolated from human and animal feces. Syst Appl Microbiol 8:159–162

König H, Kandler O, Jensen M, Rietschel ET (1983) The primary structure of the glycan moiety of pseudomurein from *Methanobacterium thermoautotrophicum*. Hoppe Seylers Z Physiol Chem 364:627–636

König H, Kralik R, Kandler O (1982) Structure and modifications of pseudomurein in Methanobacteriales. Zbl Bakt Hyg I Abt Orig C3:478–490

Lange RP, Locher HH, Wyss PC, Then RL (2007) The targets of currently used antibacterial agents: lessons for drug discovery. Curr Pharm Des 13:3140–3154

Li J, Zhang ZJ, Li ZR, Huang GY, Abe N (2006) Removal of organic matter and nitrogen from distillery wastewater by a combination of methane fermentation and denitrification/nitrification processes. J Environ Sci (China) 18:654–659

Macario AJL, Conway de Macario E (1985a) Monoclonal antibodies of predefined molecular specificity for identification and classification of methanogens and for probing their ecologic niches. In: Macario AJL, Conway de Macario E (eds) Monoclonal antibodies against bacteria, vol II. Academic Press, Orlando, FL, pp 213–247

Macario AJL, Conway de Macario E (1985b) Antibodies for methanogenic biotechnology. Trends Biotechnol 3:204–208

Macario AJL, Conway de Macario E (1985c) A preview of the uses of monoclonal antibodies against methanogens in fermentation biotechnology: significance for public health. In: Macario AJL, Conway de Macario E (eds) Monoclonal antibodies against bacteria, vol I. Academic Press, Orlando, FL, pp 269–286

Macario AJL, Dugan CB, Conway de Macario E (1987) Antigenic mosaic of *Methanogenium*: analysis with poly and monoclonal antibody probes. J Bacteriol 169:666–669

Messner P, Allmaier G, Schäffer C, Wugeditsch T, Lortal S, König H, Niemetz R, Dorner M (1997) Biochemistry of S-layers. FEMS Microbiol Rev 20:25–46

Milligan AJ, Morel FM (2002) A proton buffering role for silica in diatoms. Science 297: 1848–1850

Mukhopadhyay B, Purwantini E, Conway de Macario E, Daniels L (1991) Characterization of a *Methanosarcina* strain isolated from goat feces, and that grows on H_2-CO_2 only after adaptation. Curr Microbiol 23:165–173

Nakamura K, Terada T, Sekiguchi Y, Shinzato N, Meng XY, Enoki M, Kamagata Y (2006) Application of pseudomurein endoisopeptidase to fluorescence in situ hybridization of methanogens within the family Methanobacteriaceae. Appl Environ Microbiol 72:6907–6913

Pauly M, Albersheim P, Darvill A, York WS (1999) Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. Plant J 20:629–639

Riffat R, Krongthamchat K (2007) Anaerobic treatment of high-saline wastewater using halophilic methanogens in laboratory-scale anaerobic filters. Water Environ Res 79:191–198

Samuel BS, Gordon JI (2006) A humanized gnotobiotic mouse model of host-archaeal-bacterial mutualism. Proc Natl Acad Sci USA 103:10011–10016

Southam G, Beveridge TJ (1991) Dissolution and immunochemical analysis of the sheath of the archaeobacterium *Methanospirillum hungatei* GP1. J Bacteriol 173:6213–6222

Steenbakkers PJ, Geerts WJ, Ayman-Oz NA, Keltjens JT (2006) Identification of pseudomurein cell wall binding domains. Mol Microbiol 62:1618–1630

Vianna ME, Conrads G, Gomes BPFA, Horz HP (2006) Identification and quantification of Archaea involved in primary endodontic infections. J Clin Microbiol 44:1274–1282

Vickerman MM, Brossard KA, Funk DB, Jesionowski AM, Gill SR (2007) Phylogenetic analysis of bacterial and archaeal species in symptomatic and asymptomatic endodontic infections. J Med Microbiol 56:110–118

Chapter 11
Cell Wall Structure and Pathogenicity

T.A. Oelschlaeger, U. Dobrindt, and J. Hacker

11.1 Introduction

Components of the cell envelope are important for the interaction of pathogenic bacteria with abiotic surfaces, each another and with the host. Such components are polysaccharides and autotransporter proteins of the outer membrane in Gram-negative bacteria, which are involved in biofilm formation. Surface exposed proteins assembled into hair-like surface structures, termed fimbriae or pili, function as adhesins responsible for adherence to host surfaces. Some of them are also involved in the ability of pathogens to invade host cells. Besides fimbrial adhesins there are also afimbrial adhesins. Certain specialized surface proteins mediate internalization by host cells while others induce intracellular motility subsequent to internalization. A multipurpose tool is the bacterial flagellum. It is not just able to move the bacterial cell but in addition may function as an adhesin and an immune stimulating factor. Properties of all these cell envelope components are reviewed in this chapter. However, it must be taken into account that this review cannot be comprehensive due to space limitations and the enormous variety of prokaryotic cell wall components.

11.2 Fimbrial Adhesins/Pili of Gram-negative Bacteria. (Classification Based on Their Assembly Mechanism)

Hair-like surface structures – fimbriae or pili – were first classified according to their morphology, but more recently according to their mechanism of assembly, and this classification is also used in this chapter. These surface structures (Table 11.1)

T.A. Oelschlaeger (✉)
Institute for Molecular Infection Biology, University of Würzburg, Josef-Schneider-Str. 2, Bau D15, 97080 Würzburg, Germany
e-mail: t.oelschlaeger@mail.uni-wuerzburg.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_11,

are responsible for attachment of the bacterial cell to surfaces allowing colonization of, e.g., certain niches in a host. They are primarily composed of a single protein generically called pilin (Soto and Hultgren 1999). For many Gram-negative pathogens, disruption of fimbrial assembly results in severely reduced virulence, which highlights the crucial importance of pili for pathogenicity (Tacket et al. 1998).

11.2.1 Class 1 Fimbriae/Type IV Pili

Type IV pili (Tfp) (Fig. 11.1) are the most widespread organ of bacterial attachment and the only one present in Gram-positive as well as Gram-negative bacteria (Table 11.1 and 11.2). They are extremely thin (5–8 nm in width), long (several micrometers) and flexible filaments that often interact laterally to form characteristic bundles (for a review of type IV pili see Pelicic 2008). Tfp facilitate adhesion to host cells for enteropathogenic *Escherichia coli* (EPEC) by the bundle forming pilus (Bfp), for *Neisseria gonorrhoeae* by the GC pilus, and by the *Vibrio cholerae* toxin coregulated pilus (Tcp). Tfp mediate adhesion also to cellulose (*Ruminococcus albus*), to stainless steel (*Pseudomonas aeruginosa*) and to paper-making machines (*Deinococcus geothermalis*). In addition Tfp permit locomotion known as twitching motility (Mattick 2002), as well as DNA uptake during transformation (see below). These properties depend on the unique capacity of Tfp to retract, which generates substantial mechanical force (Maier et al. 2002). It is very likely that the *pil* genes encoding Tfp are of ancient origin and that Tfp represent a machinery for macromolecular export. For the Tfp of *Pseudomonas aeruginosa*, the PAK pili, the receptors have been identified as the glycolipids asialo-GM1 and asialo-GM2 on epithelial cell surfaces (Ramphal et al. 1991). Also, the receptor binding loop for recognition of the receptors has been mapped. The residues 128–144 of the D-region of PAK pilin, the protein of which the pilus is composed of, is the adhesive portion. However, although this region is present in each of the several thousand pilin proteins in each PAK pilus, only the D-region of the PAK pilin at the tip of the pilus filaments is exposed and able to bind the receptors (Lee et al. 1994). For pathogenic *Neisseria* spp., Tfp are required to attach to human epithelial cells in the initial stages of infection. The pilus receptor was thought to be the transmembrane glycoprotein CD46, which is present in human epithelial cells (Kallstrom et al. 1997). However, the role of CD46 as the receptor for PilC, the tip adhesin of the Tfp of *Neisseria,* has been questioned (Kirchner et al. 2005; Tobiason and Seifert 2001). Instead, a protein has been suggested to act as the receptor for PilC (Kirchner and Meyer 2005).

Conjugative fertility fimbriae (F fimbriae/F pili) are a subset of Tfp and important in the context of pathogenicity because they are essential for conjugative spread of antibiotic resistance and pathogenicity genes by transferring DNA from a donor to a recipient bacterium (Lawley et al. 2003). This mode of horizontal gene transfer is responsible for the fast spread of antibiotic resistance among bacterial populations which is not restricted to one species but able to cross species

Table 11.1 Adhesins of Gram-negative bacteria

Subtype	Examples	Characteristics	Receptor(s)
Fimbrial adhesins			
Class 1 fimbriae (Type IV pili)	Bundle forming pilus of EPEC Toxin coregulated pilus of *Vibrio cholerae* Conjugative fertility fimbriae	Pilus is anchored to the cytoplasmic membrane Major subunit at the tip functions as adhesin Tfp can rectract and thereby cause twitching motility Horizontal gene transfer (e.g., antibiotic resistance genes)	CD46 (?) for neisserial Tfp
Class 2 fimbriae (are anchored to the outer membrane)			
α Fimbriae (Fimbriae of the alternate chaperon/usher pathway)	CS1 fimbriae of ETEC	Minor subunit at tip is adhesin	Not identified
β Fimbriae	Only operons are known	No genes for tip adhesin present	
γ1 Fimbriae	Type 1 pili (e.g., of UPEC)	Minor subunit (FimH) at tip is adhesin	Mannose residues for type 1 pili
γ2 Fimbriae	F6 (987P) fimbriae of porcine isolates of ETEC	Minor subunit (FasG) at tip is adhesin	Histone H1
γ3 Fimbriae	Dr family of adhesins of UPEC and diarrhea-causing *E. coli*	The major subunit is the adhesin	DAF (CD55) and CEA, CEACAM1, CEACAM6
γ4 Fimbriae	Type 3 (mrk) fimbriae of *Klebsiella pneumoniae*	Minor subunit (MrkD) at tip is adhesin	Integrin (?)
κ Fimbriae	K99 (F4) fimbriae of porcine and bovine ETEC isolates	Minor subunit dispensable for adherence (no tip adhesin)	*N*-Glycolyl-GM3 and *N*-glycolylsialoparagloboside
π Fimbriae	P-pili or UPEC	Minor subunit (PapG) at tip is adhesin	Galα(1-4)Gal moieties
σ Fimbriae	Csu fimbriae of *Acinetobacter baumanii*	?	?
Curli (fimbriae of the extracellular nucleation/precipitation pathway)	Expressed by many members of the Enterobacteriaceae	No tip adhesion CsgA is secreted and forms a fiber (amyloid)	Curli bind to many host proteins (e.g., fibronectin, laminin, TLR2, plasminogen, t-PA)
Afimbrial adhesins			
YadA-like Adhesins; these are members of the autotransporter family	YadA of *Y. enterocolitica*, *Y. pseudotuberculosis*	Anchord to the outer membrane via its C-terminus; N-terminus forms a globular head on top of a stalk	YadA binds to collagen, laminin, fibronectin, mucus, and hydrophobic surfaces
Intimin family (>14 members)	EPEC and EHEC	Outer membrane protein with 4 protruding domains of the C-terminus	Tir, a bacterial protein translocated via a T3SS into the host cell cytoplasmic membrane

CEA: carcinoembryonic antigen; CEACAM: CEA-related cell adhesion molecules; DAF: decay accelerating factor; EHEC: enterohemorrhagic *Escherichia coli*; EPEC: enteropathogenic *E. coli*; ETEC: enterotoxigenic *E. coli*; Tfp: Type IV pili; Tir: translocated intimin receptor; TLR: Toll-like receptor; tPA: tissue plasminogen activator

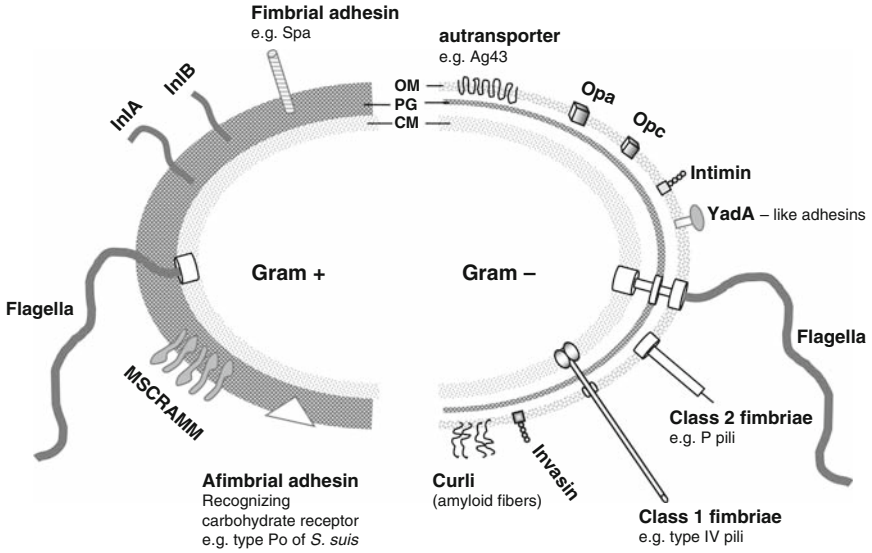

Fig. 11.1 Surface proteins and proteinaceous surface organelles of Gram-positive (Gram +) and Gram-negative (Gram−) bacteria with importance for pathogenicity. Depicted as symbols are proteins/proteinaceous surface organelles in the outer membrane (OM) and a flagellum and one class 1 fimbria, the later ones originating in the cytoplasmic membrane (CM) and penetrating through the peptidoglycan (PG) and the OM of the cell envelope of Gram-negative bacteria. All adhesins and internalin of Gram-positive bacteria shown are anchored covalently to the peptidoglycan with the exception of InlB

boundaries. These fimbriae are hollow cylinders anchored on proteins of the cytoplasmic membrane which traverse the periplasm, and penetrate the outer membrane through a specific proteinaceous pore before they stick out of the bacterial surface. The single stranded DNA is transferred through the central canal into the cytoplasm of the donor cell. In naturally competent bacteria, other members of this fimbrial class are involved in uptake of DNA from the medium (Hofreuter et al. 2001). It is speculated that DNA binds via its negatively charged backbone to the positively charged residues in the grooves of Tfp and the bound DNA is subsequently taken up by Tfp retraction (Craig and Li 2008).

Many pathogenicity (associated) genes are carried by plasmids which either encode a conjugative system including F fimbriae themselves or harbor *mob*-genes essential for conjugative transfer with the help of another plasmid with a complete Tra (transfer) system. Plasmid-encoded virulence genes include but are not limited to toxin genes (e.g., hemolysin) and adhesins and may even code for type three protein secretion systems (T3SS) for the transfer of effector proteins into host cells. These effector proteins are important for pathogenicity not only of human and animal pathogenic bacteria but even for plant pathogenic microbes. F-like fimbriae

Table 11.2 Adhesins of Gram-positive bacteria

Subtype		Expressed by	Characteristics	Receptor(s)
Fimbrial adhesins				
	Spa fimbriae	*Corynebacterium diphtheriae*	Major subunit proteins are covalently linked Minor subunit protein(s) functions as adhesin	Unknown
	PI-1 and PI-2 pilus	*Streptococcus agalactiae* (GBS)	Fimbriae are covalently linked to peptidoglycan	Unknown
	Lancefield T antigens (≥4)	*S. pyogenes* (GAS)		Unidentified part(s) of the extracellular matrix
	RlrA pilus	*S. pneumoniae*		Unknown
	Type 1 fimbriae	*Actinomyces naeslundii*	Major pilin (FimP) building the shaft and minor pilin (FimQ) at pilus tip	Proline-rich salivary proteins
	Type 2 fimbriae	*A. naeslundii*	Major pilin (FimA) building the shaft and minor pilin (FimB) at pilus tip	Glycoproteins and glycolipids
	Type IV pili	*Clostridium perfringens* and *Ruminococcus albus*	Mediates gliding motility in clostridia	Unknown
Afimbrial adhesins				
MSCRAMMs	FnbA	*Staphylococcus aureus*	All these MSCRAMMs are covalently linked to the peptidoglycan	FnbA: Fibronectin/Fibrinogen
	FnbB			FnbB: Fibronectin/Fibrinogen
	ClfA			ClfA: Fibrinogen
	ClfB			ClfB: Fibrinogen
	Cna			Cna: Collagen
	Po adhesin	*S. suis*	Present in various serotypes	Galα(1-4)Galβ1-
	Serin-rich surface proteins GspB and Hsa	*S. gordonii*	Covalently linked to peptidoglycan	Glycoprotein Iba and IIb on platelets
	Serin-aspartic repeat proteins SdrC, SdrD	*S. aureus*	Covalently linked to peptidoglycan	Unknown
			Mediate adherence to squamous cells from human nares	

GAS: group A *Streptococcus*; GBS: group B *Streptococcus*

of type four secretion systems (T4SS) are also able to secrete virulence factor proteins directly into host cells (Christie and Vogel 2000).

11.2.2 Class 2 Fimbriae

This is the so-called chaperone/usher pathway family, which is split into clades according to the sequence of the usher gene (usually only one usher gene per fimbrial operon). The following clades were recently introduced by Nuccio and Bäumler (2007).

11.2.3 Fimbriae of the Alternate Chaperone/Usher Family

The α Fimbriae. This is a rather small family, also termed the colonization factor antigen I (CFA-I)-like group, with only 15 representatives from genera belonging to the Gammaproteobacteria class and one representative from the Betaproteobacteria (Nuccio and Bäumler 2007). The best studied member of the α-fimbriae is probably the *coo* operon encoding CS1 fimbriae of human enterotoxigenic *E. coli* (ETEC) (Table 11.1). These rigid filaments do not contain a visible tip fibrillum (Gaastra and Svennerhom 1996). However, there are several other fimbriae of human ETEC that belong to this clade, including CFA/I, CS2, CS4, CS5, CS14, CS17, CS19 and PFC071 (Nuccio and Bäumler 2007). Common to all these fimbriae is an apparently tip-localized minor subunit, which mediates adherence (Gaastra and Svennerhom 1996) and is important for colonization of the small intestine (Evans et al. 1978). Most of these fimbrial determinants are located on plasmids carried by ETEC. Remarkably, all these operons present in ETEC encode human-specific colonization factors (Nuccio and Bäumler 2007). Similarly, the *tcf* operon also encoding fimbriae of the α-fimbrial clade is present in the strictly human-adapted pathogens *Salmonella enterica* serotype Typhi and *S. enterica* serotype Paratyphi (McClelland et al. 2004; Townsend et al. 2001). The receptor (structure) has not yet been identified for any of these adhesins.

11.2.4 Fimbriae of the Classical Chaperone/Usher Pathway Family

The β fimbriae. This subfamily of adhesins is the smallest one and no structural information is available because the encoding operons were all identified by whole genome sequencing. However the lack of genes resembling tip adhesins suggests that operons of this clade encode nonfimbrial or fibrillar structures, because the

absence of tip adhesins is characteristic for operons that encode proteins that assemble into thin fibrillae or nonfimbrial surface structures. Members were found in *E. coli* K-12 (*yhcADEF* operon), *Burgholderia cepacia* and *B. pseudomallei*, *S. enterica* serotype Typhimurium (*stj* operon) and *Yersinia pestis* (Nuccio and Bäumler 2007).

The γ1 fimbriae. Some of the best studied fimbriae belong to this clade and are present in several species within the Gammaproteobacteria. The most prominent examples are type 1 and type 1-like fimbriae expressed by, e.g., *Citrobacter freundii* (Fim) (Hess et al. 2004), *E. coli* (e.g., Fim, Sfm, Foc, Sfa) (Table 11.1) (Nuccio and Bäumler 2007), *Proteus mirabilis* (Atf) (Massad et al. 1996), and *S. enterica* serotype Typhimurium (Bcf, Fim). These fimbriae consist of a rigid helical shaft (7 nm in diameter) composed of several hundred FimA molecules (major subunit) and a short flexible tip fibrillum containing the minor subunit proteins FimF and FimG and at its distal end the FimH adhesin. FimH mediates mannose-sensitive heme- and yeast-agglutination (Jones et al. 1995). In uropathogenic *E. coli* (UPEC) type 1 fimbriae are important in causing cystitis. However, S fimbriae (Sfa) and F1C (Foc) fimbriae are also frequently found in UPEC. In contrast to type 1 fimbriae, S fimbriae bind to sialic acid-containing receptors and mediate neuraminidase-sensitive hemagglutination, while F1C fimbriae bind GalNAc (β1-4) β-galactoside residues (Khan et al. 2000; Parkkinen et al 1986).

The γ2 fimbriae. Apart from in *E. coli,* determinants for these fimbriae were only detected in the entomopathogenic *Photorhabdus luminescens* (Duchaud et al. 2003). The best characterized member of this clade is the operon, which encodes the F6 fimbrial antigen (987P fimbriae). This operon is commonly carried by plasmids in porcine isolates of ETEC. The encoded fimbriae are rigid filaments with a tip adhesin (FasG) which is linked by a minor subunit (FasF) to the fimbrial shaft composed of the major subunit FasA (Cao et al. 1995). 987P fimbriae bind with FasG to histone H1 protein in the brush border of the piglet intestine (Table 11.1) (Zhu et al. 2005). CS12 and CS18 fimbriae are present in human isolates of ETEC, the receptors for which are not unidentified yet. The morphology of 987P and CS18 fimbriae is a type 1 pili-like appearance with a rigid shaft and on top a fibrillum with the adhesin at the most distal end (Honarvar et al. 2003).

The γ3 fimbriae. Family members include the Dr family of adhesins common among diffusely adhering *E. coli* (DAEC) strains associated with diarrhea or UTI. Some of the γ3 family members were reported not to be assembled into fimbriae. However, most probably they are assembled into fibrillae too thin to be visualized by electron microscopy. Anderson et al. (2004) interpret the afimbrial sheath morphology for Afa-adhesins as the result of a collapse of the fine fibrillar structures onto the bacterial surface. The Dr family of adhesins have in common adherence to epithelial cells by virtue of binding to decay-accelerating factor (DAF or CD55), a glycophosphatidylinositol (GPI)-anchored glycoprotein that regulates complement activation. Some members of the Dr family are also involved in adherence to the carcinoembryonic antigen-related cell adhesion molecules CEA, CEACAM1 and CEACAM6 (Berger et al. 2004) (Table 11.1).

The fraction 1 capsule-like antigen (F1) confers antiphagocytic properties upon *Y. pestis* and is also a member of the γ3 family (Du et al. 2002). In fact, the major subunit encoded by this *caf* operon is assembled into fibers that form a large gel-like capsule on the bacterial surface (Zavialov et al. 2003).

The γ4 fimbriae. There are only three well-characterized fimbriae of this subfamily. The Mrk fimbriae or type 3 fimbriae of *Klebsiella pneumoniae* mediate mannose-resistant agglutination of tannic acid-treated erythrocytes (Table 11.1) (Tarkkanen et al. 1998). The Hif fimbriae of *Haemophilus influenzae* agglutinate erythrocytes carrying the blood group AnWj and promote colonization of the upper respiratory tract (Kubiet et al. 2000). The *fimABCD* operon of *Bordetella pertussis* encodes serotype 2 fimbriae which recognize heparin by FimD and which are involved in adherence to the laryngeal mucosa of the human respiratory tract (De Greve et al. 2007).

The κ fimbriae. These flexible fimbriae are only found among Enterobacteriaceae. Examples are K88 (F4), K99 (F5), and F107 (F18 fimbrial antigen) fimbriae of *E. coli*. They are thin (2–5 nm diameter) and can be readily distinguished morphologically from the thicker, rigid fimbriae of the γ- or π-fimbrial clade. At least for the K88 and K99 fimbriae, the major subunits (FaeG and FanC) have been implicated in determining the binding activity of their respective adhesins (Bakker et al. 1992; Jacobs et al. 1987). A receptor has not yet been identified unambiguously for K88, whereas *N*-glycolyl-GM3 and *N*-glycolylasialparagloboside were shown to be receptors for K99 fimbriae (Table 11.1) (Kyogashima et al. 1989). These *N*-glycolylsialyl residues are found in the glycoproteins and glycolipids of piglets, calves, and lambs but not in the glycoproteins and glycolipids of humans. This distribution is probably responsible for the host range of ETEC causing diarrhea in these animals (Kyogashima et al. 1989).

The π fimbriae. Members of this fimbrial subfamily are found in Betaproteobacteria and Gammaprotebacteria, e.g., in *S. enterica* serotype Paratyphi A (Ste) and *S. enterica* serotype Typhimurium (Std, Stf) (Emmerth et al. 1999; McClelland et al. 2004). The best-characterized examples are the Pap- (pyelonephritis associated pili) or P-fimbriae (Fig. 11.1) of UPEC, which bind to Galα(1–4)Gal moieties present in glycolipids expressed by kidney epithelial cells (Table 11.1) (Stapleton et al. 1998; Svenson et al. 1983). Similar to type 1 fimbriae, P-fimbriae are composed of a rigid shaft build up by the major subunit PapA and a flexible tip fibrillum with the adhesive minor subunit PapG at its very distal end. PapG is connected to the fibrillum made up of PapE by PapF. PapK serves as an adaptor to link the tip fibrillum to the helical fimbrial shaft (Kuehn et al. 1992). Other *E. coli* fimbriae closely related to P-fimbriae are Prf (P-related fimbriae) (Morschhäuser et al. 1994), Pix fimbriae (Lugering et al. 2003) and Sfp fimbriae (Brunder et al. 2001).

The σ fimbriae. This is the most widely distributed fimbrial subfamily of the chaperone/usher family found in members of Proteobacteria, Cyanobacteria and Deinococcus-Thermus. Even some plant-pathogenic bacteria express these fimbriae, such as *Erwinia carotovora* and *Xanthomonas oryzae*. Human pathogenic bacteria (e.g., *Y. pestis*, *Burgholderia pseudomallei, Vibrio parahaemolyticus*) also encode

adhesins of this subfamily (Nuccio and Bäumler 2007). Not much is known about the morphology or function of the encoded surface structures of the σ fimbrial determinants. For *Acinetobacter baumanii* it is known that the *csu* fimbrial operon is required for biofilm formation and for the elaboration of fimbriae on its surface (Table 11.1) (Tomaras et al. 2003).

11.2.5 Fimbriae of the Extracellular Nucleation/Precipitation Pathway (Curli)

Curli fimbriae (formerly designated thin aggregative fimbriae (*agf*) in *S. enterica* serovar Typhimurium) (Fig. 11.1) are produced by certain species of the family Enterobacteriaceae, form highly aggregative fibers on the bacterial surface, and bind to a variety of proteins, e.g. fibronectin (Table 11.1) (Olsen et al. 1998). Furthermore, curli mediate adhesion to host cells and inert surfaces, which is of medical importance as well (Vidal et al. 1998). Curli are critical determinants of attachment during biofilm formation and they are potent inducers of the host immune response (Bian et al. 2000; Prigent-Combaret et al. 2000). In fact curli are functional amyloid fibers, which can serve as a model for amyloid associated with debilitating human disease such as Alzheimer's and prion disease (Barnhart and Chapman 2006). The expression of two divergently transcribed operons is required for curli biogenesis. The *csgBAC* operon encodes the structural subunits of the fiber, CsgA and CsgB, whereas no role is known for CsgC, at least in *E. coli*. The products of the *csgDEFG* operon are necessary for production, secretion and assembly. The first gene encodes the transcriptional activator CsgD for this operon. CsgG is a lipoprotein localized in the outer membrane and required for the secretion of CsgA and CsgB to the cell surface (Robinson et al. 2006). CsgE and CsgF interact with CsgG at the outer membrane, modulate the stability of CsgA and CsgB, and are required for efficient curli assembly (Chapman et al. 2002).

11.3 Afimbrial Adhesins of Gram-negative Bacteria

The YadA-like adhesins are a growing family of afimbrial adhesins (Fig. 11.1). Members of this family include the Eib *E. coli* immunoglobulin-binding proteins (Sandt et al. 2002), HadA of *Haemophilus influenzae* biogroup aegyptius (Serruto et al. 2009), NadA of *Neisseria meningitidis* (Commanducci et al. 2002), the UspA1 and UspA2 adhesins of *Moraxella catarrhalis* (Hoiczyk et al. 2000), the Vomps of *Bartonella quintana*, the cause of relapsing fever ("trench fever"), endocarditis and bacillary angiomatosis (Zhang et al. 2004), and XadA of the plant pathogen *Xanthomonas oryzae* pv. *oryzae* (Ray et al. 2002). However, the first member of this family was identified in the genus *Yersinia* encoded by the pYV plasmid found

in the pathogenic *Yersinia* species (*Y. pseudotuberculosis* and *Y. enterocolitica*) (for a review see El Tahir and Skurnik 2001). YadA is a homotrimer of 45 kDa subunits that are anchored to the outer membrane via their C-termini, while their N-termini form a globular head on top of a stalk. The "lollipop"-shaped YadA structure covers the entire bacterial surface giving it hydrophobic properies. This afimbrial adhesin mediates binding to collagens, laminin, fibronectin, intestinal mucosa, and mucus, and to hydrophobic surfaces like polystyrene (Table 11.1). Furthermore, YadA-expressing bacteria autoagglutinate and agglutinate guinea pig erythrocytes. YadA is also a potent serum resistance factor by inhibiting the classical pathway of complement activation. It was demonstrated that all members of this family are virulence factors.

Recently it was reported that YadA-like proteins are also members of the autotransporter family that are characterized by their ability to cross the outer membrane without the help of accessory proteins. In classical autotransporter proteins, the monomer builds up for that purpose with their C-terminus a beta-barrel in the outer membrane composed of 12 transmembrane beta-strands. In contrast, YadA-like proteins form the beta-barrel in the outer membrane most likely after trimerization and each trimer contributes with 4 transmembrane anti-parallel beta-sheets to the oligomeric pore for export of the rest of the trimer through the outer membrane (Ackermann et al. 2008).

Another family of afimbrial adhesins of Gram-negative bacteria is the intimin (Fig. 11.1) family with at least 14 members (Jores et al. 2004). These afimbrial adhesins are expressed by enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), rabbit diarreagenic *E. coli* (RDEC) and the mouse pathogen *C. rodentium* (Frankel et al. 2001). The term intimin was chosen for these adhesins because of the close approximation of bacteria to the host cells mediated by intimin. Elecron micrographs of infected intestinal epithelia show a uniform ~10 nm gap between bacterial and host cell membranes (Frankel et al. 2001). Intimin is anchored with its N-terminus in the outer membrane whereas the 4 C-terminal domains protrude from the bacterial surface. The most distal domain binds to the intimin receptor Tir located in the host cell cytoplasmic membrane. Tir (translocated intimin receptor) is the only exception among adhesin receptors in that it is a bacterial protein translocated by the bacterial type three secretion system (T3SS) into the host cell and functioning there as the receptor for the afimbrial adhesin intimin (Table 11.1) (Kenny et al. 1997).

11.4 Fimbrial Adhesins of Gram-positive Bacteria

Fimbrial adhesins have been discovered in many Gram-positive bacteria (reviewed in Telford et al. 2006; Proft and Baker 2009). Two types can be distinguished by electron microscopy. Short, thin rods that extend between 70 nm and 500 nm in length, with diameters of 1–2 nm, were found on the cell surface of *Streptococcus salivarius*, *S. gordonii* and *S. oralis*. Much longer and flexible fimbriae of 0.3–3 μm

and with diameters of 3–10 nm have been reported for *Corynebacterium spp*., *Streptococcus pneumoniae*, *S. agalactiae* and *S. pyogenes*. These are composed of multiple copies of a major subunit protein which are covalently linked, in contrast to fimbriae of Gram-negative bacteria. In addition, minor subunit proteins are present, which are not required for pilus integrity, but which might function as adhesins and are located either at the tip, along the pilus rod or even at the pilus base. Another difference from fimbriae of Gram-negative bacteria is that they are covalently linked to the peptidoglycan mediated by a sortase enzyme, which is a specialized transpeptidase. Finally, a Gram-negative-like type IV pilus structure conferring gliding motility was found in *Clostridium perfringens* and in *Ruminococcus* (Table 11.2) (Rakotoarivonina et al. 2002; Varga et al. 2006).

In *Corynebacterium diphtheriae*, causing diphtheria, three different fimbriae were identified named according to their major subunit, sortase-mediated pilin assembly (Spa) A, D, or H (Fig. 11.1; Table 11.2). The minor subunit SpaC is located at the fimbrial tip whereas SpaB is associated along the pilus length. These minor subunits are involved in specific colonization of human pharyngeal cells. Antibodies against SpaB or SpaC block bacterial adherence and latex beads coated with one or the other minor subunit protein specifically bind to pharyngeal cells (Mandlik et al. 2007).

Genome analysis of *S. agalactiae*, the major cause of neonatal sepsis and meningitis in the developed world, led to the discovery of a gene cluster (pilus island 1, PI-1) encoding a pilus composed again of a major subunit protein (GBS80) and two minor pilins (GBS52 and GBS104) attached to the pilus fiber. A related pilus-encoding gene cluster (PI-2) was also found (Table 11.2) (Rossini et al. 2006). Because mutants devoid of the pilus shaft show normal adherence to pulmonary cells whereas deletion mutants of the minor subunit gene were significantly reduced in adherence capacity, the minor pilins seem to act as adhesins independent of the pilus structure (Krishnan et al. 2007).

Similar pilin determinants were also detected in the genome of *S. pyogenes* which colonizes the nasopharynx and skin, sometimes resulting in pharyngitis and subcutaneous skin diseases such as impetigo and cellulitis, but also sometimes leading to invasive diseases such as necrotizing fasciitis and toxic shock syndrome. The determinant detected is a highly polymorphic gene cluster that also encodes fibronectin-binding proteins (PrtF1, PrtF2), collagen-binding protein (Cpa), and the T antigen (FCT region) (Table 11.2) (Bessen and Kalia 2002). Again, the pili are composed of a major subunit protein and two minor pilins detected along the pilus shaft in all investigated *S. pyogenes* strains, one of which is Cpa (Mora et al. 2005). The pilus shaft obviously carries the two minor pilins acting as adhesins. The adhesins mediate adherence to pharyngeal cells (Detroit-562) and to human tonsil epithelial cells and skin keratinocytes (Abbot et al. 2007).

Fimbriae have also been detected in some, but not all, *Streptococcus pneumoniae* strains. Like *S. pyogenes, S. pneumoniae* can colonize the nasopharynx and subsequently may cause otitis media, sinusitis and pneumoniae. The fimbrial genes are localized on a pathogenicity islet (*rlrA*), which may be a mobile element. Besides three sortases (SrtB, C and D), three fimbrial component proteins are

encoded on RlrA: the major fimbrial shaft protein RrgB and the two minor fimbrial proteins RrgA and RrgC. RrgC is found at the pilus tip and RrgA is located mainly at the base of the fimbria but also distributed along the fimbrial shaft (Table 11.2) (LeMieux et al. 2006). The expression of these fimbriae enhances adherence to lung epithelial cells, and a fimbria expressing clinical isolate was more virulent than a nonpiliated isogenic mutant in murine models of colonization, pneumonia and bacteremia (Barrochi et al. 2006).

The dental pathogen *Actinomyces naeslundii* utilizes two structurally different fimbriae, termed Type 1 and Type 2, for colonization of the oral cavity. These heteromeric structures are build by a major subunit protein forming the shaft (FimP in Type 1 and FimA in Type 2) and a minor subunit protein (FimQ and FimB, respectively) that is localized primarily at the pilus tip (Mishra et al. 2007). Type 1 fimbriae adhere to the proline-rich salivary proteins of the tooth enamel, facilitating oral colonization (Gibbons et al. 1988). Type 2 fimbriae have lectin-like binding activity for glycoprotein or glycolipid receptors on various host cells. In addition they bind to cell wall glycans of some oral streptococci to form biofilms (dental plaque) (Table 11.2) (Mergenhagen et al. 1987).

11.5 Afimbrial Adhesins of Gram-positive Bacteria

The most prominent examples of afimbrial adhesins in Gram-positive bacteria are members of the matrix-binding proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Fig. 11.1). MSCRAMMs bind to fibronectin, collagen, vitronectin, laminin, and others (Patti et al. 1994).

Other afimbrial adhesins recognize carbohydrate receptors. In *Streptococcus suis* two most likely afimbrial adhesins were identified: type PN (Fig. 11.1) inhibitable by galactose and *N*-acetylgalactosamine, and type Po inhibitable only by galactose (Table 11.2) (Haataja et al. 1996)

Streptococcus gordonii, a member of the viridans group and a major component of the human oral flora, also harbors two afimbrial adhesins. Both of them (GspB and Hsa) are serine-rich surface glycoproteins anchored with their C-terminus to peptidoglycan. They recognize and bind to the platelet glycoprotein Iba (GPIba) and glycoprotein IIb (GPIIb) (Table 11.2) (Takamatsu et al. 2005). The ability to bind to platelets is a central interaction in the pathogenesis of infective endocarditis (Xiong et al. 2008).

Adherence of *Staphylococcus aureus* to isolated squamous cells from the nares of volunteers can be mediated by four different afimbrial adhesins. Only a mutant lacking surface proteins clumping factor B (ClfB), iron-regulated surface determinant A (IsdA), and the serin-aspartic acid repeat proteins SdrC and SdrD was completely defective in adherence (Corrigan et al. 2009). Some *S. aureus* strains express the surface protein SasG which can also promote adhesion to desquamate nasal epithelial cells in vitro. However, SasG is not expressed by many *S. aureus*

strains (Corrigan et al. 2007). Other MSCRAMMs of *S. aureus* are listed in Table 11.2.

As is the case for Gram-negative bacteria, Gram-positive bacteria also harbor genes for and express afimbrial as well as fimbrial adhesins. This also holds true for single strains. A recent genome-based analysis in *Enterococcus faecium,* for example, identified 22 predicted peptidoglycan-anchored surface proteins, of which 15 had characteristics typical of MSCRAMMs, including predicted folding into a modular architecture with multiple immunoglobulin-like domains. Strikingly, 9 of the 15 predicted MSCRAMMs showed similarity to *Enterococcus faecalis* Ebp pilus subunits and also contained motifs essential for pilus assembly (Sillanpää et al. 2008).

11.6 Flagella

Flagella are filamentous cell appendages up to 15 μm in length. They are organelles for motility in liquid media and under certain circumstance also allow movement on solid surfaces. Their activity is coupled to the chemotaxis machinery. Flagella are composed of the basal body which functions as a motor dependent on the proton gradient across the cytoplasmic membrane, a torsion hook and a helical hollow filament (the flagellum) (Fig. 11.1). The flagellin protein, which is the subunit of which the flagellum is composed, has a molecular mass of 28–80 kDa depending on the bacterial species. Flagella are essential structures for pathogenicity of bacteria because they provide motility or increase adhesion. Furthermore, the flagellin monomer is of great importance in the detection of microbes by the host and in the induction of immune responses (Ramos et al. 2004).

Motility was demonstrated to be essential for infection of the stomach and lungs by *Helicobacter pylori* and *Pseudomonas aeruginosa* respectively (Drake and Montie 1988; Ottemann and Lowethal 2002). Colonization of intestinal mucosa by *Vibrio cholerae* is strictly dependent on expression of functional flagella (Krukonis and DiRita 2003).

Besides functioning as an organelle for motility, flagella were reported to mediate adhesion. *Clostridium difficile* mutants lacking flagella associated with cecal tissue at a tenfold lower rate in comparison to the flagellated wildtype. Furthermore, crude flagella from this opportunistic pathogen bind to cecal mucus of germ-free mice (Tasteryre et al. 2001). Also the flagella of enteropathogenic *E. coli* (EPEC) mediate adherence to cultured epithelial cells which can be blocked by purified EPEC flagella and antiflagella antibodies. Most likely the mammalian cells provide a signal that triggers flagella production in EPEC (Giron et al. 2002). For another *E. coli* pathotype, enterohemorrhagic *E. coli* (EHEC), the flagella of serotype H7 is responsible for adherence of EHEC to bovine intestinal epithelium, which is a crucial initiating step for colonization of cattle, the principal reservoir of EHEC O157:H7 (Mahajan et al. 2009). In many motile pathogens, molecules with high similarity to EtpA of ETEC were identified. These EtpA-like proteins are

the secreted partner of two-partner secretion family proteins. In ETEC, EtpA is secreted and subsequently binds to FliC at the tip of the flagella and functions there as an adhesin. In this case, the flagella are used as the presenting organelles for the adhesive EtpA molecules (Roy et al. 2009). The medical importance of adherence mediated by flagella is also highlighted by a paper reporting *S. enterica* to adhere by the flagella to basil, lettuce, rocket, and spinach leaves. These observations were made with a *S. enterica* serovar Senftenberg strain which had caused an outbreak in the United Kingdom (Brown et al. 2009).

Flagella of certain pathogens are postranslationally modified by glycosylation. This was shown for, e.g., *Pseudomonas syringae* pv. *tabaci*, *Clostridium botulinum* and *Campylobacter jejeuni*. Glycosylation of flagellin is necessary for efficient adhesion mediated by the flagellum and for the ability to cause disease in tobacco (Taguchi et al. 2006). Flagellin glycosylation in *C. jejeuni* is variable and influences autoagglutination as well as adherence and invasion of INT407 cells. In addition, lack of pseudoaminic acid in flagellin resulted in attenuation in the ferret diarrheal disease model (Guerry et al. 2006).

Flagellins also exhibit some features of microbe-associate molecular patterns including sequence conservation and wide distribution among bacteria. In mammals, the Toll-like receptor 5 (TLR5) is involved in the detection of flagellin. Recognition of flagellin by TLR5 stimulates transcription of proinflammatory genes that are dependent on NF-κB and MAPKs (Ramos et al. 2004).

In *C. jejeuni* as well as in *Yersinia enterocolitica* the flagellar export apparatus secretes certain virulence factors. For *Y. enterocolitica* the secretion of several proteins was demonstrated including the virulence-associated phospholipase YplA (Young et al. 1999). Similarly, a set of proteins is synthesized by *C. jejeuni* during coculture with epithelial cells, a subset of which is secreted via the flagellar export system (Konkel et al. 2004).

As was shown for the glycosylated flagella of *C. jejuni*, flagella of other pathogenic bacteria also mediate invasion of host cells. Certain UPECs, in particular strain AL511, are able to enter and persist within mouse collecting duct mpkCCDcl4 cells depending on the expression of a functional flagella. For strain AL511, both flagellum filament assembly and the motor proteins MotA and MotB appear to be required for uptake into collecting duct cells (Pichon et al. 2009).

11.7 Invasins

Certain pathogenic bacteria even invade host cells. The intracellular niche provides several advantages to invasive bacteria. Extracellularly located bacteria might be exposed to physical stresses such as low pH or shear stress and many other host defense mechanisms, in contrast to intracellular bacteria. In addition, a spectrum of antibiotics will not affect bacteria inside host cells.

The first bacterial surface proteins identified to mediate uptake by host cells were invasins expressed by enteropathogenic *Y. enterocolitica* (MW: 91.3 kDa) and

Yersinia pseudotuberculosis (103 kDa) (Fig. 11.1). Invasin is an outer membrane protein which binds with extraordinarily high affinity to at least five different integrins that have the β1 chain. Such integrin receptors are present at the apical surface of M cells in the intestine. Binding via invasin to β1 integrins on M cells allows bacterial internalization and subsequent translocation into regional lymph nodes (Isberg and Barnes 2001). In order to reach the intracellular niche, some bacteria use adhesins or proteins encoded by an adhesion determinant. Pathogenic *E. coli* (e.g., UPEC) might enter host cells either via type 1 fimbriae or by expressing AfaE or DraE. The DraE or AfaE-III adhesin subunits are necessary and sufficient to promote the receptor-mediated bacterial internalization into epithelial cells (CHO-B2) expressing human decay-accelerating factor (DAF), CEACAM1, CEA, or CEACAM6. This internalization occurs through a microfilament-independent, microtubule-dependent, and lipid raft-dependent mechanism (Korotkova et al. 2008; Guignot et al. 2009). CEACAM receptors are also manipulated by *Neisseria gonorrhoeae* and *Neisseria meningitidis* to induce bacterial internalization. Interaction of these *Neisseria* spp. with CEACAM1, CEACAM3, CEACAM5, and/or CEACAM6 occurs by binding of variants of the integral outer membrane proteins of the Opa family (Dehio et al. 1998) (Fig. 11.1). Opa proteins are predicted to span the lipid bilayer eight times with four surface-exposed loops (Malorny et al. 1998). Individual gonococcal strains possess ~11 different *opa* alleles, each of which may encode functionally and/or antigenically distinct variants (Dehio et al. 1998). Only Opa_{50} of *N. gonorrhoeae* strain MS11 binds either to cell surface-associated heparan sulfate proteoglycans (HSPG) or to a combination of HSPG receptor(s) and the serum-derived extracellular matrix proteins fibronectin and vitronectin. In the latter case, the extracellular matrix proteins bridge Opa_{50} with the fibronectin/vitronectin-specific integrin receptors and ligate these with HSPG-containing syndecan receptors, thereby triggering their engulfment by host cells (McCaw et al. 2004).

At least in vitro, curli, a member of the growing family of naturally occurring amyloids, not only mediate adherence but are also able to mediate invasion of *E. coli* and *S. enterica* serovar Typhimurium (Gophna et al. 2002; Uhlich et al. 2009). Invasion of host cells by *S. enterica* serovars and *Shigella* spp. is the result of translocation of protein effector molecules from the bacterial cytoplasm into the host cell cytoplasm via an "injectosome" of the type three protein secretion system (T3SS). The part of the "injectosome" crossing the cytoplasmic membrane, the periplasm and the outer membrane is highly homologous to the basal body of the flagella. However, instead of the extruding flagella a straight and rigid hollow cylinder extends from the basal body forming a needle through which the effectors are translocated into the host cell after contact of the needle tip with the host cell cytoplasmic membrane.

Invasiveness is not a property restricted to Gram-negative bacteria. Bacterial mediated entry into host cells was also reported for many pathogenic Gram-positive bacteria. The best studied example is *Listeria monocytogenes* which is able to cross three host barriers: the intestinal, the blood–brain and the materno-fetal barrier. This species expresses two surface located proteins – InlA and InlB – which function as the main invasins (Fig. 11.1). InlA is a protein of 88 kDa with a

carboxyterminal sorting signal consisting of a LPXTG motif followed by a hydrophobic domain comprising ~20 amino acids and a tail of positively charged amino acids. This signal is responsible for the covalent connection of InlA to the peptidoglycan. Like all members of the so-called internalin multigene family, InlA contains also an amino-terminal leucin-rich repeat (LRR) domain followed by a conserved inter-repeat region (IR). The LRR domain consists of tandem repeats of 20–22 amino acids with conserved leucin residues. These repeats are known to be involved in protein–protein interactions. In InlA the LRR and IR regions are necessary and sufficient for bacterial entry into permissive cells (Lecuit et al. 1997). Genome analysis of *L. monocytogenes* revealed the presence of 14 genes encoding internalin-like proteins with an LPXTG motif (Cabanes et al. 2002).

In contrast to InlA, InlB is not covalently linked to the peptidoglycan but is only loosely associated with the bacterial cell wall via its different C-terminus lacking the LPXTG motif (Schubert et al. 2001). Instead, the 231 carboxyterminal amino acids of InlB are necessary and sufficient for binding to lipoteichoic acids on the bacterial surface and for interaction with glycosaminoglycans on mammalian cells. Important for the interactions are the three highly conserved modules of ~80 amino acids containing the dipeptide Gly-Trp (GW modules) (*L. monocytogenes* surface proteins are reviewed in Cabanes et al. 2002).

The glycosaminoglycans, as well as gC1qR/p32 and Met/HGFR (hepatocyte growth factor receptor), are receptors for InlB. The gC1qR/p3 receptor can trimerize and is mostly located at mitochondria and can be detected in the nucleus and on the cell surface. It has been reported to interact with many viral proteins (Cossart and Toledo-Arana 2008). Met is a tyrosine kinase and the receptor for the hepatocyte growth factor (Shen et al. 2000). Full Met activation requires not only binding of the LRR region but also C-terminal domains of InlB, which induce heparin-mediated receptor clustering and potent signaling (Cossart and Toledo-Arana 2008). It should be noted that InlB plays no role in crossing of the intestinal barrier in an oral infection model with transgenic mice (Khelef et al. 2006).

The receptor used by InlA for entry is E-cadherin (Mengaud et al. 1996). Like InlB, InlA interacts with its receptor via the N-terminal LRR containing domain. InlA binds to the N-terminal part of the ecto-domain of E-cadherin which is totally different from the homotypic E-cadherin-E-cadherin interactions, responsible for the physiological role of E-cadherin, ensuring tight adhesion of neighboring epithelial cells through homotopic interactions in adherence junctions on their basolateral side (Uemura 1998). InlA is the major factor promoting crossing of the intestinal barrier. Both InlA and InlB are necessary for the crossing of the maternofetal barrier (Disson et al. 2008).

After internalization of *L. monocytogenes* by host cells, this bacterium escapes from the vacuole and replicates in the host cell cytoplasm. Other surface proteins now come into play, the most prominent of which is ActA. ActA recruits and activates Arp2/3 and induces the formation of a network of branched actin filaments, mimicking WASP family proteins in the leading edge of motile cells. The formation of branched filaments propels *L. monocytogenes* through the host cell and finally into a neighboring host cell without an extracellular intermediate step (Smith

and Portnoy 1997). This strategy is suited to protect this pathogen during its spread from cell to cell from the host's immune system.

Although *L. monocytogenes* is the best studied model organism for invasive Gram-positive bacteria, there are many reports regarding other invasive Gram-positive microorganisms, e.g., cocci. Invasiveness has been demonstrated for group A streptococci (GAS), group B streptococci (GBS; *S. agalactiae*), group C streptococci, group G streptococci, and *S. pneumoniae,* as well as staphylococci (reviewed in Nitsche-Schmitz et al. 2007). The presence of viable intracellular streptococci in tonsils removed from patients with recurrent tonsillitis was an important indication of the in-vivo relevance of GAS invasiveness (Österlund et al. 1997). Internalization mediated by streptococcal and staphylococcal fibronectin-binding proteins is a major mechanism common to streptococci and staphylococci. In GAS, a key player of this internalization mechanism is SfbI or its allelic variant protein F1 (Jadoun et al. 1997). In addition, the surface-exposed M protein of streptococci is obviously also an invasion protein (Hagman et al. 1999). Besides these nonfimbrial invasins, the pilus of GBS, a major causative agent of meningitis in newborns, is involved not only in adherence to but also in invasion of brain microvascular endothelial cells (Maisey et al. 2007).

11.8 Cell Surface-associated Proteins and Polysaccharides in Biofilm Formation

Biofilm formation is a biphasic process, where bacteria initially adhere to biotic or abiotic surfaces, and subsequently accumulate into a multilayer biofilm (Davey and O'Toole 2000; Donlan and Costerton, 2002; Hall-Stoodley et al. 2004). The ability to form biofilms on foreign materials such as permanent catheters is considered an essential patho-mechanism for catheter-associated infections caused by different bacterial pathogens (Trautner and Darouiche 2004). In the early stages of biofilm formation, the surface attachment is crucial.

In the case of biofilm-forming staphylococci, nonspecific factors such as surface charge and hydrophobicity are supposed to mediate initial adherence, but specific cell surface-associated factors such as teichoic acids and proteins which interact with matrix proteins (e.g., fibronectin and collagen) also play an important role. In addition, the surface-associated protein Aap (accumulation-associated protein) and Bap/Bhp (biofilm-associated protein) as well as protein A contribute to biofilm formation by *Staphylococcus epidermidis* and *S. aureus* (Cucarella et al. 2001; Merino et al. 2009; Rohde et al. 2005). For biofilm accumulation, the production of factors that mediate a close cell-to-cell contact is crucial (Götz et al. 2000; Heilmann et al. 2004; Hussain et al. 2001a, b, c). So-called micro-colonies are formed, which subsequently develop into heterogeneous, complex three-dimensional structures which are characterized by an exopolysaccharide matrix (Davey and O'Toole 2000; Donlan and Costerton 2002). At this stage, the "polysaccharide intercellular adhesin" (PIA), which consists of β-1,6-linked glucose aminoglycan units and represents a considerable fraction of

the extracellular matrix (Mack et al. 1996), is essential for *S. epidermidis* biofilm formation.

Pseudomonas aeruginosa biofilms are important issues in the pathogenesis of the bacterium in ventilator-associated pneumonia, urinary and peritoneal dialysis catheter infections, bacterial keratitis, otitis externa, and burn wound infections (Costerton et al. 1999). Chronic lung infection by *P. aeruginosa* leads to a decline of lung function, respiratory failure, and, ultimately, death in cystic fibrosis (CF) patients (Tümmler and Kiewitz, 1999). The mechanisms involved in bacterial adhesion have been increasingly investigated over the last decade. Genes coding for cell surface-associated factors such as flagella and type IV pili (O'Toole and Kolter, 1998), Cup fimbriae (Vallet et al. 2001), and the *pel* genes (Vasseur et al. 2005) are the most frequently cited *P. aeruginosa* determinants among those shown to be implicated in various stages of biofilm formation. Establishment of a chronic CF lung infection by *P. aeruginosa* coincides with production of the exopolysaccharide alginate involved in the formation of persistent biofilms which protect cells from antibiotics and the host immune response (Costerton 2001; Lyczak et al. 2002).

Escherichia coli biofilms are often associated with catheter-associated urinary tract infections. The ability of *E. coli* to form biofilms contributes to colonization of catheter surfaces and protects the bacteria against mechanical shearing forces by urine flow as well as against the host immune system or antibiotics. Biofilms of UPEC may also participate in the establishment of recurrent and chronic urinary tract infections in which intracellular biofilm-like structures are involved (Anderson et al. 2003; Hall-Stoodley et al. 2004). Biofilm formation in *E. coli* is a multifactorial process. Flagella have been reported to play an important role for initial attachment (Prigent-Combaret et al. 2000). Additionally, different cell surface-exposed fimbrial and afimbrial adhesins mediate the binding of the bacterial cells to each other and to other cells as well as to the substrate surface (Pratt and Kolter 1998; Schembri and Klemm 2001). Furthermore, cell surface-associated factors, such as flagella, different fimbrial adhesins, curli, autotransporters (Fig. 11.1), and various components of the extracellular matrix, contribute to the establishment of three-dimensional cell aggregates embedded in an extracellular matrix. The latter is formed by various adhesins such as curli and fimbriae as well as by cell surface-associated polysaccharides including colanic acid and other capsular material, poly-β-1, 6-*N*-acetyl-D-glucosamine, and cellulose (Danese et al. 2000a, b, 2001; Donlan and Costerton 2002; Römling 2005; Wang et al. 2004; Zogaj et al. 2001).

The cell surface-associated polysaccharide poly-β-1,6-GlcNAc (PGA) was originally described as polysaccharide intercellular adhesin (PIA) from *S. epidermidis* and later also from *S. aureus* (Joyce et al. 2003). Many Gram-negative bacteria produce the same polysaccharide. Besides mediating cell-to-cell and cell-to-surface adhesion in biofilms, PGA is essential for the formation of the biofilm architecture of *E. coli* (Agladze et al. 2003; Agladze et al. 2005). PGA has considerable effects on host–microbe interactions as it promotes the transmission of *Y. pestis* from the flea vector to the mammalian host (Hinnebusch and Erickson 2008) as well as colonization, virulence, and immune evasion in infections caused by Gram-positive and

Gram-negative pathogens (Cerca et al. 2007a, b; Izano et al. 2007; Sloan et al. 2007; Vuong et al. 2004). Like many bacterial cell surface compounds, PGA is an effective target for opsonic and protective antibodies. Antibodies raised against staphylococcal PGA protected mice against lethality from different extraintestinal pathogenic *E. coli* strains expressing PGA. Consequently, PGA could be a promising antigen and conserved vaccine target for multiple bacterial pathogens (Cerca et al. 2007a, b). Similarly, other cell surface-associated factors have been suggested as potential targets for rational design of UTI vaccines, due to their immunogenicity. The careful analysis of outer membrane-associated protein expression during growth in human urine or during UTI of *E. coli* demonstrated that expression of various siderophore receptors is induced under these conditions and that these proteins, as well as bacterial flagellin, elicit a strong antibody response in the host during urinary tract infection (Alteri and Mobley 2007; Hagan and Mobley 2007). Accordingly, flagellin (FliC) is a pathogen-associated molecular pattern (PAMP) universally recognized by plants and animals. In *S. enterica*, monomeric flagellin is recognized by Toll-like receptor 5 (TLR5) and is about 100-fold more active than polymerized FliC (Gewirtz et al. 2001; Smith et al. 2003). Upon *S. enterica* sv Typhimurium gastrointestinal infection, the epithelium is partially stimulated by monomeric FliC to secrete cytokines such as IL-8 (Lyons et al. 2004). Interestingly, monomeric flagellin (FliC) is able to elicit an immuno-stimulatory response in the gastrointestinal epithelial cell line HT-29 when bound to curli fimbriae (Rochon and Römling 2006). Curli, also known as thin aggregative fimbriae, bind to a variety of extracellular matrix proteins such as fibronectin and laminin, as well as to serum proteins, plasminogen, human contact phase proteins, and congo red. Furthermore, curli fimbriae mediate invasion of *E. coli* into epithelial cells (Gophna et al. 2001; Uhlich et al. 2002).

The expression of such biofilm matrix components alters the host–bacterium interaction of enterobacteria: co-expression of curli and cellulose decreases adherence, invasion, and IL-8 induction of HT29 cells. A partial correlation exists between curli expression, enhanced invasion of *E. coli* commensals, and IL-8 production (Wang et al. 2006). However, cellulose production of *E. coli* strain Nissle 1917 is required for bacterial adhesion to a gastrointestinal epithelial cell line in vitro and mouse epithelium *in vivo* and for enhanced cytokine production (Monteiro et al. 2009).

Autotransporter proteins represent another class of important cell surface-exposed factors and have been identified in a wide variety of Gram-negative pathogens (Fig. 11.1). Diverse functions of autotransporters have been described ranging from cell-associated adhesins to secreted toxins. Their primary structure is composed of three domains (Henderson et al. 1998): the signal sequence, the passenger, and the translocation domain. The N-terminal signal sequence allows targeting of the protein to the inner membrane for its export into the periplasmic space. The surface-exposed passenger domain (α-domain) is responsible for different effector functions, e.g., autoaggregation, adhesion, invasion, or biofilm formation (Henderson et al. 2004). The C-terminal translocation domain (β-domain) forms a transmembrane pore in the outer membrane and is required for translocation of the passenger domain through the outer membrane (Desvaux et al. 2004).

The major phase-variable *E. coli* outer membrane protein antigen 43 (Ag43) belongs to the autotransporter family (Henderson et al. 2004). The *agn43* gene coding for Ag43 is widespread among the species *E. coli* and often multiple identical or different alleles exist in the genome of individual isolates. Pathogenic *E. coli* isolates in particular frequently contain more than one similar but not always identical *agn43* gene coding for Ag43 variants with differences in their passenger domains (Al-Hasani et al. 2001; Beloin et al. 2006; Grozdanov et al. 2004; Owen et al. 1996; Roche et al. 2001; Torres et al. 2002). The correlation between functional properties and the structure of different Ag43 variants is not yet completely understood. Ag43 has been reported to affect colony morphology, promote bacterial autoaggregation, and thus contribute to cell-to-cell adhesion and biofilm formation (Danese et al. 2000a, b; Hasman et al. 2000; Henderson et al. 1997; Klemm et al. 2004). It has also been proposed that certain Ag43 variants may contribute to long-term persistence of UPEC in the murine urinary tract (Ulett et al. 2007). Recently, it has been shown that glycosylated Ag43 confers binding to eukaryotic cells (Sherlock et al. 2006). Several other autotransporter proteins of *E. coli* have also been described to function as adhesins that mediate aggregation, biofilm formation, and adhesion to various ECM proteins or eukaryotes (Reidl et al. 2009; Valle et al. 2008; Wells et al. 2008).

Similarly, so-called trimeric autotransporters represent an entire family of virulence factors of many Gram-negative bacteria (reviewed in Linke et al. 2006). Expression of trimeric autotransporter YadA in pathogenic yersiniae is essential for the establishment of infections. YadA mediates adherence to proteins of the extracellular matrix such as collagen, fibronectin, and laminin, as well as binding to epithelial and polymorphonuclear cells, and is also essential for autoagglutination. Additionally, binding of YadA triggers interleukin-8 (IL-8) secretion from host cells and contributes to serum resistance by inhibiting complement activation (El Tahir and Skurnik 2001). NadA of *Neisseria meningitidis* mediates binding to and invasion of human epithelial cells (Commanducci et al. 2002). BadA, the key virulence factor of *Bartonella henselae,* is involved in the induction of vasculoproliferations and is also crucial for binding to extracellular matrix proteins (Riess et al. 2004). The VompABCD proteins of *B. quintana* are close relatives of BadA with similar properties (Zhang et al. 2004). UspA1 and A2 are important pathogenicity factors that mediate cell adherence and serum resistance (Aebi et al. 1998; Lafontaine et al. 2000) in *Moraxella catarrhalis*, a major agent of respiratory tract infections (RTIs) in humans. Hsf and Hia mediate adherence to host cells and are prominent in the pathogenicity of *Haemophilus influenzae* (St Geme and Cutter 2000).

11.9 Conclusions

The prokaryotic cell envelope does not just shield the interior of the bacteria from the surrounding, rather it is the interface for communication between bacterial cells as well as with the cellular and acellular components of the host. For pathogenic

bacteria, components of the cell wall are essential for establishing a successful infection. This involves getting a foothold on the host surface(s) by expressing fimbrial and/or afimbrial adhesins. The kind of adhesins to be expressed might be important for determining host and/or tissue specificity. Motility by flagella is often essential for pathogenic bacteria to reach certain host niches, e.g., the upper urinary tract and kidneys. Protection from the host's immune system is based on the formation of biofilms by (among other means) synthesizing a protective extracellular matrix, expression of a capsule, invasion of host cells, variation of exposed surface proteins (e.g., fimbrial or afimbrial adhesins and invasins), production and secretion of toxins, and translocation of effectors into immune cells by type three or type four protein secretion systems. In all these processes, components of the prokaryotic cell wall are involved. Understanding the synthesis, assembly, delivery, and functioning as well as identifying the targets for these components will form the basis for the development of new preventive and therapeutic measures which are urgently needed to fight infectious diseases caused by pathogenic prokaryotes.

References

Abbot EL, Smith WD, Siou GP, Chiriboga C, Smith RJ, Wilson JA, Hirst BH, Kehoe MA (2007) Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsils and skin. Cell Microbiol 9:1822–1833

Ackermann N, Tiller M, Anding G, Roggenkamp A, Heesemann J (2008) Contribution of trimeric autotransporter C-terminal domains of oligomeric coiled-coil adhesion (Oca) family members YadA, UpsA1, EibA, and Hia to translocation of the YadA passenger domain and virulence of *Yersinia enterocolitica*. J Bacteriol 190:5031–5043

Aebi C, Lafontaine ER, Cope LD, Latimer JL, Lumbley SL, McCracken GH Jr, Hansen EJ (1998) Phenotypic effect of isogenic *uspA*1 and *uspA*2 mutations on *Moraxella catarrhalis* 035E. Infect Immun 66:3113–3119

Agladze K, Jackson D, Romeo T (2003) Periodicity of cell attachment patterns during *Escherichia coli* biofilm development. J Bacteriol 185:5632–5638

Agladze K, Wang X, Romeo T (2005) Spatial periodicity of *Escherichia coli* K-12 biofilm microstructure initiates during a reversible, polar attachment phase of development and requires the polysaccharide adhesin PGA. J Bacteriol 187:8237–8246

Al-Hasani K, Rajakumar K, Bulach D, Robins-Browne R, Adler B, Sakellaris H (2001) Genetic organization of the *she* pathogenicity island in *Shigella flexneri* 2a. Microb Pathog 30:1–8

Alteri CJ, Mobley HL (2007) Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. Infect Immun 75:2679–2688

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ (2003) Intracellular bacterial biofilm-like pods in urinary tract infections. Science 301:105–107

Anderson KL, Billington J, Pettigrew D, Cota E, Simpson P, Roversi P, Chen HA, Urvil P, du Merle L, Barlow PN, Medof ME, Smith RAG, Nowicki B, Le Bougenec C, Lea SM, Matthews S (2004) An atomic resolution model for assembly, architecture, and function of the Dr adhesins. Mol Cell 15:647–657

Bakker D, Willemsen PT, Simons LH, van Zijderveld FG, de Graf FK (1992) Characterization of the antigenic and adhesive properties of FaeG, the major subunit of K88 fimbriae. Mol Microbiol 6:247–255

Barnhart MM, Chapman MR (2006) Curli biogenesis and function. Annu Rev Microbiol 60:131–147

Barrochi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, Dahlberg S, Fernebro J, Moschioni M, Masignani V, Hultenby K, Taddei AR, Beiter K, Wartha F, von Euler A, Covacci A, Holden DW, Normark S, Rappuoli R, Henriques-Normark B (2006) A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci USA 103:2857–2862

Beloin C, Michaelis K, Lindner K, Landini P, Hacker J, Ghigo JM, Dobrindt U (2006) The transcriptional antiterminator RfaH represses biofilm formation in *Escherichia coli*. J Bacteriol 188:1316–1331

Berger CN, Billker O, Meyer TF, Servin AL, Kansau I (2004) Differential recognition of members of the carcinoembryonic antigen family by Afa/Dr adhesins of diffusely adhering *Escherichia coli* (Afa/Dr DAEC). Mol Microbiol 52:963–983

Bessen DE, Kalia A (2002) Genomic localization of a T serotype locus to a recombinatorial zone encoding extracellular matrix-binding proteins in *Streptococcus pyogenes*. Infect Immun 70:1159–1167

Bian Z, Brauner A, Li Y, Normark S (2000) Expression of and cytokine activation by *Escherichia coli* curli fibers in human sepsis. J Infect Dis 181:602–612

Brown DJ, Mather H, Clare S, Dougan G, Pallen MJ, Frankel G (2009) Interaction of *Salmonella enterica* with basil and other salad leaves. ISME J 3:261–265

Brunder W, Khan AS, Hacker J, Karch H (2001) Novel type of fimbriae encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H$^-$. Infect Immun 69:4447–4457

Cabanes D, Dhoux P, Dussurget O, Frangeul L, Cossart P (2002) Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol 10:238–245

Cao J, Khan AS, Bayer ME, Schifferli DM (1995) Ordered translocation of 987P fimbrial subunits through the outer membrane of *Escherichia coli*. J Bacteriol 177:3704–3713

Cerca N, Jefferson KK, Maira-Litran T, Pier DB, Kelly-Quintos C, Goldmann DA, Azeredo J, Pier GB (2007a) Molecular basis for preferential protective efficacy of antibodies directed to the poorly acetylated form of staphylococcal poly-N-acetyl-beta-(1–6)-glucosamine. Infect Immun 75:3406–3413

Cerca N, Oliveira R, Azeredo J (2007b) Susceptibility of *Staphylococcus epidermidis* planktonic cells and biofilms to the lytic action of staphylococcus bacteriophage K. Lett Appl Microbiol 45:313–317

Chapman MR, Robinson LS, Pinker JS, Roth R, Heuser J, Hammer M, Normark S, Hultgren SJ (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. Science 295:851–855

Christie PJ, Vogel JP (2000) Bacterial type IV secretion: conjugation. systems adapted to deliver effector molecules to host cells. Trends Microbiol 8:354–360

Commanducci M, Bambini S, Brunelli B, Adu-Bobie J, Arico B, Capecchi B, Guiliani MM, Masignani V, Santini L, Savino S, Granoff DM, Caugant DA, Pizza M, Rappuoli R, Mora M (2002) NadA, a novel vaccine candidate of *Neisseria meningitidis*. J Exp Med 195:1445–1454

Corrigan RM, Miajlovic H, Foster TJ (2009) Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. BMC Microbiol 9:22

Corrigan RM, Rigby D, Handley P, Foster TJ (2007) The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. Microbiol 153:2435–2446

Cossart P, Toledo-Arana A (2008) *Listeria monocytogenes*, a unique model in infection biology: an overview. Microb Infect 10:1041–1050

Costerton JW (2001) Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. Trends Microbiol 9:50–52

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Craig L, Li J (2008) Type IV pili: paradoxes in form and function. Curr Opin Struct Biol 18:267–277

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol 183:2888–2896

Danese PN, Pratt LA, Dove SL, Kolter R (2000a) The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. Mol Microbiol 37:424–432

Danese PN, Pratt LA, Kolter R (2000b) Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J Bacteriol 182:3593–3596

Danese PN, Pratt LA, Kolter R (2001) Biofilm formation as a developmental process. Methods Enzymol 336:19–26

Davey ME, O'Toole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867

De Greve H, Wyns L, Bouckaert J (2007) Combining sites of bacterial fimbriae. Curr Opin Struct Biol 17:506–512

Dehio C, Gray-Owen SD, Meyer TF (1998) The role of neisserial Opa proteins in interactions with host cells. Trends Microbiol 6:489–495

Desvaux M, Parham NJ, Henderson IR (2004) The autotransporter secretion system. Res Microbiol 155:53–60

Disson O, Grayo S, Huillet E, Nikitas G, Langa-Vives F, Dussurget O, Ragon M, Le Monnier A, Babinet C, Cossart P, Lecuit M (2008) Conjugated action of two species-specific invasion proteins for fetoplacental listeriosis. Nature 455:1114–1118

Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193

Drake D, Montie TC (1988) Flagella, motility and invasive virulence of *Pseudomonas aeruginosa*. J Gen Microbiol 134:43–52

Du Y, Rosquist R, Forsberg A (2002) Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis. Infect Immun 70:1453–1460

Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taourit S, Bocs S, Boursaux-Eude C, Chandler M, Charles JF, Dassa E, Derose R, Derzelle S, Freyssinet G, Gaudriault S, Medigue C, Lanois A, Powel K, Danchin A, Kunst F (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. Nat Biotechnol 21:1307–1313

El Tahir Y, Skurnik M (2001) YadA, the multifaceted *Yersinia* adhesin. Int J Med Microbiol 291:209–218

Emmerth M, Goebel W, Miller SI, Hueck CJ (1999) Genomic subtraction indentifies *Salmonella typhimurium* prophages, F-related plasmid sequences, and a novel fimbrial operon, *stf*, which are absent in *Salmonella typhi*. J Bacteriol 181:5652–5661

Evans DG, Satterwhite TK, Evans DJ, DuPont HL (1978) Differences in serological responses and excretion patterns of volunteers challenged with enterotoxigenic *Escherichia coli* with and without the colonization factor antigen. Infect Immun 19:883–888

Frankel G, Phillips AD, Trabulsi LR, Knutton S, Dougan G, Matthews S (2001) Intimin and the host cell – is it bound to end in Tir(s)? Trends Microbiol 9:214–218

Gaastra W, Svennerhom AM (1996) Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). Trends Microbiol 4:444–452

Gewirtz AT, Simon PO Jr, Schmitt CK, Taylor LJ, Hagedorn CH, O'Brien AD, Neish AS, Madara JL (2001) *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. J Clin Invest 107:99–109

Gibbons RJ, Hay DI, Cisar JO, Clark WB (1988) Adsorbed salivary proline-rich protein 1 and statherin: receptors for type 1 fimbriae of *Actinomyces viscosus* T14V–J1 on apatitic surfaces. Infect Immun 56:2990–2903

Giron JA, Torres AG, Freer E, Kaper JB (2002) The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Mol Microbiol 44:361–379

Gophna U, Barlev M, Seijffers R, Oelschläger TA, Hacker J, Ron EZ (2001) Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. Infect Immun 69:2659–2665

Gophna U, Oelschlaeger TA, Hacker J, Ron EZ (2002) Role of fibronectin in curli-mediated internalization. FEMS Microbiol Lett 212:55–58

Götz F, Heilmann C, Cramton SE (2000) Molecular basis of catheter associated infections by staphylococci. Adv Exp Med Biol 485:103–111

Grozdanov L, Raasch C, Schulze J, Sonnenborn U, Gottschalk G, Hacker J, Dobrindt U (2004) Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. J Bacteriol 186:5432–5441

Guerry P, Ewing CP, Schirm M, Lorenzo M, Kelly J, Pattarini D, Majam G, Thibault P, Logan S (2006) Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. Mol Microbiol 60:299–311

Guignot J, Hudault S, Kansau I, Chau I, Servin AL (2009) Human deca-accelerating factor and CEACAM receptor-mediated internalization and intracellular lifestyle of Afa/Dr diffusely adhering *Escherichia coli* in epithelial cells. Infect Immun 77:517–531

Haataja S, Tikkanen K, Hytönen J, Finne J (1996) The Galα(1–4)Gal-binding adhesion of *Streptococcus suis*, a Gram-positive meningitis associated bacterium. Adv Exp Med Biol 408:25–34

Hagan EC, Mobley HL (2007) Uropathogenic *Escherichia coli* outer membrane antigens expressed during urinary tract infection. Infect Immun 75:3941–3949

Hagman MM, Dale JB, Stevens DL (1999) Comparison of adherence to and penetration of a human laryngeal epithelial cell line by group A streptococci of various M protein types. FEMS Immunol Med Microbiol 23:195–204

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108

Hasman H, Schembri MA, Klemm P (2000) Antigen 43 and type 1 fimbriae determine colony morphology of *Escherichia coli* K-12. J Bacteriol 182:1089–1095

Heilmann C, Niemann S, Sinha B, Herrmann M, Kehrel BE, Peters G (2004) *Staphylococcus aureus* fibronectin-binding protein (FnBP)-mediated adherence to platelets, and aggregation of platelets induced by FnBPA but not by FnBPB. J Infect Dis 190:321–329

Henderson IR, Meehan M, Owen P (1997) Antigen 43, a phase-variable bipartite outer membrane protein, determines colony morphology and autoaggregation in *Escherichia coli* K-12. FEMS Microbiol Lett 149:115–120

Henderson IR, Navarro-Garcia F, Nataro JP (1998) The great escape: structure and function of the autotransporter proteins. Trends Microbiol 6:370–378

Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D (2004) Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev 68:692–744

Hess P, Altenhöfer A, Khan AS, Daryab N, Kim KS, Hacker J, Oelschlaeger TA (2004) A *Salmonella fim* homologue in *Citrobacter freundii* mediates invasion in vitro and crossing of the blood-brain barrier in the rat pub model. Infect Immun 72:5298–5307

Hinnebusch BJ, Erickson DL (2008) *Yersinia pestis* biofilm in the flea vector and its role in the transmission of plague. Curr Top Microbiol Immunol 322:229–248

Hofreuter D, Odenbreit S, Haas R (2001) Natural transformation competence in *Helicobacter pylori* is mediated by the basic components of a type IV secretion system. Mol Microbiol 41:379–391

Hoiczyk E, Roggenkamp A, Reichenbecher M, Lupas A, Heesemann J (2000) Structure and sequence analysis of *Yersinia* YadA and *Moraxella* UspAs reveal a novel class of adhesins. EMBO J 19:5989–5999

Honarvar S, Choi BK, Schifferli DM (2003) Phase variation of the 987P-like CS18 fimbriae of human enterotoxigenic *Escherichia coli* is regulated by site-specific recombinases. Mol Microbiol 48:157–171

Hussain M, Becker K, von Eiff C, Peters G, Herrmann M (2001a) Analogs of Eap protein are conserved and prevalent in clinical *Staphylococcus aureus* isolates. Clin Diagn Lab Immunol 8:1271–1276

Hussain M, Becker K, von Eiff C, Schrenzel J, Peters G, Herrmann M (2001b) Identification and characterization of a novel 38.5-kilodalton cell surface protein of *Staphylococcus aureus* with extended-spectrum binding activity for extracellular matrix and plasma proteins. J Bacteriol 183:6778–6786

Hussain M, Heilmann C, Peters G, Herrmann M (2001c) Teichoic acid enhances adhesion of *Staphylococcus epidermidis* to immobilized fibronectin. Microb Pathog 31:261–270

Isberg RR, Barnes P (2001) Subversion of integrins by enteropathogenic *Yersinia*. J Cell Science 114:21–28

Izano EA, Sadovskaya I, Vinogradov E, Mulks MH, Velliyagounder K, Ragunath C, Kher WB, Ramasubbu N, Jabbouri S, Perr MB, Kaplan JB (2007) Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. Microb Pathog 43:1–9

Jacobs AAC, Simons BH, de Graaf FK (1987) The role of lysine-132 and arginine-136 in the receptor-binding domain of the K99 fibrillar subunit. EMBO J 6:1805–1808

Jadoun J, Burstein E, Hanski E, Sela S (1997) Proteins M6 and F1 are required for efficient invasion of group A streptococci into cultured epithelial cells. Adv Exp Med Biol 418:511–515

Jones CH, Pinker JS, Roth R, Heuser J, Nicholes AV, Abraham SN, Hultgren SJ (1995) FimH adhesion of type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. Proc Natl Acad Sci USA 92:2081–2085

Jores J, Rumer L, Wieler LH (2004) Impact of the locus of enterocyte effacement pathogenicity island on the evolution of pathogenic *Escherichia coli*. Int J Med Microbiol 294:103–113

Joyce JG, Abeygunawardana C, Xu Q, Cook JC, Hepler R, Przysiecki CT, Grimm KM, Roper K, Ip CC, Cope L, Montgomery D, Chang M, Campie S, Brown M, McNeely TB, Zorman J, Maira-Litran T, Pier GB, Keller PM, Jansen KU, Mark GE (2003) Isolation, structural characterization, and immunological evaluation of a high-molecular-weight exopolysaccharide from *Staphylococcus aureus*. Carbohydr Res 338:903–922

Kallstrom H, Liszewski MK, Atkinson JP, Jonsson AB (1997) Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. Mol Microbiol 25:639–647

Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB (1997) Enteropathogenic *E. coli* (EPEC) transfer its receptor for intimate adherence into mammalian cells. Cell 91:511–520

Khan AS, Kniep B, Oelschlaeger TA, Van Die I, Korhonen T, Hacker J (2000) Receptor structure for F1C fimbriae of uropathogenic *Escherichia coli*. Infect Immun 68:3541–3547

Khelef N, Lecuit M, Bierne H, Cossart P (2006) Species specificity of the *Listeria monocytogenes* InlB protein. Cell Microbiol 8:457–470

Kirchner M, Hener D, Meyer TF (2005) CD46 independent binding of neisserial type IV pili and the major pilus adhesin PilC to human epithelial cells. Infect Immun 73:3072–3082

Klemm P, Hjerrild L, Gjermansen M, Schembri MA (2004) Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from *Escherichia coli*. Mol Microbiol 51:283–296

Konkel ME, Klena JD, Rivera-Amill V, Monteville MR, Biswas D, Raphael B, Mickelson J (2004) Secretion of virulence proteins from *Campylobacter jejeuni* is dependent on a functional flagellar export apparatus. J Bacteriol 186:3296–3303

Korotkova N, Yarova-Yarovaya Y, Tchesnokova V, Yazvenko N, Carl MA, Stapleton AE, Moseley SL (2008) *Escherichia coli* DraE adhesion-associated bacterial internalization by epithelial cells is promoted independently by decay-accelerating factor and carcinoembryonic antigen-related cell adhesion molecule binding and does not require the DraD invasion. Infect Immun 76:3869–3880

Krishnan V, Gaspar AH, Ye N, Mandlik A, Ton-That H, Narayana SV (2007) An IgG-like domain in the minor pilin GBS52 of *Streptococcus agalactiae* mediates lung epithelial cell adhesion. Structure 15:893–903

Krukonis ES, DiRita VJ (2003) From motility to virulence: sensing and responding to environmental signals in *Vibrio cholerae*. Curr Opin Microbiol 6:186–190

Kubiet M, Ramphal R, Weber A, Smith A (2000) Pilus-mediated adherence of *Haemophilus influenzae* to human respiratory mucins. Infect Immun 68:3362–3367

Kuehn MJ, Heuser J, Normark S, Hultgren SJ (1992) P pili in uropathogenic *E. coli* are composite fibres with distinct fibrillar adhesive tips. Nature 356:252–255

Kyogashima M, Ginsburg V, Krivan HC (1989) *Escherichia coli* K99 binds to N-glycolylsialoparagloboside and N-glycolyl-GM3 found in piglet small intestine. Arch Biochem Biophys 270:391–397

Lafontaine ER, Cope LD, Aebi C, Latimer JL, McCracken GH Jr, Hansen EJ (2000) The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella catarrhalis* to human epithelial cells *in vitro*. J Bacteriol 182:1364–1373

Lawley TD, Klimke WA, Gubbins MJ, Frost LS (2003) F factor conjugation is a true type IV secretion system. FEMS Microbiol Lett 224:1–5

Lecuit M, Ohayon H, Braun L, Mengaud J, Cossart P (1997) Internalin of *Listeria monocytogenes* with an intact leucin-rich repeat region is sufficient to promote internalization. Infect Immun 65:5309–5319

Lee KK, Sheth HB, Wong WY, Sherburne R, Paranchych W, Hodges RS, Lingwood CA, Krivan H, Irvin RT (1994) The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. Mol Microbiol 11:705–713

LeMieux J, Hava DL, Basset A, Camilli A (2006) RrgA and RrgB are components of a multi-subunit pilus encoded by the *Streptococcus pnaumoniae rlrA* pathogenicity islet. Infect Immun 74:2453–2456

Linke D, Riess T, Autenrieth IB, Lupas A, Kempf A (2006) Trimeric autotransporter adhesins: variable structure, common function. Trends Microbiol 14:264–270

Lugering A, Benz I, Knochenhauer S, Ruffing M, Schmidt MA (2003) The Pix pilus adhesin of the uropathogenic *Escherichia coli* strain X2194 (O2:K(-):H6) is related to Pap pili but exhibits a truncated regulatory region. Microbiol 149:1387–1397

Lyczak JB, Cannon CL, Pier GB (2002) Lung infections associated with cystic fibrosis. Clin Microbiol Rev 15:194–222

Lyons S, Wang L, Casanova JE, Sitaraman SV, Merlin D, Gewirtz AT (2004) *Salmonella typhimurium* transcytoses flagellin via an SPI2-mediated vesicular transport pathway. J Cell Sci 117:5771–5780

Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, Egge H, Laufs R (1996) The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1, 6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 178:175–183

Mahajan A, Currie CG, Mackie S, Tree J, McAteer S, McKendrick I, McNeilly T, Roe A, La Ragione RM, Woodward MJ, Gally DL, Smith DGE (2009) An investigation of the expression and adhesion function of H7 flagella in the interaction of *Escherichia coli* O157:H7 with bovine intestinal epithelium. Cell Microbiol 11:121–137

Maier B, Potter L, So M, Long CD, Seifert HS, Sheetz MP (2002) Single pilus motor forces exceed 100 pN. Proc Natl Acad Sci USA 99:16012–16017

Maisey HC, Hensler M, Nizet V, Doran KS (2007) Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. J Bacteriol 189:1464–1467

Malorny B, Morelli G, Kusecek B, Kolberg J, Achtman M (1998) Sequence diversity, predicted two-dimensional protein structure, and epitope mapping of neisserial Opa proteins. J Bacteriol 180:1323–1330

Mandlik A, Swierczynski A, Das A, Ton-That H (2007) *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. Mol Microbiol 64:11–124

Massad G, Fulkerson JF, Watson C, Mobley HL (1996) *Proteus mirabilis* ambient-temperature fimbriae: cloning and nucleotide sequence of the *aft* gene cluster. Infect Immun 64:4390–4395

Mattick JS (2002) Type IV pili and twitching motility. Annu Rev Microbiol 56:289–314

McCaw SE, Liao EH, Gray-Owen SD (2004) Engulfment of *Neisseria gonorrhoeae*: revealing distinct processes of bacterial entry by individual carcinoembryonic antigen-related cellular adhesion molecule family receptors. Infect Immun 72:2742–2752

McClelland M, Sanderson KE, Clifton SW, Latreille P, Prowollik S, Sabo A, Meyer R, Bierei T, Ozersky P, Mclelan Harkins CR, Wang C, Nguyen C, Berghoff A, Elliot G, Kohlberg S, Strong C, Du F, Carter J, Kremizki C, Layman D, Leonhadr S, Sun H, Fulton C, Magrini V, Nhan M, Warren W, Florea L, Spith J, Wilson RK (2004) Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. Nat Genet 36:1268–1274

Mengaud J, Ohayon H, Gounon P, Mege RM, Cossart P (1996) E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. Cell 84:923–932

Mergenhagen SE, Sandberg AL, Chassy BM, Brennan MJ, Yeung MK, Donkersloot JA, Cisar JO (1987) Molecular basis of bacterial adhesion in the oral cavity. Rev Infect Dis 9(Suppl 5): S467–S274

Merino N, Toledo-Arana A, Vergara-Irigaray M, Valle J, Solano C, Calvo E, Lopez JA, Foster TJ, Penades JR, Lasa I (2009) Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J Bacteriol 191:832–843

Mishra A, Das A, Cisar JO, Ton-That H (2007) Sortase-catalyzed assembly of distinct heteromeric fimbriae in *Actinomyces naeslundii*. J Bacteriol 189:3156–3165

Monteiro C, Saxena I, Wang X, Kader A, Bokranz W, Simm R, Nobles D, Chromek M, Brauner A, Brown RM Jr, Römling U (2009) Characterization of cellulose production in *Escherichia coli* Nissle 1917 and its biological consequences. Environ Microbiol 11:1105–1116

Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, Maggi T, Taddei AR, Grandi G, Telford JL (2005) Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci USA 102:15641–15646

Morschhäuser J, Vetter V, Emödy L, Hacker J (1994) Adhesion regulatory genes within large, unstable DNA regions of pathogenic *Escherichia coli*: cross-talk between different adhesin gene clusters. Mol Microbiol 11:555–566

Nitsche-Schmitz DP, Rohde M, Chhatwal GS (2007) Invasion mechanisms of Gram-positive pathogenic cocci. Thromb Haemost 98:488–496

Nuccio S-P, Bäumler AJ (2007) Evolution of the chaperone/usher assembly pathway: fimbrial classification goes greek. Microbiol Mol Biol Rev 71:551–575

Olsen A, Wick MJ, Mörgelin M, Björck L (1998) Curli, fibrous surface proteins of *Escherichia coli*, interact with major histocompatiblity complex class I moelcules. Infect Immun 66:944–949

Österlund A, Popa R, Nikkilä T, Scheynius A, Engstrand L (1997) Intracellular reservoir of *Streptococcus pyogenes* in vivo: a possible explanation for recurrent pharyngotonsilitis. Laryngoscope 107:640–647

O'Toole GA, Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304

Ottemann KM, Lowethal AD (2002) *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. Infect Immun 70:1984–1990

Owen P, Meehan M, de Loughry-Doherty H, Henderson I (1996) Phase-variable outer membrane proteins in *Escherichia coli*. FEMS Immunol Med Microbiol 16:63–76

Parkkinen J, Rogers GN, Korhonen T, Dahr W, Finne J (1986) Identification of the O-linked sialyloligosaccharides of glycophorin A as the erythrocyte receptors for S-fimbriated *Escherichia coli*. Infect Immun 54:37–42

Patti JM, Allen BL, McGavin MJ, Hook M (1994) Mscramm-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol 48:585–617

Pelicic V (2008) The type IV pili: e pluribus unum? Mol Microbiol 68:827–837

Pichon C, Hechard C, du Merle L, Chaudray C, Bonne I, Gaudagnini S, Vandewalle A, Le Bouguenec C (2009) Uropathogenic *Escherichia coli* AL511 requires flagellum to enter renal collecting duct cells. Cell Microbiol 11:616–628

Pratt LA, Kolter R (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 30:285–293

Prigent-Combaret C, Prensier G, Le Thi TT, Vidal O, Lejeune P, Dorel C (2000) Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli, and colanic acid. Environ Microbiol 2:450–464

Proft T, Baker EN (2009) Pili in Gram-negative and Gram-positive bacteria – structure, assembly and their role in disease. Cell Mol Life Sci 66:613–635

Rakotoarivonina H, Jubelin G, Hebraud M, Gaillar-Martinie B, Forano E, Mosoni P (2002) Adhesion to cellulose of the Gram-positive bacterium *Ruminococcus albus* involves type IV pili. Microbiol 148:1871–1880

Ramos HC, Rumbo M, Sirard J-C (2004) Bacterial flagellins: mediators of pathogenicity and host immune response in mucosa. Trends Microbiol 12:509–517

Ramphal R, Carnoy C, Fievre S, Michalski J-C, Houdret N, Lamblin G, Stercker G, Roussel P (1991) *Pseudomonas aeruginosa* recognizes carbohydrate chains containing type 1 (Galβ1-3GlcNAc) or type 2 (Galβ1-4GlcNAc) disaccharide units. Infect Immun 59:700–704

Ray SK, Rajeshwari R, Sharma Y, Sonti RV (2002) A high-molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. *oryzae* exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence. Mol Microbiol 46:637–647

Reidl S, Lehmann A, Schiller R, Khan SA, Dobrindt U (2009) Impact of O-glycosylation on the molecular and cellular adhesion properties of the *Escherichia coli* autotransporter protein Ag43. Int J Med Microbiol 299:398–401

Riess T, Andersson SG, Lupas A, Schaller M, Schafer A, Kyme P, Martin J, Walzlein JH, Ehehalt U, Lindroos H, Schirle M, Nordheim A, Autenrieth IB, Kempf VA (2004) *Bartonella adhesin* a mediates a proangiogenic host cell response. J Exp Med 200:1267–1278

Robinson LS, Ashman EM, Hultgren SJ, Chapman MR (2006) Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein. Mol Microbiol 59:870–881

Roche A, McFadden J, Owen P (2001) Antigen 43, the major phase-variable protein of the *Escherichia coli* outer membrane, can exist as a family of proteins encoded by multiple alleles. Microbiology 147:161–169

Rochon M, Römling U (2006) Flagellin in combination with curli fimbriae elicits an immune response in the gastrointestinal epithelial cell line HT-29. Microbes Infect 8:2027–2033

Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JK, Heilmann C, Herrmann M, Mack D (2005) Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol 55:1883–1895

Römling U (2005) Characterization of the rdar morphotype, a multicellular behaviour in *Enterobacteriaceae*. Cell Mol Life Sci 62:1234–1246

Rossini R, Rinaudo CD, Soriani M, Lauer P, Mora M, Maione D, Taddei A, Santi I, Ghezzo C, Brettoni C, Buccato S, Margarit I, Grandi G, Telford JL (2006) Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. Mol Microbiol 61:126–141

Roy K, Hilliard GM, Hamilton DJ, Luo J, Ostmann MM, Fleckenstein JM (2009) Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between flagella and host cells. Nature 457:594–560

Sandt CH, Hopper JE, Hill CW (2002) Activation of prophage *eib* genes for immunoglobulin-binding proteins by genes from the IbrAB genetic island of *Escherichia coli* ECOR-9. J Bacteriol 184:3640–3648

Schembri MA, Klemm P (2001) Coordinate gene regulation by fimbriae-induced signal transduction. Embo J 20:3074–3081

Schubert W-D, Göbel G, Diepholz M, Darji A, Kloer D, Hain T, Chakraborty T, Wehland J, Domman E, Heinz DW (2001) Internalins from the human pathogen *Listeria monocytogenes* combine threee distinct folds into a contiguous internalin domain. J Mol Biol 312:783–794

Serruto D, Spadafina T, Scarselli M, Bambini S, Comaducci M, Höhle S, Kilian M, Veiga E, Cossart P, Oggioni MR, Savino S, Ferlenghi I, Taddei AR, Rappuoli R, Pizza M, Masignani V, Arico B (2009) Had A is an atypical new multifunctional trimeric coiled-coil adhesion of *Haemophilus influenzae* biogroup aegypticus, which promotes entry into host cells. Cell Microbiol 11:1044–1063

Shen Y, Naujokas M, Park M, Ireton K (2000) InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. Cell 103:501–510

Sherlock O, Dobrindt U, Jensen JB, Munk V, Klemm P (2006) Glycosylation of the self-recognizing *Escherichia coli* Ag43 autotransporter protein. J Bacteriol 188:1798–1807

Sillanpää J, Nallapareddy SR, Prakash VP, Qin X, Höök M, Weinstock GM, Murray BE (2008) Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*. Microbiol 154:3199–3211

Sloan GP, Love CF, Sukumar N, Mishra M, Deora R (2007) The *Bordetella* Bps polysaccharide is critical for biofilm development in the mouse respiratory tract. J Bacteriol 189:8270–8276

Smith GS, Portnoy DA (1997) How the *Listeria monocytogenes* ActA protein converts actin polymerization into a motile force. Trends Microbiol 5:272–276

Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, Cookson BT, Aderem A (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 4:1247–1253

Soto GE, Hultgren SJ (1999) Bacterial adhesins: common themes and variations in architecture and assembly. J Bacteriol 181:1059–1071

St Geme JW 3rd, Cutter D (2000) The *Haemophilus influenzae* Hia adhesin is an autotransporter protein that remains uncleaved at the C terminus and fully cell associated. J Bacteriol 182:6005–6013

Stapleton AE, Stroud MR, Hakomori SI, Stamm WE (1998) The globoseries glycosphingolipid sialosyl galactosyl globoside is found in urinary tissue and is a preferred binding receptor in vitro for uropathogenic *Escherichia coli* expressing *pap*-encoded adhesins. Infect Immun 66:3856–3861

Svenson SB, Hultberg H, Kallenius G, Korhonen K, Mollby R, Winberg J (1983) P-fimbriae of pyelonephritogenic *Escherichia coli*: identification and chemical characterization of receptors. Infection 11:61–67

Tacket CO, Taylor RD, Losonsky G, Lim Y, Nataro JP, Kaper JB, Levine MM (1998) Investigation of the roles of toxin-coregulated pili and mannose sensitive hemagglutinin pili in the pathogenesis of *Vibrio cholerae* O139 infection. Infect Immun 66:692–695

Taguchi F, Takeuchi K, Katoh E, Murata K, Suzuki T, Marutani M, Kawasaki T, Eguchi M, Katoh S, Kaku H, Yasuda C, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y (2006) Identification of glycosylation genes and glycosylated amino acids of flagellin in *Pseudomonas syringae* pv. *tabaci*. Cell Microbiol 8:923–938

Takamatsu D, Bensing BA, Cheng H, Jarvis GA, Siboo IR, Lopez JA, Griffiss JM, Sullam PM (2005) Binding of the *Streptococcus gordonii* surface glycoproteins GspB and Hsa to specific carbohydrate structures on platelet membrane glycoprotein Ibalpha. Mol Microbiol 58:380–392

Tarkkanen A-M, Westerlund-Wikström B, Erkkilä L, Korhonen TK (1998) Immunohistological localization of the MrkD adhesion in the type 3 fimbriae of *Klebsiella pneumoniae*. Infect Immun 66:2356–2361

Tasteryre A, Barc M-C, Collignon A, Boureau H, Karjalainen T (2001) Role of FliC and FliD flagellar proteins of *Clostridium difficile* in adherence and gut colonization. Infect Immun 69:937–7940

Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G (2006) Pili in gram-positive pathogens. Nat Rev Microbiol 4:509–519

Tobiason DM, Seifert HS (2001) Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. Microbiology 147:2333–2340

Tomaras AP, Dorsey CW, Edelmann RE, Actis LA (2003) Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumanii*: involvement of a novel chaperon-usher pili assembly system. Microbiol 149:3473–3484

Torres AG, Perna NT, Burland V, Ruknudin A, Blattner FR, Kaper JB (2002) Characterization of Cah, a calcium-binding and heat-extractable autotransporter protein of enterohaemorrhagic *Escherichia coli*. Mol Microbiol 45:951–966

Townsend SM, Kramer NE, Edwards R, Baker S, Hamlin N, Simmonds M, Stevens K, Maloy S, Parkhill J, Dougan G, Bäumler AJ (2001) *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infect Immun 69:2894–2901

Trautner BW, Darouiche RO (2004) Role of biofilm in catheter-associated urinary tract infection. Am J Infect Control 32:177–183

Tümmler B, Kiewitz C (1999) Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. Mol Med Today 5:351–358

Uemura T (1998) The cadherin superfamily at the synapse: more members, more missions. Cell 93:1095–1098

Uhlich GA, Keen JE, Elder RO (2002) Variations in the *csgD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. Infect Immun 70:395–399

Uhlich GA, Gunther NW, Bayles DO, Mosier DA (2009) The CsgA and Lpp proteins of an *Escherichia coli* O157:H7 strain affect Hep-2 cell invasion, motility, and biofilm formation. Infect Immun 77:1543–1552

Ulett GC, Valle J, Beloin C, Sherlock O, Ghigo JM, Schembri MA (2007) Functional analysis of antigen 43 in uropathogenic *Escherichia coli* reveals a role in long-term persistence in the urinary tract. Infect Immun 75:3233–3244

Valle J, Mabbett AN, Ulett GC, Toledo-Arana A, Wecker K, Totsika M, Schembri MA, Ghigo JM, Beloin C (2008) UpaG, a new member of the trimeric autotransporter family of adhesins in uropathogenic *Escherichia coli*. J Bacteriol 190:4147–4161

Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. Proc Natl Acad Sci USA 98:6911–6916

Varga JJ, Nguyen V, O'Brien DK, Rodgers K, Walker RA, Melville SB (2006) Type IV pili-dependent gliding motility in the Gram-positive pathogen *Clostridium perfringens* and other *Clostridia*. Mol Microbiol 62:680–694

Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A (2005) The *pel* genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology 151:985–997

Vidal O, Longin R, Prigent-Combaret C, Dorel C, Hooreman M, Lejeune P (1998) Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new ompR allele that increases curli expression. J Bacteriol 180:2442–2449

Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 6:269–275

Wang X, Preston JF 3rd, Romeo T (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J Bacteriol 186: 2724–2734

Wang X, Rochon M, Lamprokostopoulou A, Lunsdorf H, Nimtz M, Römling U (2006) Impact of biofilm matrix components on interaction of commensal *Escherichia coli* with the gastrointestinal cell line HT-29. Cell Mol Life Sci 63:2352–2363

Wells TJ, Sherlock O, Rivas L, Mahajan A, Beatson SA, Torpdahl M, Webb RI, Allsopp LP, Gobius KS, Gally DL, Schembri MA (2008) EhaA is a novel autotransporter protein of enterohemorrhagic *Escherichia coli* O157:H7 that contributes to adhesion and biofilm formation. Environ Microbiol 10:589–604

Xiong YQ, Bensing BA, Bayer AS, Chambers HF, Sullam PM (2008) Role of the serin-rich surface glycoprotein GspB of *Streptococcus gordonii* in the pathogenesis of infective endocarditis. Microb Pathog 45:297–301

Young GM, Schmiel DH, Miller VL (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein secretion system. Proc Natl Acad Sci USA 96:6456–6461

Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacINyre S, Knight SD (2003) Structure and biogenesis of the capsular F1 antigen from *Yersinia pestis*: preserved folding energy drives fiber formation. Cell 113:587–596

Zhang P, Schau CBB, MK GJS, Kelminson KL, George SS, Lerche NW, Koehler JE (2004) A family of variably expressed outer-membrane proteins (Vomp) mediates adhesion and autoaggregation in *Bartonella quintana*. Proc Natl Acad Sci USA 101:13630–13635

Zhu G, Chen H, Choi BK, Del Piero F, Schifferli DM (2005) Histone H1 proteins act as receptors for the 987P fimbriae of enterotoxigenic *Escherichia coli*. J Biol Chem 280:23057–23065

Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling U (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39:452–1463

Part IV
Cell Wall Growth and Inhibition

Chapter 12
Cell Wall Targeted Antibiotics

Regine Hakenbeck, Reinhold Brückner, and Bernhard Henrich

12.1 Introduction

Peptidoglycan or murein is a unique cell-sized macromolecule named the sacculus located outside the cytoplasmic membrane (Weidel and Pelzer 1964; Vollmer et al. 2008a). It protects the cell against osmotic pressure, and is involved in maintenance of the specific cell shape. Moreover it serves as the anchoring platform for proteins and polysaccharides that are part of the cell envelope. Biochemically it is a heteropolymer whose long glycan chains are made of alternating units of *N*-acetylmuramoyl-(MurNAc-)peptides and *N*-acetylglucosamine (GlcNAc) that are crosslinked via short peptide side chains. Murein is an essential component for eubacteria, and its complex biosynthetic machinery has no equivalent in mammalian cells. Since the discovery that penicillin interferes with murein biosynthesis, the bacterial cell wall has become the center of interest for the identification of biosynthetic steps that are specific targets for known antibiotics and potential ones for novel antimicrobial substances (Rogers et al. 1980; Green 2002; van Heijenoort 2007).

Murein biosynthesis can be divided into three stages according to the cellular compartment where they take place: cytoplasmic steps responsible for the synthesis of muropeptide precursors, reactions that occur on the inner side of the cytoplasmic membrane involving the undecaprenyl-phosphate carrier lipid (C_{55}-P; bactoprenol), and the final polymerization and assembly of the macromolecule in the periplasm (see Breukink and de Kruijff 2006; Bouhss et al. 2008; Barreteau et al. 2008; Zapun et al. 2008 for review).

During the first stage, UPD-MurNAc is produced from fructose-6-phosphate by the GlmS, GlmM and GlmU enzymes. Then, the soluble murein nucleotide precursor UPD-MurNAc-pentapeptide is synthesized by a series of enzymes, the

R. Hakenbeck (✉)
Department of Microbiology, University of Kaiserslautern, Paul Ehrlich Str., Geb. 23, 67663 Kaiserslautern, Germany
e-mail: hakenb@rhrk.uni-kl.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_12,

Mur ligases MurC–F which are responsible for the respective addition of L-Ala, D-Glu, *m*-DAP or L-Lys, and the dipeptide D-Ala-D-Ala (Wang et al. 2009) (Fig. 12.1a). Inhibitors of GlmS-, GlmU- and MurB-catalyzed reactions will not be considered here since none of them is used therapeutically; they have been reviewed recently (Barreteau et al. 2008). In Gram-negative bacteria, the nonessential MpI (murein peptide ligase) adds the tripeptide L-Ala-D-Glu-*m*DAP (*m*-Lys) to UDP-MurNAc, thus participating in recycling of murein (Mengin-Lecreulx et al. 1996; Herve et al. 2007). The UPD-MurNAc-pentapeptide is then transferred to the C_{55}-P lipid carrier undecaprenylphosphate by the transferase/translocase MraY yielding lipid I, followed by MurG-catalyzed transfer of GlcNAc from UDP-GlcNAc resulting in lipid II (Bouhss et al. 2008). Lipid II has to be transferred through the membrane by a still unknown proposed flippase, to serve as the substrate for the polymerization reactions carried out by membrane-associated enzymes, the multidomain penicillin-binding proteins (Fig. 12.1b). After dephosphorylation, the C_{55}-PP can enter the cycle again. Whether this reaction, which is carried out by the BacA (Upp) phosphatase, occurs on both sides on the membrane or only on one side is unknown. It is noteworthy that the lipid carrier is essential in the assembly of all important cell wall polysaccharides including teichoic acid, capsule and LPS.

There are many naturally produced antibiotics identified in early biological tests whose characterization later identified the bacterial cell wall as the target molecule. The focus of many recent screens for new antimicrobial compounds is directed to specific target-based screening programs based on enzyme activities of the murein biosynthesis pathway. The following sections will describe the main families of therapeutically relevant inhibitors specific for individual steps of the murein biosynthetic pathway, their target molecules and resistance mechanisms. Moreover, two bacterial proteins – colicin M and BLIP – will briefly be discussed since they reveal novel mechanisms of interference with important target molecules relevant for antibiotic action.

12.2 D-Cycloserine

D-Cycloserine (Fig. 12.2a) (oxamycin or D-4-amino-3-isoxazolidone) is an oxazolidinone antibiotic produced by various *Streptomyces* species such as *S. garyphalus*, *S. archidaceus* or *S. lavendulae*. It was the first antibiotic whose mechanism of action was established at the molecular level (Neuhaus 1967). Technically, it can be synthesized from the amino acid D-serine.

D-Cycloserine acts as a cyclic analogue of D-Ala which is an essential compound for peptidoglycan crosslinking. It competitively inhibits the enzymatic activities of both D-Ala racemase (DadX/Alr) and D-Ala-D-Ala ligase (Ddl) (David et al. 1969; Lambert and Neuhaus 1972). Recent data confirmed that Ddl is also essential for growth in *Mycobacterium smegmatis* in medium lacking D-Ala, similar to other bacteria (Milligan et al. 2007). However in *Mycobacterium* spp., which are among the most sensitive bacteria to D-cycloserine, Ddl is probably not significantly

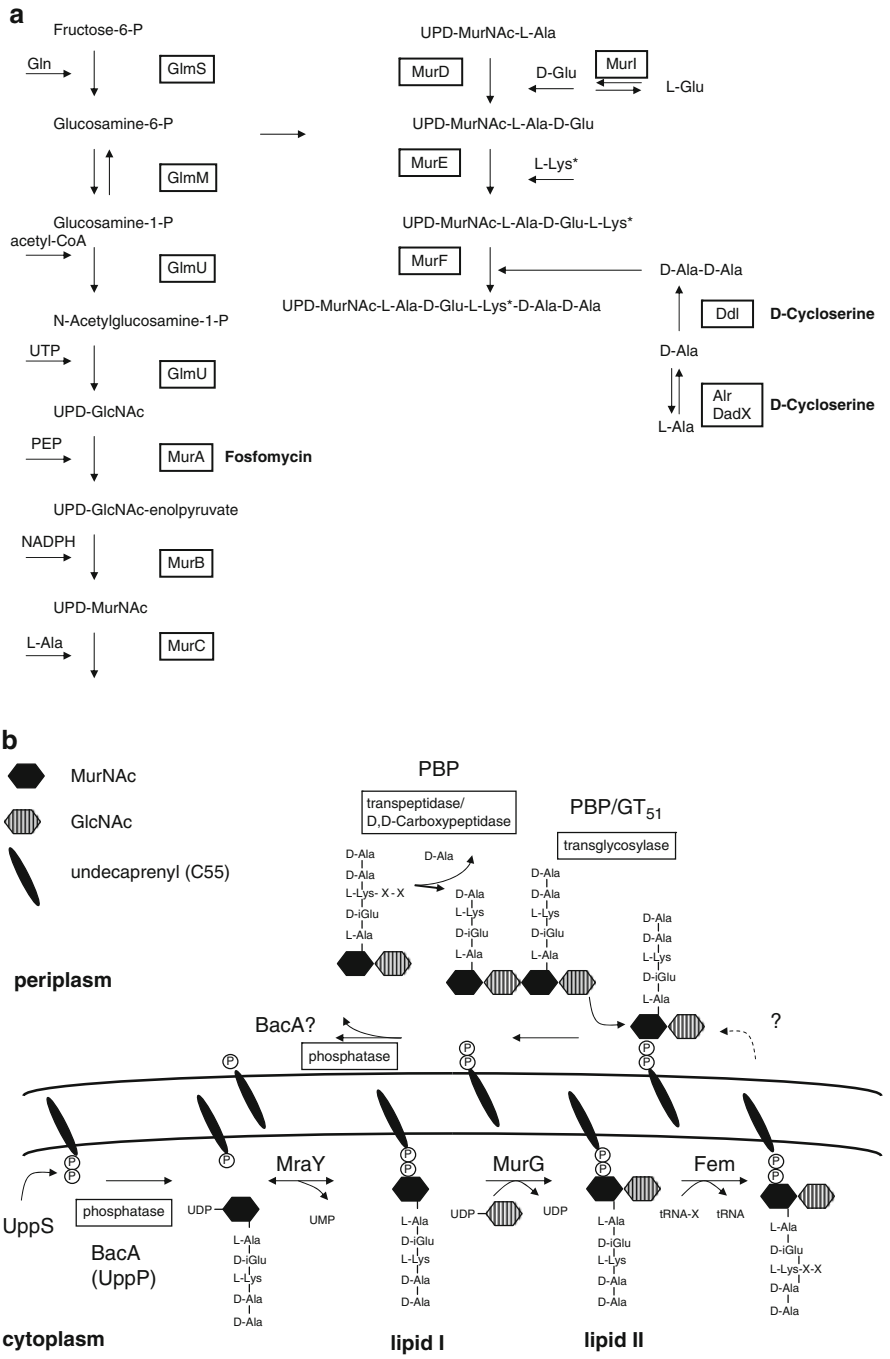

Fig. 12.1 Peptidoglycan biosynthesis. (**a**) Cytoplasmic steps. (**b**) Membrane-associated steps and periplasmic polymerization reactions

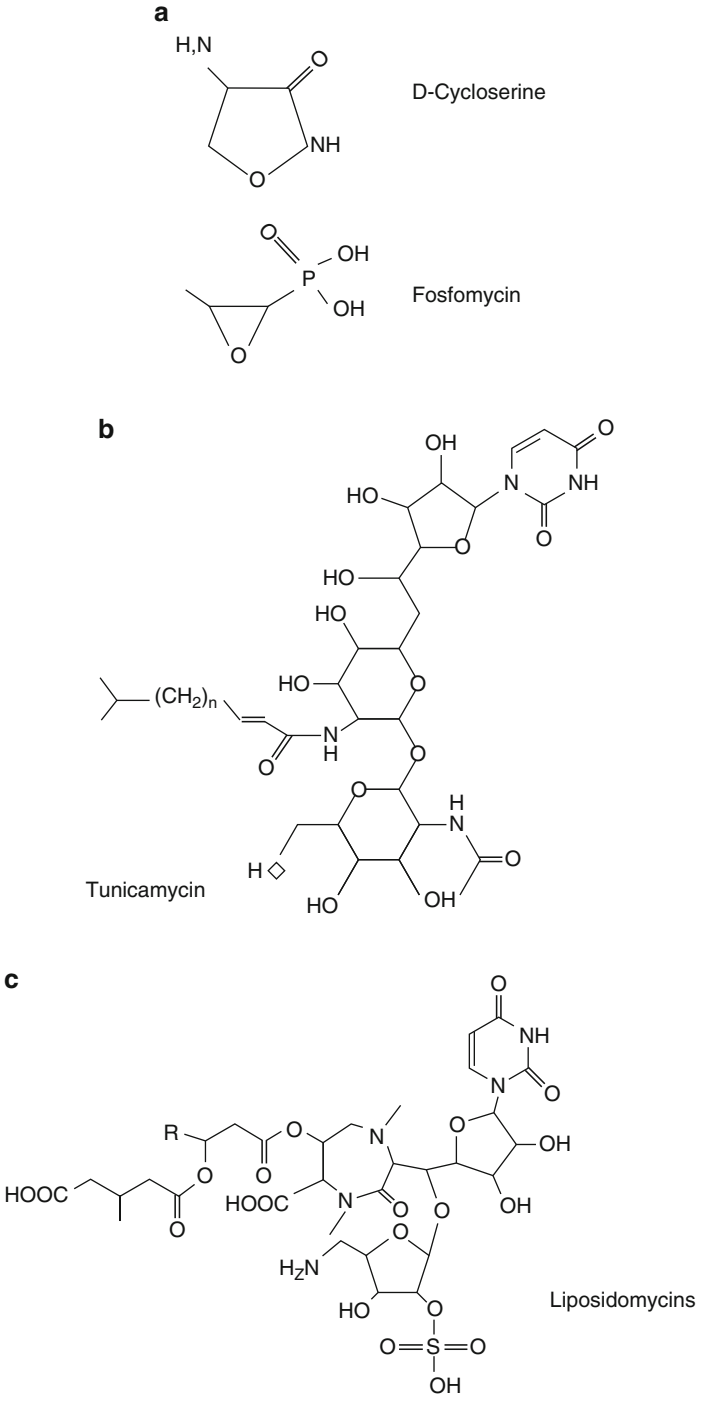

Fig. 12.2 (continued)

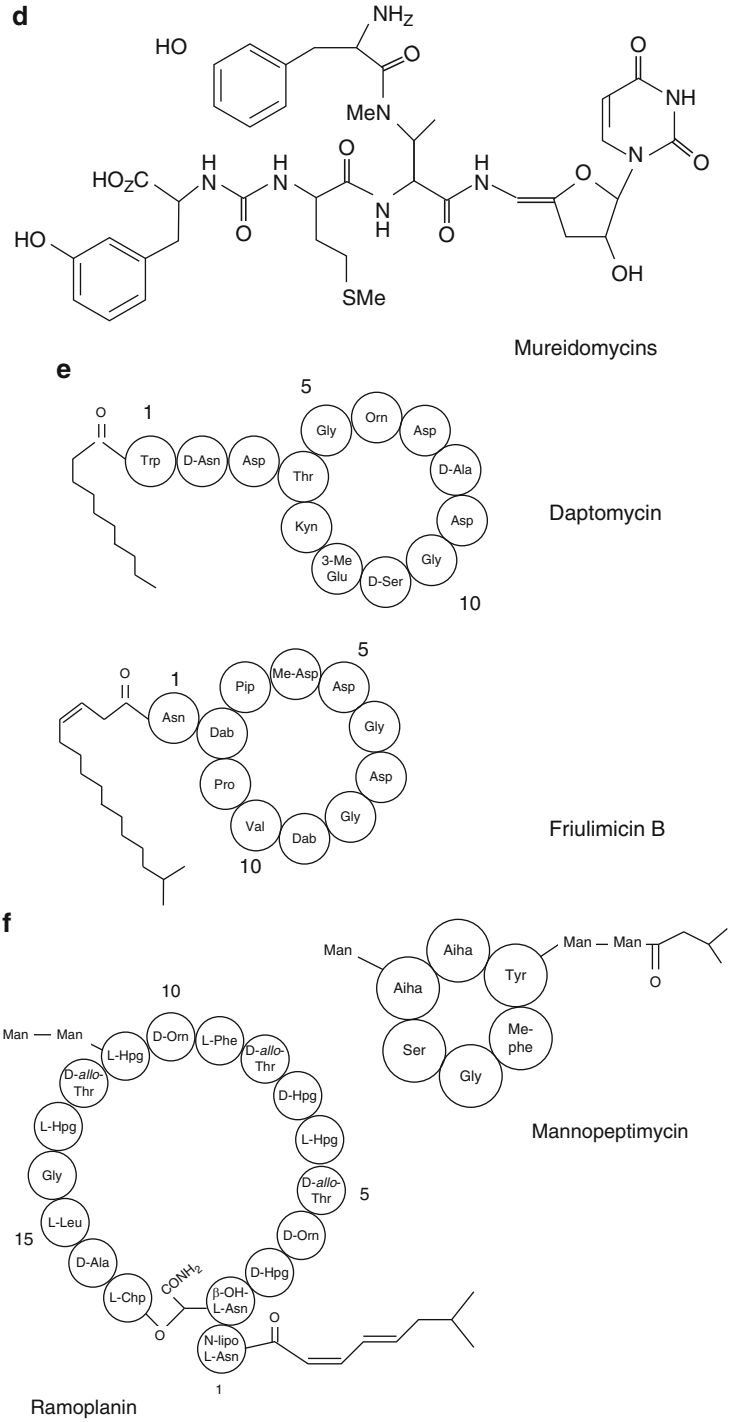

Fig. 12.2 (continued)

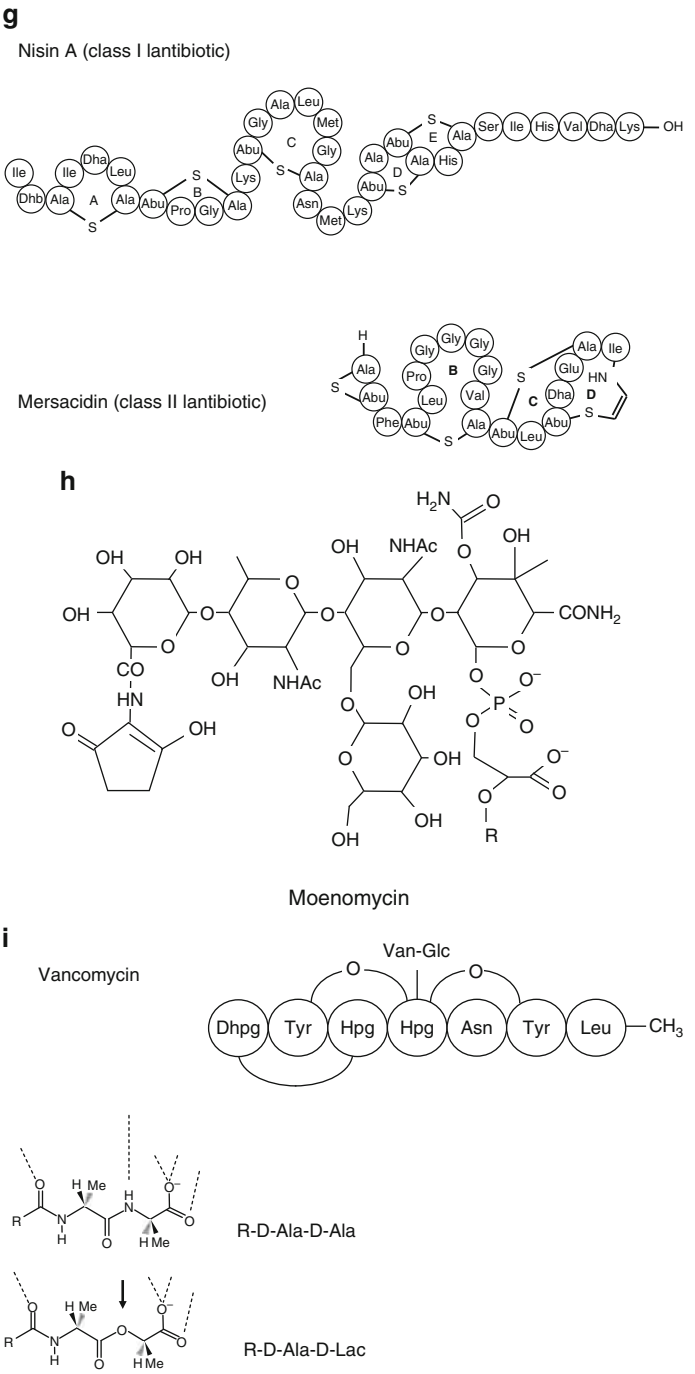

Fig. 12.2 (continued)

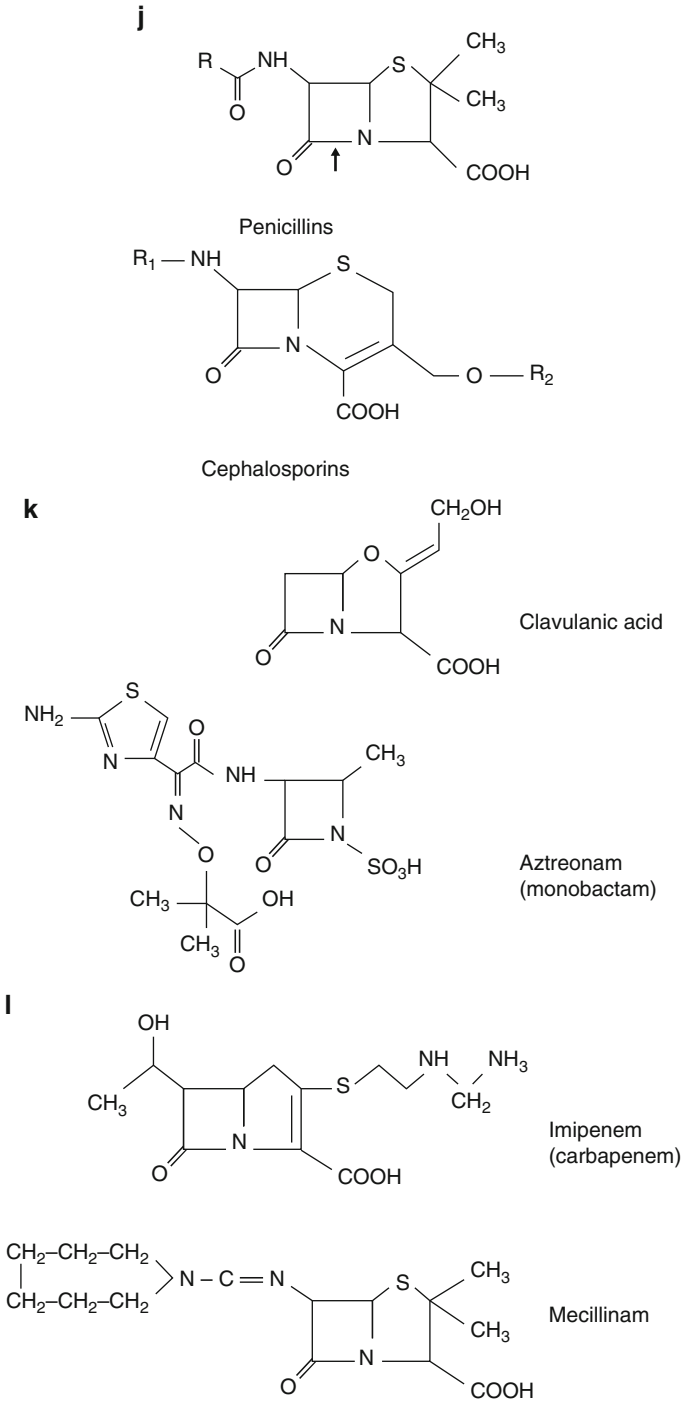

Fig. 12.2 (continued)

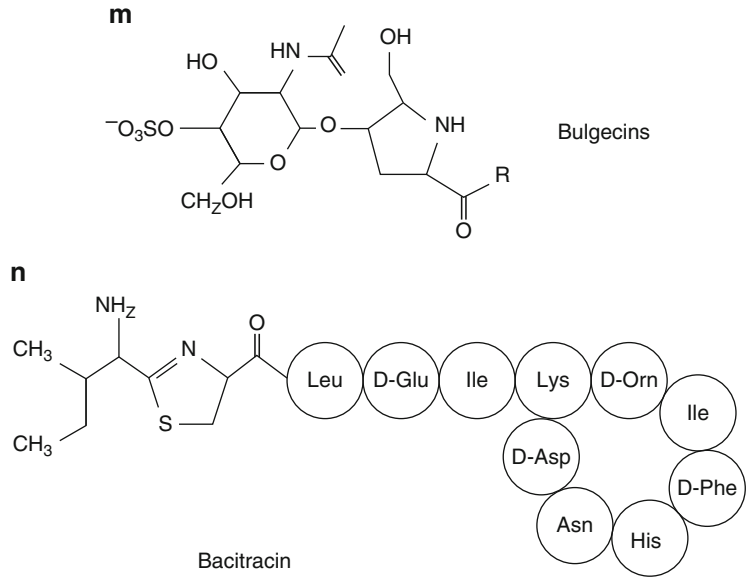

Fig. 12.2 Cell wall targeting antibiotics. (**a**) D-Cycloserine and fosfomycin. (**b**) Tunicamycin. (**c**) Liposidomycin. (**d**) Mureidomycin. (**e**) Daptomycin and friulimicin. (**f**) Mannopeptimycin and ramoplanin. (**g**) Nisin and mersacidin. (**h**) Moenomycin. (**i**) Vancomycin. (**j**) Penicillin and cephalosporin. (**k**) Clavulanic acid and aztreonam. (**l**) Imipenem and mecillinam. (**m**) Bulgecin. (**n**) Bacitracin. Amino acids are indicated by the three-letter code. Unusual amino acids and other molecules: Aiha, α-amino-β-[4′-(2′-iminoimidazolidinyl)]-β-hydroxypropionic acid; Chp, 3-chloro-4-hydroxy-phenylglycine; Dab: diaminobutyric acid; Dha: didehydroalanine; Dhb: (Z)-2,3-didehydrobutyrine; Dhpg, dihydroxyphenylglycine; Glc, glucose; HAsn, β-hydroxyasparagine; HAsp, β-hydroxyaspartic acid; Hleu, β-hydroxyleucine; Hpg, hydroxyphenylglycine; HPro, 3-hydroxyproline; Kyn: kynurenin; Man, mannose; Me-Asp: methylasparatic acid; Me-phe, β-methyl-phenylalanine; Orn, ornithine; PhSer, β-phenylserine; pip: pipecolinic acid; Van, vancosamine

inhibited by D-cycloserine at concentrations that inhibit Alr (Feng and Barletta 2003). Treatment with D-cycloserine leads to the accumulation of the peptidoglycan precursor UDP-glycolylmuramyl tripeptide in mycobacteria (Takayama et al. 1970; Mahapatra et al. 2005). D-Cycloserine also inhibits the synthesis of D-Ala-containing mycobacterial cell wall peptidoglycolipids, such as wax-D and mycoside-C (David et al. 1988).

Although in principle active against all types of eubacteria, D-cycloserine is mainly used as a second-line antibiotic for the treatment of (chronic) tuberculosis caused by multidrug-resistant strains of *M. tuberculosis* (Johnson et al. 2006). Use of D-cycloserine in clinical medicine is restricted because, at effective doses, it causes severe psychotic side effects such as seizures, schizophrenia, depression, and suicidal tendencies. Agonist activity of D-cycloserine against *N*-methyl-D-aspartate receptors in the amygdala may contribute to these effects (Pitkanen et al. 1995). In fact, D-cycloserine has only recently been investigated as an augmentation therapeutic for psychological treatment of anxiety disorders (Hofmann et al. 2006).

In *Escherichia coli*, D-cycloserine is taken up via the D-alanine-glycine transporter CycA, and mutations in the *cycA* gene mediate first-step resistance to the drug (Wargel et al. 1971; Russell 1972). Similarly, a single transport system accumulates L- and D-Ala, Gly and D-Ser in *M. tuberculosis*, and D-cycloserine-resistant mutants defective in this system have been described (David 1971). Moreover, mutations causing the overproduction of D-alanine racemase decrease the susceptibility of mycobacteria to the antibiotic (Caceres et al. 1997). A novel mechanism involving peptidoglycan assembly at the cell surface has been proposed to explain simultaneous resistance of an *M. smegmatis* mutant to both D-cycloserine and vancomycin (Peteroy et al. 2000).

12.3 Fosfomycin

Fosfomycin, a phosphonic acid derivative (*cis*-1,2-epoxypropyl phosphonic acid, [(2*R*,3*S*)-3-methyloxiran-2-yl]phosphonic acid (Fig. 12.2a) also known as phosphomycin or phosphonomycin) (Christensen et al. 1969), was originally isolated from cultures of *Streptomyces* species (Hendlin et al. 1969) and has a broad-spectrum activity against Gram-positive and Gram-negative bacteria. Today, synthetically prepared fosfomycin tromethamine, a soluble salt with improved bioavailability, is indicated in the treatment of uncomplicated urinary tract infections (UTIs) caused by *E. coli* and *Enterococcus faecalis*. Additional applications, especially against multidrug-resistant pathogens, have recently been proposed (Falagas et al. 2008).

Fosfomycin has a bactericidal mechanism of action. It is an antimetabolite of phosphoenolpyruvate in the enzymatic synthesis of the *N*-acetylmuramic acid component of the bacterial cell wall. In detail, the epoxide ring of fosfomycin covalently and irreversibly alkylates the thiol group of a cysteine residue (Cys-115 in *E. coli*) in the active site of UDP-*N*-acetylglucosamine-enolpyruvyltransferase (MurA) which catalyzes the transfer of enolpyruvate from phosphoenolpyruvate to UDP-*N*-acetylglucosamine (Kahan et al. 1974). The MurA enzymes of, e.g., *M. tuberculosis* and *Chlamydia trachomatis*, species which are naturally resistant to fosfomycin, contain an Asp instead of the Cys residue (De Smet et al. 1999; McCoy and Maurelli 2005). In contrast to Gram-negative organisms, Gram-positive bacteria contain two copies of *murA* which have probably arisen by gene duplication, both of which may be fosfomycin-sensitive (Du et al. 2000). A number of novel inhibitors of MurA have been discovered through various screening efforts (Barreteau et al. 2008).

Fosfomycin enters the cells of almost all sensitive bacteria by active transport through the nonessential, partially constitutive L-α-glycerophosphate uptake system GlpT. The antibiotic can also be transported via the hexose phosphate uptake system Uhp which, however, is present only when induced by glucose-6-phosphate (Kahan et al. 1974).

Loss of a functional GlpT transport protein is the primary cause for the emergence of fosfomycin-resistant mutants (Kahan et al. 1974). Other chromosomally

encoded resistance mechanisms observed in *E. coli* include defects in the Uhp transporter (Kadner and Winkler 1973), low affinity of the MurA target enzyme towards fosfomycin (Venkateswaran and Wu 1972), and overproduction of MurA (Marquardt et al. 1992). In clinical settings, frequent development of bacterial resistance under fosfomycin treatment is due to resistance proteins which catalyze the inactivation of the antibiotic by opening the epoxide ring. The corresponding genes were initially identified on plasmids isolated from clinical isolates of *Serratia marcescens* (Mendoza et al. 1980), *Klebsiella pneumoniae* (O′Hara 1993), and *Staphylococcus epidermidis* (Etienne et al. 1989). Chromosomally located genes encoding fosfomycin resistance proteins have also been identified, and the structure of two such proteins from *Pseudomonas aeruginosa* and *Listeria monocytogenes* have been obtained (Rife et al. 2002; Fillgrove et al. 2007). Four different fosfomycin resistance genes have been described. The *fosA* and *fosB* genes both encode thioltransferases which catalyze the addition of either glutathione (FosA) (Bernat et al. 1997) or L-cysteine (FosB) (Cao et al. 2001) to fosfomycin. The *fosD* gene was recently detected by its similarity to *fosB* (Nakaminami et al. 2008), and FosX proteins inactivate the antibiotic by hydration (Fillgrove et al. 2007). Two additional resistance genes (*fomA*, *fomB*) conferring high-level fosfomycin resistance on *E. coli* are located in the fosfomycin biosynthetic clusters of *Streptomyces wedmorensis* (Kobayashi et al. 2000) and *Streptomyces fradiae* (Woodyer et al. 2006). Both genes specify ATP-dependent protein kinases. FomA catalyzes phosphorylation of fosfomycin, and FomB converts fosfomycin phosphate to fosfomycin diphosphate. These functions are probably involved in self-resistance mechanisms of the fosfomycin-producing bacteria (Kobayashi et al. 2000).

12.4 Nucleoside Antibiotics

The integral membrane protein phospho-*N*-acetylmuramyl-pentapeptide translocase (MraY, translocase I) catalyzes the reaction of UDP-Mur*N*Ac-pentapeptide with undecaprenylphosphate to give undecaprenyl-P-P-Mur*N*Ac-pentapeptide (lipid intermediate I) and UMP in the first step of the lipid-linked cycle of bacterial peptidoglycan synthesis. MraY is the target for several classes of uridine-containing nucleoside natural antibiotics, some of which have been studied in detail since they have a so far unexploited mode of action (Kimura and Bugg 2003; Dini 2005; Bugg et al. 2006; Bouhss et al. 2008). Translocase I is not the target of any agents in current clinical use.

12.5 Tunicamycins

Tunicamycins (Fig. 12.2b) are fatty acid nucleosides composed of uracil, *N*-acetylglucosamine, an amide-linked fatty acid, and the unique 11-carbon 2-aminodialdose sugar tunicamine. The naturally occurring tunicamycin, produced by several *Streptomyces* species, is a mixture of ten or more individual components which differ

only by the *N*-linked acyl chains. Tunicamycin specifically inhibits the formation of the *N*-acetylglucosamine-P-P-lipid intermediates in the biosynthetic pathways for a variety of glycoconjugates (Tamura 1982).

In bacteria, tunicamycin acts as an analogue of the UDP-Mur*N*Ac-pentapeptide substrate which reversibly and competitively inhibits the MraY enzyme (Brandish et al. 1996). Its use as therapeutic antibiotic, however, is precluded, since it is also toxic to mammals due to potent inhibition of GlcNAc-1-phosphotransferase in the dolichol cycle of glycoprotein biosynthesis (Heifetz et al. 1979). Accordingly, bacterial resistance to tunicamycin has not been thoroughly investigated. Resistance of *Bacillus subtilis* was related to overproduction of an ATP-binding membrane protein (TmrB) which has the ability to bind tunicamycin (Noda et al. 1992, 1995).

12.6 Liposidomycins

Liposidomycins (Fig. 12.2c) are fatty acyl nucleosides, originally isolated from *Streptomyces griseosporeus*, whose unique structures contain a sulfated aminoglycoside residue (Isono et al. 1985). In contrast to tunicamycin, they selectively inhibit the biosynthesis of undecaprenyl-P-P-MurNAc-pentapeptide, whereas glycoconjugate formation (e.g., in the biosynthesis of glycoproteins or teichoic acids) is only scarcely affected (Muroi et al. 1997; Kimura et al. 1998). Liposidomycin B was characterized as a slow-binding inhibitor of solubilized *E. coli* MraY, meaning that it acts noncompetitively against UDP-MurNAc-pentapeptide and competitively with the dodecaprenyl phosphate lipid substrate used here, which the *E. coli* MraY translocase accepts in vitro with K_m values similar to that of the natural substrate (Brandish et al. 1996).

Variants of liposidomycin were isolated which, due to the loss of the hydrophilic sulfate moiety, showed improved membrane permeability and increased antimicrobial activity (Kimura et al. 1998). Based on structure–function studies, simplified analogues of liposidomycin (named riburamycins) were developed which are powerful MraY inhibitors and possess antibacterial activity against Gram-positive bacteria including *Staphylococcus aureus* (Dini et al. 2000, 2002; Stachyra et al. 2004). Bacterial resistance to liposidomycin has not yet been documented.

12.7 Mureidomycins

Mureidomycins (Fig. 12.2d) (related to the pacidamycins and napsamycins) are peptidyl nucleosides containing a 3′-deoxyuridine sugar attached via an enamide linkage to an unusual peptide chain. The peptide chain contains an *N*-methyl-2,3-diaminobutyric acid residue, and a urea linkage to a C-terminal aromatic amino acid (Isono et al. 1989a). The mureidomycins A–D, isolated from *Streptomyces flavidoviridens* (Inukai et al. 1989), can protect mice against *Pseudomonas*

aeruginosa infection (Isono et al. 1989b). They are selectively active against species belonging to rRNA groups I and III of the genus *Pseudomonas* (Isono et al. 1992). A uridinyl dipeptide analogue of mureidomycin A retained biological activity against *Pseudomonas* (Howard and Bugg 2003). No other antibiotics have been reported to have such a selective antipseudomonal activity.

Mureidomycins selectively and specifically inhibit bacterial translocase I (MraY), while not inhibiting bacterial teichoic acid or mammalian glycoprotein biosynthesis (Isono and Inukai 1991; Inukai et al. 1993). Kinetic studies with mureidomycin A showed that it acts as a slow-binding inhibitor of MraY. Inhibition is competitive against both the soluble UDP-MurNAc-pentapeptide substrate and the undecaprenyl phosphate lipid substrate (Brandish et al. 1996). The enamide functional group is not primarily responsible for MraY inhibition (Boojamra et al. 2001), and it was proposed that binding of the amino-terminus of mureidomycin A to the Mg^{2+} cofactor-binding site is involved in inhibition of the enzyme (Howard and Bugg 2003).

12.8 Pacidamycins

The mureidomycins and the related pacidamycins suffer from the limitation that potential target pathogens such as *P. aeruginosa* develop resistance at a high frequency (Fernandes et al. 1989; Isono et al. 1989b). High intrinsic resistance of *E. coli* to mureidomycins has been explained by differential expression of appropriate efflux systems (Gotoh et al. 2003).

12.9 Muraymycins and Capuramycins

Other compounds such as the muraymycins (McDonald et al. 2002) and capuramycins (Muramatsu et al. 2003) reported only a few years ago are also potent MraY inhibitors. They are of particular interest because they are active against the "problem bacteria" staphylococci and enterococci, and *Mycobacterium* spp., respectively.

12.10 FEM Inhibitors

Most Gram-positive bacteria produce so-called branched peptidoglycan precursors due to the addition of amino acids to the diamino acid Lys or Dap (Schleifer and Kandler 1972). The side chain is also highly variable with respect to its mode of synthesis. Aspartate ligases that belong to the ATPGrasp superfamily incorporate D-Asp into the side chain by an ATP-dependent reaction (Staudenbauer and

Strominger 1972; Bellais et al. 2006), and D-Glu is probably incorporated by the same mechanism. In contrast, L-amino acids and glycine are activated as aminoacyl-t-RNAs and transferred independently from the ribosome to the murein precursor (Matsuhashi et al. 1965; Plapp and Strominger 1970). The genes encoding the transferases were identified first in *S. aureus* (Berger-Bächi et al. 1989), and were named *fem* for "factors for methicillin resistance". The Fem family belongs to the GCN5-related *N*-acetyltransferase (GNAT) protein superfamily (Berger-Bächi and Tschierske 1998). In *S. aureus*, a pentaglycin bridge is synthesized by the activity of three enzymes, FemA, B, and X, whereas in *Streptococcus pneumoniae*, for example, L-Ser and L-Ala containing peptide branches result from the activity of the MurMN (FibAB) enzymes (Mainardi et al. 2008 and references within).

Fem transferases are considered to be attractive targets especially for antimicrobial agents active against multiresistant bacteria. However, mainly due to the complexity of their nucleotide substrates, only a few studies have been performed. Inhibitors of *S. pneumoniae* MurM have recently been described: synthetic phosphonate analogues of a putative transition state intermediate (Cressina et al. 2007). A completely different type of inhibitor is represented by an analogue of Ala-$tRNA_{Ala}$ which has been shown to inhibit *S. aureus* FemX efficiently (Chemama et al. 2007); (see Mainardi et al. 2008 for details).

12.11 Cyclic Lipopeptides

The characterization of this class of antibiotics began in the 1950s with the isolation of amphomycin produced by *Streptomyces canus* (Heinemann et al. 1953) and various closely related lipopeptides (Baltz et al. 2005) including daptomycin (Fig. 12.2e) produced by *Streptomyces roseosporus* (Debono et al. 1987) and friulimicins isolated from *Actinoplanes friuliensis* (Aretz et al. 2000). Lipopeptides are secondary metabolites produced by actinomycetes as complex mixtures of compounds with variation in their fatty acid substituent. They all share a 10-membered ring structure with identical positioning of D-amino acids or achiral amino acids. Their chemistry can be manipulated by feeding with amino acid precursors of fatty acid biosynthesis during the fermentation; e.g., friumilicin B (Fig. 12.2e) is the major compound when the medium is substituted with Val. They have excellent activity against Gram-positive bacteria including MRSA, vancomycin-resistant Gram-positive cocci and penicillin-resistant *S. pneumoniae*. Daptomycin has been approved for the treatment of complicated skin and skin structure infections caused by Gram-positive pathogens and for the treatment of bacteremia and endocarditis caused by *S. aureus*; friulimicin B is still in the clinical trial phase.

The most extensively studied antibiotic of the lipopeptide family is daptomycin containing *n*-decanoyl which requires Ca^{2+} for full antibacterial activity. Early studies indicated that it inhibits peptidoglycan synthesis due to interference with precursor formation indirectly as a result of dissipation of the membrane potential (Alborn et al. 1991; Allen et al. 1991). Probably this is the result of a calcium-

dependent insertion into the cytoplasmic membrane of the fatty acid tail resulting in pore formation which results in a potassium efflux (Silverman et al. 2003). It is bactericidal in the absence of cell lysis in *S. aureus*, and an impairment of the cell division machinery has been suggested (Cotroneo et al. 2008). Other lipopeptides may act by different mechanisms. Amphomycin blocks cell wall synthesis by interfering with the transfer of UDP-MurNAc-pentapeptide to the lipid carrier undecaprenylphosphate. It was shown that it inhibited noncompetitively MraY transferase (Tanaka et al. 1982), and formed a complex with undecaprenylmonophosphate (Banerjee 1989). Considering the structural similarity of friulimicin B and amphomycin, one is tempted to speculate that both act in a similar way.

There are no known resistance mechanisms to daptomycin, although there are a few reports on resistance development during treatment (Baltz et al. 2005). So far, resistance to amphomycin or friulimicin B in pathogenic bacteria has not been reported. However, overexpression of a membrane component of an ABC transporter from the friulimicin B biosynthetic gene cluster of the producer *A. friuliensis* in *Streptomyces lividans* substantially decreased susceptibility to friulimicin B (Müller et al. 2007).

12.12 Ramoplanin

The peptide antibiotic ramoplanin (Fig. 12.2f) (ramoplanin factor A2, ramoplanin A2) is produced by the actinomycete *Actinoplanes* sp. ATCC33076 and is the predominant component of ramoplanins A1–3 (Cavalleri et al. 1984; Parenti et al. 1990). Ramoplanins are cyclic lipodepsipeptides carrying a dimannosyl group (α-D-mannoxyl-(1→2)-α-D-mannose in ramoplanin) and are differentiated by their various acylamide moieties. They contain numerous hydroxyphenylglycines and β-hydroxylated amino acids and are covalently linked at their N- and C-termini by a lactone bridge, forming a 49-membered ring (McCafferty et al. 2002).

Ramoplanin is active against a wide range of Gram-positive bacteria including enterococci, staphylococci, streptococci and also anaerobes such as *Clostridium difficile* (McCafferty et al. 2002). Ramoplanin belongs to the growing group of antibiotics targeting the essential cell wall precursor lipid II (Breukink and de Kruijff 2006). It has been shown that ramoplanin binds to lipid I as well to lipid II (Cudic et al. 2002a), and is thus able to inhibit MurG-mediated conversion of lipid I to lipid II and the transglycosylase reaction using lipid II as substrate. However, the affinity of ramoplanin for lipid II is much greater than for lipid I (Fang et al. 2006). In addition, lipid I remains at the internal surface of the cytoplasmic membrane, whereas lipid II is translocated to the external side of the membrane and can be targeted by the large water-soluble antibiotic. Consequently, inhibition of the transglycosylation step of peptidoglycan synthesis is the major mode of action of ramoplanin (Walker et al. 2005; Fang et al. 2006). Ramoplanin was also shown to interact directly with MurG, but the exact mechanism of inhibition is not known (Fang et al. 2006).

For efficient binding of ramoplanin to lipid II, the pyrophosphate moiety appears to be essential (Cudic et al. 2002b), a region distinctly different to that of vancomycin. Therefore, ramoplanin is highly effective even against vancomycin-resistant Gram-positive bacteria. Fortunately, resistance to ramoplanin has not yet been reported.

12.13 Mannopeptimycins

Mannopeptimycins (Fig. 12.2f) are antibacterial glycopeptides produced by *Streptomyces hygroscopicus* LL-AC98 (He et al. 2002; He 2005). A mixture of five mannopeptimycins is found in the fermentation broth of that strain, which was originally described as the AC98 complex and later designated mannopeptimycins (1–5) (He et al. 2002). Mannopeptimycins are glycosylated cyclic hexapeptides containing two stereoisomers of an unusual amino acid, αα-amino-β-[4′-(2′-iminoimidazolidinyl)]-β-hydroxypropionic acid (Aiha), which is a distinguishing feature for these antibiotics. Four of the five mannopeptimycins contain mannosyl disaccharide moieties attached to the cyclic core of peptides, while one harbors a mannosyl monosaccharide. In three mannopeptimycins, an isovaleryl group is found in the terminal mannose apparently enhancing antibacterial potency (Singh et al. 2003).

Mannopeptimycins are active against a wide variety of Gram-positive bacteria, among them methicillin-resistant staphylococci and vancomycin-resistant enterococci. The antibiotics were found to inhibit cell wall biosynthesis (Singh et al. 2003) most likely by binding to the cell wall precursor lipid II (Ruzin et al. 2004). Although molecular details of this interaction have not been worked out, it is different from binding of other lipid II-interacting antibiotics such as vancomycin (Breukink and de Kruijff 2006). Binding of mannopeptimycins to teichoic acids has also been observed, but the biological significance of this interaction remains to be elucidated (Ruzin et al. 2004). Resistance to mannopeptimycins has not been described.

12.14 Lantibiotics

The lantibiotics such as nisin or mersacidin (Fig. 12.2g) are a large family of peptide antibiotics (bacteriocins) characterized by the presence of the unusual thioether amino acids lanthionine and 3-methyllanthionine (Hechard and Sahl 2002; Chatterjee et al. 2005; Willey and van der Donk 2007). Since the discovery of the first lantibiotic nisin produced by a *Lactococcus lactis* strain (Rogers 1928), a large number of different lantibiotics have been identified that are secreted from numerous Gram-positive bacteria. Despite the wealth of different lantibiotics, none has made it to clinical applications and only nisin is applied as a preservative in foods (Delves-Broughton et al. 1996).

The family of lantibiotics has been subdivided into type A and type B groups comprising peptides of elongated and globular structures, respectively. Nisin (type A) and mersacidin, a type B lantibiotic produced by a *Bacillus* strain HIL Y-85

(Chatterjee et al. 1992), can be considered as the prototypes for these groups. However, the recent characterization of lantibiotics with intermediate properties or novel features makes their classification difficult (Chatterjee et al. 2005).

It has been shown that a number of lantibiotics bind to lipid II (Breukink and de Kruijff 2006). The best studied examples of this interaction are nisin and mersacidin, but the consequences of these interactions are not identical. While mersacidin binding to lipid II resulted in the block of the transglycosylase reaction (Brötz et al. 1998), nisin is able to induce pores in the cytoplasmic membrane by interacting with lipid II (Breukink et al. 1999). In vitro studies of cell wall synthesis and experiments with nisin variants unable to induce pores showed that nisin is also able to inhibit peptidoglycan synthesis (Linnett and Strominger 1973; Wiedemann et al. 2001; Hasper et al. 2006). However, the major bacteriocidal effect of nisin appears to be receptor-mediated pore formation using lipid II as docking molecule in the cell membrane. On the other hand, mersacidin is not pore-forming and relies on its inhibitory effect on cell wall biosynthesis as its mode of action.

The structure of the nisin-lipid II complex revealed a new motif, a pyrophosphate cage, that binds the pyrophosphate moiety of lipid II (Hsu et al. 2004). The pyrophosphate cage is formed by the lanthionine rings A and B, providing a rationale for the conservation of this structure in other lipid II-binding lantibiotics (Hsu et al. 2004).

The two-peptide lantibiotic lacticin 3147, also produced by a *Lactococcus lactis* strain, is of interest as in this case the lipid II targeting and antibiotic activity are separated (Wiedemann et al. 2006). One component of lacticin 3147, the subunit LtnA1, is able to bind to lipid II but was not able to substantially inhibit cell wall biosynthesis. Recruitment of LtnA2 to form a three-component complex results in the inhibition of cell wall biosynthesis combined with pore formation. The separation of lipid II targeting and inhibiting activity onto two peptides may offer possibilities for the design of new antibiotics.

Resistance to nisin and other lantibiotics appears to be mediated by shielding lipid II from the lantibiotic rather than changing the amount of lipid II or its structure (Kramer et al. 2004). Modification of teichoic acids by D-alanine or sugars thereby reducing the negative charge of the cell wall is a common mechanism by which bacteria protect themselves from lantibiotics (Peschel et al. 1999). But other mechanisms changing the cell wall or the membrane (Mazzotta and Montville 1999) have been described, including increasing the thickness of the septum (Kramer et al. 2008).

12.15 Moenomycin

Moeomycins (Fig. 12.2h) are a group of phosphoglycolipid antibiotics produced by various species of *Streptomyces* (Huber 1979). Moenomycin A is the main constituent produced from *S. bambergensis*. Its structure was determined on the basis of

spectroscopic studies and degradation (Welzel et al. 1981, 1983). Moenomycin is not effective in humans because of poor pharmacokinetic properties, including a long half-life and minimal oral bioavailability (Goldman and Gange 2000). Despite its extensive use as a growth promoter in animal feed, no plasmid-borne resistance to moenomycin has been detected so far (Butaye et al. 2001). The recent completion of the total synthesis of moenomycin (Taylor et al. 2006; Welzel 2007) and the identification of the genes involved in the biosynthesis of the drug (Ostash et al. 2007) represent a valuable basis from which to explore structural changes for the first time.

Moenomycin is active only against Gram-positive organisms since it cannot penetrate the outer membrane of Gram-negative bacteria. It was soon recognized that moenomycin probably interferes with peptidoglycan synthesis. Sublethal concentrations result in the accumulation of UDP-MurNAc-pentapeptides in *S. aureus* (Huber 1979). It inhibited in vitro peptidoglycan synthesis and led to the accumulation of the lipid intermediate (Lugtenberg et al. 1971; Linnett and Strominger 1973), and inhibited the transglycosylation reaction performed by *E. coli* PBP1b using lipid intermediate (Suzuki et al. 1980; van Heijenoort and van Heijenoort 1980; Matsuhashi et al. 1984).

Moenomycin is the only well-characterized inhibitor of the PBP-associated transglycosylase activity located in the N-terminal domain of high-molecular-weight class A PBPs (peptidoglycan glycosyltransferase family GT_{51}; www.cazy.org). Predicted monofunctional glycosyltransferases (MGT) which are homologues of the PBP-TG domain were first identified in Gram-negative species (Spratt et al. 1996; Paik et al. 1999). A soluble and active form of *S. aureus* MGT catalyzes a moenomycin-sensitive glycan chain polymerization from lipid II, whereas an *E. coli* MGT was insensitive to moenomycin (Sauvage et al. 2008).

It has been proposed, based on chemical similarity, that ring C and E of moenomycin would bind in the same way as a MurNAc-GlcNAc disaccharide of the substrate, and the recent crystal structure of *S. aureus* PBP2 (class A PBP) in complex with moenomycin supports this view (Lovering et al. 2007). Moenomycin is not competitive with respect to the lipid II substrate (Chen et al. 2003). The interactions between the protein and inhibitor are extensive, consistent with moenomycin and related compounds possessing some of the lowest median inhibitory concentration values in the nanomolar range known for antibiotics (Halliday et al. 2006). It also provides direct evidence that lipid II is the acceptor for the transglycosylation reaction and the growing chain is the donor (Lovering et al. 2007), supporting numerous experimental sources summarized by Welzel (2005). Several new generation glycopeptides that inhibit the transglycosylation process have been reviewed recently (Halliday et al. 2006). Detailed analysis of the 2.3 Å structure of the GT-domain of *Aquifex aeolicus* PBP1a with a moenomycin derivative and description of these binding interactions provided further information to enable structure-based design of moenomycin analogues (Yuan et al. 2008).

12.16 Vancomycin and Other Glycopeptides

Already in the early 1950s, *S. aureus* strains with multiple drug resistance, e.g. to erythromycin, tetracycline and penicillin, were isolated with increased frequency. This initiated a large-scale screening program aimed at the identification of antibiotics with high antistaphylococcal activity, resulting in the isolation of the first glycopeptide-producing *Streptomyces orientalis* from soil samples from the jungle in Borneo. The active compound, first named "Mississippi mud" and later vancomycin, was highly active against Gram-positive bacterial species (McCormick et al. 1956). Early toxicity problems were largely overcome by improved purification procedures. Other glycopeptides (e.g., avoparcin) were used as animal feed additives (see Barna and Williams 1984 and references within). Ristocetin, isolated from *Nocardia lurida* (Grundy et al. 1957) in the 1950s, causes aggregation of blood platelets and could therefore not be used therapeutically (Nikaido and Rosenberg 1983). Teicoplanin produced by *Actinoplanes teichomyceticus* nov. sp. (Parenti et al. 1978) has been introduced for the treatment of serious infections in humans due to resistant Gram-positive bacteria or in cases of *beta*-lactam allergy. Glycopeptides are inactive against Gram-negative bacteria since they cannot penetrate the outer membrane.

Features common to all members of the glycopeptide group include a central heptapeptide backbone with five highly conserved amino acids (Jones 2006). The amino acids are linked via several phenolic acid residues, resulting in the formation of complex tetra- or tricyclic structures. The core structure is referred to as the aglycone, to which a variable number of sometimes unusual sugars or amino sugars are attached which are not essential for antibiotic activity. In the teicoplanin complex, an amino sugar carries the various fatty acids characteristic for each member, rendering the molecule more hydrophobic than vancomycin (Parenti 1986).

Vancomycin and teicoplanin are now widely used and recommended for treatment of severe infections, especially those caused by multiple-drug-resistant Gram-positive pathogens (teicoplanin is used only outside North America). The glycopeptide avoparcin has been introduced as a growth promoter in animal husbandry in the past, and represents the main reservoir for the VanA type of vancomycin resistance in enterococci (see below) (Klare et al. 2003). Semisynthetic derivatives of vancomycin and teicoplanin, lipoglycopeptides, show an extended spectrum of activity against multiresistant and partly vancomycin-resistant bacteria and may be approved for clinical treatment soon (Reynolds 1989; Nordmann et al. 2007). Surprisingly, vancomycin-derived compounds carrying substituents on the disaccharide portion are active against vancomycin-resistant bacteria. Recent results confirmed that such compounds have a second mechanism of action directed against the transglycosylase activity, e.g. of *E. coli* PBP1b (Chen et al. 2003).

Vancomycin binds with high affinity to the C-terminal D-Ala-D-Ala moiety of the MurNAc-pentapeptide, and thus inhibits transglycosylation and subsequent transpeptidation of muropeptide precursors in the periplasm (Reynolds 1989).

A key step in elucidating the interaction of glycopeptides with the target site was the determination of the three-dimensional structure of a vancomycin degradation product, CDP-1, and its complex with acetyl-D-Ala-D-Ala by X-ray analysis (Sheldrick et al. 1978; Molinari et al. 1990). Recent studies using a new water-soluble derivative of lipid II showed, however, that glycopeptides interact differently with this synthetic target compared with D-Ala-D-Ala (Vollmerhaus et al. 2003).

Resistance against glycopeptides had been considered to be highly improbable, and thus resistance mechanisms developed by enterococci and staphylococci in the 1980s came completely unexpectedly. Resistance to vancomycin in enterococci is due to the presence of gene clusters encoding up to nine proteins whose activity finally results in target modification via the synthesis of new cell wall precursors with low affinity to the glycopeptide antibiotic replacing the C-terminal D-Ala residue by D-lactate or D-serine (Courvalin 2006; Werner et al. 2008). Most prominent worldwide are *vanA* and *vanB* types whose reservoir are enterococci. The VanA-type is characterized by inducible high levels of resistance to vancomycin and teicoplanin, whereas VanB mediates inducible, moderate vancomycin resistance levels only. Meanwhile, another three acquired resistance phenotypes (VanC, E and G) have been described mainly in *Enterococcus* spp.; VanC represents an intrinsic resistance phenotype in *E. gallinarum* and *E. casseliflavus*. The six types of resistance can be distinguished by the location of the corresponding genes (plasmid or chromosomal) and by the mode of regulation of gene expression (inducible or not).

Crucial for the target modification are a dehydrogenase VanH which reduces pyruvate to D-Lac, and a new ligase VanA resulting in the formation of the depsipeptide D-Ala and D-Lac, replacing the C-terminal D-Ala-D-Ala in the muropeptide precursor. The new muropeptide shows a considerably decrease in its affinity for glycopeptides due to diminishing of the hydrogen bond network (see Fig. 12.2i). The sophisticated *vanA* resistance machinery located on the transposon Tn*1546* includes the two-component system VanR/S responsible for induction of the gene cluster, and two enzymes which remove the usual D-Ala-D-Ala-terminating precursors: the vanX D,D-dipeptidase which hydrolyzes D-Ala-D-Ala, and the VanY D,D-carboxypeptidase which removes the C-terminal D-Ala residue. The function of VanZ (*vanA*-type) and VanW (*vanB*-type) are unknown. A curious but clinically important phenomenon is the development of glycopeptide-dependent strains in some VanA and VanB-type enterococci. This is due to the presence of a degenerate *ddl*-ligase, and thus the strains depend on the induction of the VanA ligase (Van Bambeke et al. 1999).

The transfer of *van* resistance genes from *Enterococcus* species to *S. aureus,* which results in high levels of resistance to vancomycin, was obtained in vitro and in an animal model (Noble et al. 1992). The recent acquisition of the *vanA* cluster by clinical isolates of methicillin-resistant *S. aureus* (VRSA) is especially worrisome; meanwhile seven cases have been identified in the USA (Werner et al. 2008). A completely different mechanism is apparent in intermediate-resistant *S. aureus* (VISA), and various loci have been implicated as the genetic basis for reduced glycopeptide susceptibility, as reviewed recently by Werner et al. (2008).

12.17 β-Lactam Antibiotics

In 1928, Alexander Fleming observed the production of an antibacterial substance by *Penicillum notatum* which accidentally contaminated his plate of *S. aureus,* and which he later called "penicillin" (Fleming 1929). Mainly due to the instability of the active substance, attempts to isolate and purify penicillin were largely unsuccessful, and interest in penicillin had almost disappeared. However, in 1938 Florey and Chain took up a systematic investigation of antimicrobial components, resulting in the purification of penicillin, and the demonstration of its therapeutic properties (Chain et al. 1940). These results justified the initiation of a gigantic Anglo-American collaboration during World War II to improve methods of production and isolation of penicillin. Its structure was finally established by X-ray crystallography in 1945. It is remarkable that Fleming stated in 1940: "the trouble of making it seemed not worth while" (Abraham 1981). To this date, β-lactams belong to the most widely used antibiotics worldwide. The only, rare, side effects observed during therapy are hypersensitive reactions and allergies that can lead to anaphylactic shock syndrome.

In 1953, Cephalosporin C was purified from *Cephalosporium acremonium* isolated by Giuseppe Brotzu in Sardinia, and shown to be resistant to penicillinase action (Newton and Abraham 1956). The identification of the penicillin core, 6-amino-penicillanic acid (6-APA) (Batchelor et al. 1959), and that of cephalosporin C (7-ACA) (Morin et al. 1962), enabled the production of semisynthetic β-lactams by introducing new side chains, resulting in derivatives of improved activity in the following decades (Mitsuhashi 1981; Rolinson 1998) (Fig. 12.2j). The discovery that bacteria, most notably *Streptomyces* spp., produce special classes of β-lactams – carbapenems and monobactams – further revolutionized the design of new β-lactams (Kahan et al. 1979; Sykes and Bonner 1985; Ward and Hodgson 1993). Since then, thousands of natural, semisynthetic and synthetic β-lactam antibiotics have been produced, making them one of the most successful natural product derivatives. The new β-lactams have been produced with the aim of obtaining β-lactamase-resistant compounds, those with high activity against Gram-negative pathogens and broad-spectrum activity, and with improved pharmacological properties.

β-Lactams are targeted by penicillin-binding proteins (PBPs), membrane-associated proteins involved in late stages of murein biosynthesis. PBPs function as D,D-peptidases (D,D-transpeptidase, D,D-carboxypeptidase, or D,D-endopeptidase) acting on the terminal D-Ala-D-Ala moiety of the muropeptide. They share a common domain with an active site Ser which is part of the SXXK motif. Large PBPs of class A contain, in addition to the transpeptidase domain, a transglycosylase domain (see Section on moenomycin), and most small PBPs are D,D-carboxypeptidases (Goffin and Ghuysen 2002; Sauvage et al. 2008). PBPs are easily be detected after interaction with radioactive or fluorescent β-lactams followed by SDS-polyacrylamide gel electrophoresis, and visualization after fluorography. The number of PBPs varies between three to over ten depending on the species, and they

are named according to their apparent molecular size on the gels. The structure of several PBPs has been solved (for review, see Sauvage et al. 2008).

β-Lactams form a covalent complex with the active site Ser residue of PBPs due to their structural similarity with the D-Ala-D-Ala dipeptide of the substrate muropeptide (Tipper and Strominger 1965; Ghuysen 1991). The interaction with substrate and inhibitor follows a three-step scheme: the rapid and reversible formation of the noncovalent Michaelis complex, followed by formation of a covalent acyl-enzyme intermediate, and the final deacylation step. Interaction with β-lactams generally leads to a covalent intermediate with a long half life, but for each β-lactam and each PBP large differences in the kinetic parameters can be observed. This can be very useful for studying properties of individual PBPs that interact exclusively at certain concentrations with one particular β-lactam. When mecillinam, which targets only PBP2 is added to growing *E. coli*, cells become round, whereas addition of cefotaxime, which interacts over a wide range of concentrations only with *E. coli* PBP3, results in filament formation (Spratt and Pardee 1975). These observations were crucial for the identification of PBPs as central components of the bacterial cell elongation and division process. On the other hand, aztreonam (Fig. 12.2k) and cefotaxime do not interact with *S. pneumoniae* PBP2b, revealing a central role of PBP2b for β-lactam-induced autolysis (Hakenbeck et al. 1987).

Unexpectedly the spectrum of activity of β-lactams extends to include transpeptidases of the L,D specificity which have been recently identified in *Enterococcus faecium*, *B. subtilis* and *E. faecalis* (Mainardi et al. 2007; Magnet et al. 2007a). Imipenem (Fig. 12.2l) totally inhibits the L,D-transpeptidase activity of *E. faecium* Ldt_{fm} at a concentration equal to the MIC of the drug (0.5 μg/ml) (Mainardi et al. 2007). Cys442, the catalytic residue of Ldt_{fm}, is the site of acylation: the enzyme "commits suicide" by catalyzing formation of a thioester bond between its catalytic residue and the β-lactam ring of carbapenems. Homologues of the Ldt_{fm} family were recently found to be responsible for anchoring the Braun lipoprotein to peptidoglycan (Magnet et al. 2007b). In spite of its wide distribution, the physiological role of peptidoglycan L,D-transpeptidases remains unknown.

The first β-lactamase-producing *E. coli* were discovered as early as 1940 by Abraham and Chain (Abraham and Chain 1940), and subsequently penicillinase activity was found in a number of pathogens including *S. aureus*, *B. cereus* and other Gram-negative organisms. The history of the development of β-lactams reflects in an amazing way the cycles of successful treatment with an antibiotic, followed by failure due to resistance mechanisms, overcoming such problems by newly synthesized compounds active against the resistant organisms, again followed by selection of bacteria resistant to such novel antibiotics, and so on. The paradigm for this development is represented by *S. aureus* (Gardam 2000). β-Lactamase-producing *S. aureus* were already recognized before the onset of therapeutic use, and became a problem in hospitals soon after the introduction of penicillin in the early 1950s. Within a year after the introduction of methicillin, a penicillinase-resistant β-lactam, methicillin-resistant *S. aureus* (MRSA) appeared and is now a problem in hospitals worldwide and common in the community as

well. Although MRSA-targeting β-lactams have been described, narrow-spectrum antibiotics are generally not produced by pharmaceutical companies for commercial reasons. The occurrence of vancomycin-resistance genes in MRSA isolates has been recognized as an alarming signal in the "postantibiotic era" (Courvalin 2006). The current race is marked by ceftobiprole, the first β-lactam antibiotic with MRSA activity to complete phase III therapeutic trials (Bush et al. 2007).

PBPs and class A, C and D β-lactamases belong to the superfamily of acyltransferases with the active site motif SerXxxXxxLys (Goffin and Ghuysen 2002; Sauvage et al. 2008). β-Lactamases of class B comprise metalloenzymes in which Zn^{2+} atoms interact with one cysteine and three serine residues (Sutton et al. 1987). Among β-lactamases, the carbapenemases represent the most versatile group that, despite their name, hydrolyze almost all β-lactams, and commercial β-lactamase inhibitors are inefficient for most of these enzymes (Queenan and Bush 2007, and references within). Extended-spectrum β-lactamases (ESBL) include cephalosporin-hydrolyzing TEM and SHV enzymes (Livermore 2008). In addition to β-lactamase production, resistance against β-lactams is due to alteration of the outer membrane permeability (Nikaido 1989), or changes in PBPs due to gene transfer in clinical isolates of point mutations that result in low-affinity variants (Denapaite et al. 2007; Sauvage et al. 2008; Maurer et al. 2008). Overproduction of a PBP is still another phenomenon associated with β-lactam resistance in *S. aureus* and *Enterococcus faecium* (Zapun et al. 2008). Other mechanisms assisting or accompanying PBP alterations have been implicated in the resistance process in *S. pneumoniae*, including the two-component regulatory system CiaRH, a glycosyltransferase, the MurMN enzymes, and others (for review, see Denapaite et al. 2007). Mecillinam (Fig. 12.2l) resistance in *E. coli* also follows unusual routes (Vinella et al. 2005).

12.18 BLIP

The inhibition of β-lactamases represents a curious situation with respect to cell wall inhibitors. Inhibitors against β-lactamases in fact inhibit a resistance mechanism directed against β-lactams. One particular example outside the antibiotic world is the BLIP protein (β-lactamase inhibitory protein), a small 165-amino-acid protein produced by *Streptomyces* clavuligerus (Strynadka et al. 1994, 1996). It potently inhibits TEM-1 β-lactamases, the most prevalent class of this enzymes in Gram-negative bacteria which are of major concern due to their broad specificity profile (Bush et al. 1995).

BLIP has a tandem repeat structure of a 76-amino-acid αβ domain. These two tandem domains form a β saddle whose concave surface interacts with the substrate binding pocket of TEM-1. The Asp49 residue of BLIP occupies the catalytic site of TEM-1 (Strynadka et al. 1996). Probably because of a relatively poor shape

complementarity of BLIP and the enzyme, BLIP can bind to many β-lactamases (Zhang and Palzkill 2004). A detailed study of the interactions between BLIP and TEM-1 β-lactamases indicated surface residues involved in hydrogen bond formation as attractive targets for inhibitor design (Wang et al. 2009).

12.19 Bulgecin

In addition to monocyclic β-lactam antibiotics, *Pseudomonas acidophila* strain G6302 and *Pseudomonas mesoacidophila* strain SB-72310 produce the *O*-sulfonated glycopeptides bulgecins (Fig. 12.2m), containing an unusual cyclic imino acid. In addition to bulgecin A, the major component, bulgecin B and C are also produced (Shinagawa et al. 1985). Their names originated from their ability to induce bulges in cooperation with β-lactam antibiotics in *E. coli* (Imada et al. 1982); by themselves they have no antibacterial activity.

A clue to the mechanism of action came from the observation that mutants in the lytic transglycosylase SLT70 show the same phenotype, i.e., bulge formation, in the absence of β-lactams. The discovery that SLT70 is specifically inhibited by bulgecin explained these results (Templin et al. 1992).

SLT70 belongs to a group of exomuramidases first described in *E. coli*, catalyzing the cleavage of the P-1,4-glycosidic bond between MurNAc and GlcNAc residues. Slt70 is a periplasmic enzyme, whereas the other two *E. coli* lytic transglycosylases are lipoproteins attached to the inner leaflet of the outer membrane (Vollmer et al. 2008b). They are distinct from endo-hydrolytic lysozymes in that they carry out an intramolecular glycosyl transferase reaction resulting in the formation of 1,6-anhydromuropeptides (Höltje et al. 1975). Their precise role during cell growth and division, as well the regulation of their potential dangerous activity, is not understood.

The crystal structures of two soluble lytic transglycosylases have been solved with bound inhibitor bulgecin A: *E. coli* Slt70 (Thunnissen et al. 1995) and *E. coli* Slt35, a proteolytic degradation product of the membrane bound MltB (van Asselt et al. 2000). The data identified an important Glu residue in the proteins, and suggested that bulgecin acts as an analogue of an oxocarbonium ion intermediate in the reaction catalyzed by Slt70. This structural information will permit the rational design of more effective inhibitors.

12.20 Bacitracin

Bacitracin (Fig. 12.2n) is an antibiotic composed of a complex mixture of branched cyclic dodecylpeptides first isolated from *B. licheniformis* (Johnson et al. 1945), but is also produced by some strains of *B. subtilis*. To exert its biological activity,

bacitracin needs divalent metal ions (Stone and Strominger 1971). The antibiotic blocks peptidoglycan synthesis by binding to undecaprenylpyrophosphate (UPP) (Siewert and Strominger 1967; Stone and Strominger 1971). This sequestration prevents UPP dephosphorylation and therefore recycling of the lipid carrier. Bacitracin may be applied topically to treat skin infections, quite often in conjunction with other antibiotics. It is used extensively for prophylaxis and therapy in food animals.

Two major bacitracin resistance mechanisms have been described in *B. subtilis* and *E. coli*. One involves the dephosphorylation of UPP by overexpression of UPP phosphatase (BacA; also named UppP), thereby providing the essential cell wall precursor undecaprenylphosphate (El Ghachi et al. 2004; Bernard et al. 2005). The second mechanism, and apparently the more efficient one, involves expression of an ABC transporter system and a two-component regulatory system that responds to bacitracin, as identified in *B. subtilis*, *B. licheniformis*, and *Streptococcus mutans* (Podlesek et al. 1995; Tsuda et al. 2002; Ohki et al. 2003). While it is clear that the ABC transporter is not only involved in bacitracin resistance but also in bacitracin sensing, the exact role of transport, i.e., bacitracin export or import, is still the subject of discussions (Bernard et al. 2007; Rietkötter et al. 2008).

12.21 Colicin M

Colicins are plasmid-encoded cytotoxic proteins produced by certain *E. coli* that kill other *E. coli* strains lacking this plasmid. Colicin M has a unique mode of action in that it inhibits murein and O-antigen biosynthesis by interfering with the regeneration of bactoprenol (Schaller et al. 1982; Harkness and Braun 1989a, b). Colicin M was in fact identified as an enzyme catalyzing the specific degradation of lipids I and II peptidoglycan intermediates (El Ghachi et al. 2006). Specifically it hydrolyzes the pyrophosphate linkage between bactoprenol and the murein precursor (1-pyrophospho-MurNAc-(pentapeptide)-GlcNAc) in the periplasm. The resulting bactoprenol cannot serve as acceptor of the murein and O-antigen precursors.

Its export mechanism is unknown. Import into the periplasm of target cells requires the Ton system similar to other colicins (Braun et al. 2002), and the partially unfolded colicin M is presumably refolded into the active form by the FkpA, a periplasmic chaperone (Hullmann et al. 2008).

Colicin M is the smallest colicin, composed of 271 amino acid residues. Its structure has been solved at 1.7 Å resolution (Zeth et al. 2008). It consists of three domains (like all colicins) whose architectures show no similarity to known structures. The N-terminal domain is required for translocation across the outer membrane, the receptor binding domain is represented by the central domain, and the phosphatase activity is represented by the C-terminal domain. Predicted bacteriocins with homology to the C-terminal domain have been discovered in *Pseudomonas* spp. and *Burkholderia* spp., suggesting that these organisms also possess a phosphatase-based killing mechanism (Zeth et al. 2008).

12.22 Conclusions

What started with the accidental observation that a fungus produces antibacterial substances has become an enormous enterprise in pharmaceutical industry and biological research. Enzymes involved in peptidoglycan biosynthesis represent tremendously attractive antimicrobial targets due to the fact that they are essential and unique to the eubacterial world. The lipid carrier also plays a central role in the biosynthesis of other cell wall polymers. Due to improved biochemical techniques, this molecule has been only recently approached as a promising target. Bacteria have evolved a vast spectrum of unpredictable resistance mechanisms against cell wall inhibitors. These include MRSA and VRE, methicillin-resistant *S. aureus* and vancomycin-resistant enterococci, where new genes ensure the synthesis of novel types of cell walls. Trying to understand the action of new cell wall inhibitors and resistance mechanisms against them might help to unravel the remaining mysteries of cell wall biosynthesis.

Acknowlegement This work was supported by the EU (Intafar, LSHM-CT-2004-512138) and the Deutsche Forschungsgemeinschaft (Ha1011/11-1).

References

Abraham EP (1981) A short history of the *beta*-lactam antibiotics. In: Mitsuhashi S (ed) *Beta*-lactam antibiotics. Japan Scientific Press and Springer, Tokyo and Berlin, pp 3–11

Abraham EP, Chain EB (1940) Enzymes from bacteria able to destroy penicillin. Nature (London) 146:837

Alborn WE Jr, Allen NE, Preston DA (1991) Daptomycin disrupts membrane potential in growing *Staphylococcus aureus*. Antimicrob Agents Chemother 35:2282–2287

Allen NE, Alborn WE Jr, Hobbs JN Jr (1991) Inhibition of membrane potential-dependent amino acid transport by daptomycin. Antimicrob Agents Chemother 35:2639–2642

Aretz W, Meiwes J, Seibert G, Vobis G, Wink J (2000) Friulimicins: novel lipopeptide antibiotics with peptidoglycan synthesis inhibiting activity from *Actinoplanes friuliensis* sp. nov. I. Taxonomic studies of the producing microorganism and fermentation. J Antibiot (Tokyo) 53:807–815

Baltz RH, Miao V, Wrigley SK (2005) Natural products to drugs: daptomycin and related lipopeptide antibiotics. Nat Prod Rep 22:717–741

Banerjee DK (1989) Amphomycin inhibits mannosylphosphoryldolichol synthesis by forming a complex with dolichylmonophosphate. J Biol Chem 264:2024–2028

Barna JC, Williams DH (1984) The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Annu Rev Microbiol 38:339–357

Barreteau H, Kovac A, Boniface A, Sova M, Gobec S, Blanot D (2008) Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol Rev 32:168–207

Batchelor FR, Doyle FP, Nayler JH, Rolinson GN (1959) Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature 183:257–258

Bellais S, Arthur M, Dubost L, Hugonnet JE, Gutmann L, van Heijenoort J, Legrand R, Brouard JP, Rice L, Mainardi JL (2006) Aslfm, the D-aspartate ligase responsible for the addition of D-aspartic acid onto the peptidoglycan precursor of *Enterococcus faecium*. J Biol Chem 281:11586–11594

Berger-Bächi B, Tschierske M (1998) Role of *fem* factors in methicillin resistance. Drug Resist Updat 1:325–335

Berger-Bächi B, Barberis-Maino L, Strassle A, Kayser FH (1989) FemA, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. Mol Gen Genet 219:263–269

Bernard R, El Ghachi M, Mengin-Lecreulx D, Chippaux M, Denizot F (2005) BcrC from *Bacillus subtilis* acts as an undecaprenyl pyrophosphate phosphatase in bacitracin resistance. J Biol Chem 280:28852–28857

Bernard R, Guiseppi A, Chippaux M, Foglino M, Denizot F (2007) Resistance to bacitracin in *Bacillus subtilis*: unexpected requirement of the BceAB ABC transporter in the control of expression of its own structural genes. J Bacteriol 189:8636–8642

Bernat BA, Laughlin LT, Armstrong RN (1997) Fosfomycin resistance protein (FosA) is a manganese metalloglutathione transferase related to glyoxalase I and the extradiol dioxygenases. Biochemistry 36:3050–3055

Boojamra CG, Lemoine RC, Lee JC, Leger R, Stein KA, Vernier NG, Magon A, Lomovskaya O, Martin PK, Chamberland S, Lee MD, Hecker SJ, Lee VJ (2001) Stereochemical elucidation and total synthesis of dihydropacidamycin D, a semisynthetic pacidamycin. J Am Chem Soc 123:870–874

Bouhss A, Trunkfield AE, Bugg TD, Mengin-Lecreulx D (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol Rev 32:208–233

Brandish PE, Kimura KI, Inukai M, Southgate R, Lonsdale JT, Bugg TD (1996) Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-*N*-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. Antimicrob Agents Chemother 40: 1640–1644

Braun V, Patzer SI, Hantke K (2002) Ton-dependent colicins and microcins: modular design and evolution. Biochimie (Paris) 84:365–380

Breukink E, de Kruijff B (2006) Lipid II as a target for antibiotics. Nat Rev Drug Discov 5: 321–332

Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl H, de Kruijff B (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science 286:2361–2364

Brötz H, Bierbaum G, Leopold K, Reynolds PE, Sahl HG (1998) The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrob Agents Chemother 42: 154–160

Bugg TD, Lloyd AJ, Roper DI (2006) Phospho-MurNAc-pentapeptide translocase (MraY) as a target for antibacterial agents and antibacterial proteins. Infect Disord Drug Targets 6:85–106

Bush K, Jacoby GA, Medeiros AA (1995) A functional classification scheme for *beta*-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 39:1211–1233

Bush K, Heep M, Macielag MJ, Noel GJ (2007) Anti-MRSA *beta*-lactams in development, with a focus on ceftobiprole: the first anti-MRSA *beta*-lactam to demonstrate clinical efficacy. Expert Opin Investig Drugs 16:419–429

Butaye P, Devriese LA, Haesebrouck F (2001) Differences in antibiotic resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from farm and pet animals. Antimicrob Agents Chemother 45:1374–1378

Caceres NE, Harris NB, Wellehan JF, Feng Z, Kapur V, Barletta RG (1997) Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. J Bacteriol 179:5046–5055

Cao M, Bernat BA, Wang Z, Armstrong N, Helmann JD (2001) FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in *Bacillus subtilis*. J Bacteriol 183:2380–2383

Cavalleri B, Pagani H, Volpe G, Selva E, Parenti F (1984) A-16686, a new antibiotic from *Actinoplanes*. I. Fermentation, isolation and preliminary physico-chemical characteristics. J Antibiot (Tokyo) 37:309–317

Chain E, Florey HW, Gardner HW, Heatley NG, Jennings MA, Orr-Erwing J et al (1940) Penicillin as a chemotherapeutic agent. Lancet 2:226–228

Chatterjee S, Lad SJ, Phansalkar MS, Rupp RH, Ganguli BN, Fehlhaber HW, Kogler H (1992) Mersacidin, a new antibiotic from *Bacillus*. Fermentation, isolation, purification and chemical characterization. J Antibiot (Tokyo) 45:832–838

Chatterjee C, Paul M, Xie L, van der Donk WA (2005) Biosynthesis and mode of action of lantibiotics. Chem Rev 105:633–684

Chemama M, Fonvielle M, Villet R, Arthur M, Valery JM, Etheve-Quelquejeu M (2007) Stable analogues of aminoacyl-tRNA for inhibition of an essential step of bacterial cell wall synthesis. J Am Chem Soc 129:12642–12643

Chen L, Walker D, Sun B, Hu Y, Walker S, Kahne D (2003) Vancomycin analogues active against *vanA*-resistant strains inhibit bacterial transglycosylase without binding substrate. Proc Natl Acad Sci USA 100:5658–5663

Christensen BG, Leanza WJ, Beattie TR, Patchett AA, Arison BH, Ormond RE, Kuehl FA Jr, bers-Schonberg G, Jardetzky O (1969) Phosphonomycin: structure and synthesis. Science 166:123–125

Cotroneo N, Harris R, Perlmutter N, Beveridge T, Silverman JA (2008) Daptomycin exerts bactericidal activity without lysis of *Staphylococcus aureus*. Antimicrob Agents Chemother 52:2223–2225

Courvalin P (2006) Vancomycin resistance in gram-positive cocci. Clin Infect Dis 42(Suppl 1): S25–S34

Cressina E, Lloyd AJ, De Pascale G, Roper DI, Dowson CG, Bugg TD (2007) Adenosine phosphonate inhibitors of lipid II: alanyl tRNA ligase MurM from *Streptococcus pneumoniae*. Bioorg Med Chem Lett 17:4654–4656

Cudic P, Behenna DC, Kranz JK, Kruger RG, Wand AJ, Veklich YI, Weisel JW, McCafferty DG (2002a) Functional analysis of the lipoglycodepsipeptide antibiotic ramoplanin. Chem Biol 9:897–906

Cudic P, Kranz JK, Behenna DC, Kruger RG, Tadesse H, Wand AJ, Veklich YI, Weisel JW, McCafferty DG (2002b) Complexation of peptidoglycan intermediates by the lipoglycodepsipeptide antibiotic ramoplanin: minimal structural requirements for intermolecular complexation and fibril formation. Proc Natl Acad Sci USA 99:7384–7389

David HL (1971) Resistance to D-cycloserine in the tubercle bacilli: mutation rate and transport of alanine in parental cells and drug-resistant mutants. Appl Microbiol 21:888–892

David HL, Takayama K, Goldman DS (1969) Susceptibility of mycobacterial D-alanyl-D-alanine synthetase to D-cycloserine. Am Rev Respir Dis 100:579–581

David HL, Rastogi N, Clavel-Sérès S, Clément F (1988) Alterations in the outer wall architecture caused by the inhibition of mycoside C biosynthesis in *Mycobacterium avium*. Curr Microbiol 17:61–68

De Smet KA, Kempsell KE, Gallagher A, Duncan K, Young DB (1999) Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from *Mycobacterium tuberculosis*. Microbiology 145(Pt 11):3177–3184

Debono M, Barnhart M, Carrell CB, Hoffmann JA, Occolowitz JL, Abbott BJ, Fukuda DS, Hamill RL, Biemann K, Herlihy WC (1987) A21978C, a complex of new acidic peptide antibiotics: isolation, chemistry, and mass spectral structure elucidation. J Antibiot (Tokyo) 40:761–777

Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J (1996) Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek 69:193–202

Denapaite D, Chi F, Maurer P, Nolte O, Hakenbeck R (2007) Mechanism of penicillin resistance in *Streptococcus pneumoniae*: targets, gene transfer, and mutations. In: Hakenbeck R, Chhatwal GS (eds) Molecular biology of Streptococci. Horizon Bioscience, Wymondham, Norfolk UK, pp 290–303

Dini C (2005) MraY inhibitors as novel antibacterial agents. Curr Top Med Chem 5:1221–1236

Dini C, Collette P, Drochon N, Guillot JC, Lemoine G, Mauvais P, Aszodi J (2000) Synthesis of the nucleoside moiety of liposidomycins: elucidation of the pharmacophore of this family of MraY inhibitors. Bioorg Med Chem Lett 10:1839–1843

Dini C, Didier-Laurent S, Drochon N, Feteanu S, Guillot JC, Monti F, Uridat E, Zhang J, Aszodi J (2002) Synthesis of sub-micromolar inhibitors of MraY by exploring the region originally occupied by the diazepanone ring in the liposidomycin structure. Bioorg Med Chem Lett 12:1209–1213

Du W, Brown JR, Sylvester DR, Huang J, Chalker AF, So CY, Holmes DJ, Payne DJ, Wallis NG (2000) Two active forms of UDP-*N*-acetylglucosamine enolpyruvyl transferase in gram-positive bacteria. J Bacteriol 182:4146–4152

El Ghachi M, Bouhss A, Blanot D, Mengin-Lecreulx D (2004) The *bacA* gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. J Biol Chem 279:30106–30113

El Ghachi M, Bouhss A, Barreteau H, Touze T, Auger G, Blanot D, Mengin-Lecreulx D (2006) Colicin M exerts its bacteriolytic effect *via* enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors. J Biol Chem 281:22761–22772

Etienne J, Gerbaud G, Courvalin P, Fleurette J (1989) Plasmid-mediated resistance to fosfomycin in *Staphylococcus epidermidis*. FEMS Microbiol Lett 52:133–137

Falagas ME, Giannopoulou KP, Kokolakis GN, Rafailidis PI (2008) Fosfomycin: use beyond urinary tract and gastrointestinal infections. Clin Infect Dis 46:1069–1077

Fang X, Tiyanont K, Zhang Y, Wanner J, Boger D, Walker S (2006) The mechanism of action of ramoplanin and enduracidin. Mol Biosyst 2:69–76

Feng Z, Barletta RG (2003) Roles of *Mycobacterium smegmatis* D-alanine:D-alanine ligase and D-alanine racemase in the mechanisms of action of and resistance to the peptidoglycan inhibitor D-cycloserine. Antimicrob Agents Chemother 47:283–291

Fernandes PB, Swanson RN, Hardy DJ, Hanson CW, Coen L, Rasmussen RR, Chen RH (1989) Pacidamycins, a novel series of antibiotics with anti-*Pseudomonas aeruginosa* activity. III. Microbiologic profile. J Antibiot (Tokyo) 42:521–526

Fillgrove KL, Pakhomova S, Schaab MR, Newcomer ME, Armstrong RN (2007) Structure and mechanism of the genomically encoded fosfomycin resistance protein, FosX, from *Listeria monocytogenes*. Biochemistry 46:8110–8120

Fleming A (1929) J Exp Pathol 10:226–236

Gardam MA (2000) Is methicillin-resistant *Staphylococcus aureus* an emerging community pathogen? A review of the literature. Can J Infect Dis 11:202–211

Ghuysen J-M (1991) Serine β-lactamases and penicillin-binding proteins. Annu Rev Microbiol 45:37–67

Goffin C, Ghuysen J-M (2002) Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins *versus* lack of efficiency of penicillin as therapeutic agent. Microbiol Mol Biol Rev 66:706–738

Goldman RC, Gange D (2000) Inhibition of transglycosylation involved in bacterial peptidoglycan synthesis. Curr Med Chem 7:801–820

Gotoh N, Murata T, Ozaki T, Kimura T, Kondo A, Nishino T (2003) Intrinsic resistance of *Escherichia coli* to mureidomycin A and C due to expression of the multidrug efflux system AcrAB-TolC: comparison with the efflux systems of mureidomycin-susceptible *Pseudomonas aeruginosa*. J Infect Chemother 9:101–103

Green DW (2002) The bacterial cell wall as a source of antibacterial targets. Expert Opin Ther Targets 6:1–19

Grundy WE, Sinclair AC, Theriault RJ, Goldstein AW, Rickcher CJ, Warren Jr HB, OliverTJ, Sylvester JC (1957) Ristocetin, microbiologic properties. Antibiot Annu 1956–1957:687–692

Hakenbeck R, Tornette S, Adkinson NF (1987) Interaction of non-lytic β-lactams with penicillin-binding proteins in *Streptococcus pneumoniae*. J Gen Microbiol 133:755–760

Halliday J, McKeveney D, Muldoon C, Rajaratnam P, Meutermans W (2006) Targeting the forgotten transglycosylases. Biochem Pharmacol 71:957–967

Harkness RE, Braun V (1989a) Colicin M inhibits peptidoglycan biosynthesis by interfering with lipid carrier recycling. J Biol Chem 264:6177–6182

Harkness RE, Braun V (1989b) Inhibition of lipopolysaccharide O-antigen synthesis by colicin M. J Biol Chem 264:14716–14722

Hasper HE, Kramer NE, Smith JL, Hillman JD, Zachariah C, Kuipers OP, de Kruijff B, Breukink E (2006) An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science 313:1636–1637

He H (2005) Mannopeptimycins, a novel class of glycopeptide antibiotics activeagainst gram-positive bacteria. Appl Microbiol Biotechnol 67:444–452

He H, Williamson RT, Shen B, Graziani EI, Yang HY, Sakya SM, Petersen PJ, Carter GT (2002) Mannopeptimycins, novel antibacterial glycopeptides from *Streptomyces hygroscopicus*, LL-AC98. J Am Chem Soc 124:9729–9736

Hechard Y, Sahl HG (2002) Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie 84:545–557

Heifetz A, Keenan RW, Elbein AD (1979) Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl-phosphate Glc-NAc-1-phosphate transferase. Biochemistry 18:2186–2192

Heinemann B, Kaplan MA, Muir RD, Hooper IR (1953) Amphomycin, a new antibiotic. Antibiotic Chemother 3:1239–1242

Hendlin D, Stapley EO, Jackson M, Wallick H, Miller AK, Wolf FJ, Miller TW, Chaiet L, Kahan FM, Foltz EL, Woodruff HB, Mata JM, Hernandez S, Mochales S (1969) Phosphonomycin, a new antibiotic produced by strains of streptomyces. Science 166:122–123

Herve M, Boniface A, Gobec S, Blanot D, Mengin-Lecreulx D (2007) Biochemical characterization and physiological properties of *Escherichia coli* UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase. J Bacteriol 189:3987–3995

Hofmann SG, Pollack MH, Otto MW (2006) Augmentation treatment of psychotherapy for anxiety disorders with D-cycloserine. CNS Drug Rev 12:208–217

Höltje JV, Mirelman D, Sharon N, Schwarz U (1975) Novel type of murein transglycosylase in *Escherichia coli*. J Bacteriol 124:1067–1076

Howard NI, Bugg TD (2003) Synthesis and activity of 5′-uridinyl dipeptide analogues mimicking the amino terminal peptide chain of nucleoside antibiotic mureidomycin A. Bioorg Med Chem 11:3083–3099

Hsu ST, Breukink E, Tischenko E, Lutters MA, de Kruijff B, Kaptein R, Bonvin AM, van Nuland NA (2004) The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat Struct Mol Biol 11:963–967

Huber G (1979) Moenomycins. In: Hahn FE (ed) Antibiotic V/I. Mechanism of action of antibacterial agents. Springer, Berlin, pp 135–153

Hullmann J, Patzer SI, Romer C, Hantke K, Braun V (2008) Periplasmic chaperone FkpA is essential for imported colicin M toxicity. Mol Microbiol 69:926–937

Imada A, Kintaka K, Nakao M, Shinagawa S (1982) Bulgecin, a bacterial metabolite which in concert with *beta*-lactam antibiotics causes bulge formation. J Antibiot (Tokyo) 35:1400–1403

Inukai M, Isono F, Takahashi S, Enokita R, Sakaida Y, Haneishi T (1989) Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. I. Taxonomy, fermentation, isolation and physico-chemical properties. J Antibiot (Tokyo) 42:662–666

Inukai M, Isono F, Takatsuki A (1993) Selective inhibition of the bacterial translocase reaction in peptidoglycan synthesis by mureidomycins. Antimicrob Agents Chemother 37:980–983

Isono F, Inukai M (1991) Mureidomycin A, a new inhibitor of bacterial peptidoglycan synthesis. Antimicrob Agents Chemother 35:234–236

Isono K, Uramoto M, Kusakabe H, Kimura K, Isaki K, Nelson CC, McCloskey JA (1985) Liposidomycins: novel nucleoside antibiotics which inhibit bacterial peptidoglycan synthesis. J Antibiot (Tokyo) 38:1617–1621

Isono F, Inukai M, Takahashi S, Haneishi T, Kinoshita T, Kuwano H (1989a) Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. II. Structural elucidation. J Antibiot (Tokyo) 42:667–673

Isono F, Katayama T, Inukai M, Haneishi T (1989b) Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. III. Biological properties. J Antibiot (Tokyo) 42:674–679

Isono F, Kodama K, Inukai M (1992) Susceptibility of *Pseudomonas* species to the novel antibiotics mureidomycins. Antimicrob Agents Chemother 36:1024–1027

Johnson BA, Anker H, Meleney FL (1945) Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. Science 102:376–377

Johnson R, Streicher EM, Louw GE, Warren RM, van Helden PD, Victor TC (2006) Drug resistance in *Mycobacterium tuberculosis*. Curr Issues Mol Biol 8:97–111

Jones RN (2006) Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict clinical outcomes or detect resistant strains. Clin Infect Dis 42(Suppl 1):S13–S24

Kadner RJ, Winkler HH (1973) Isolation and characterization of mutations affecting the transport of hexose phosphates in *Escherichia coli*. J Bacteriol 113:895–900

Kahan FM, Kahan JS, Cassidy PJ, Kropp H (1974) The mechanism of action of fosfomycin (phosphonomycin). Ann NY Acad Sci 235:364–386

Kahan JS, Kahan FM, Goegelman R, Currie SA, Jackson M, Stapley EO, Miller TW, Miller AK, Hendlin D, Mochales S, Hernandez S, Woodruff HB, Birnbaum J (1979) Thienamycin, a new *beta*-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J Antibiot (Tokyo) 32:1–12

Kimura K, Bugg TD (2003) Recent advances in antimicrobial nucleoside antibiotics targeting cell wall biosynthesis. Nat Prod Rep 20:252–273

Kimura K, Ikeda Y, Kagami S, Yoshihama M, Suzuki K, Osada H, Isono K (1998) Selective inhibition of the bacterial peptidoglycan biosynthesis by the new types of liposidomycins. J Antibiot (Tokyo) 51:1099–1104

Klare I, Konstabel C, Badstubner D, Werner G, Witte W (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. Int J Food Microbiol 88:269–290

Kobayashi S, Kuzuyama T, Seto H (2000) Characterization of the *fomA* and *fomB* gene products from *Streptomyces wedmorensis*, which confer fosfomycin resistance on *Escherichia coli*. Antimicrob Agents Chemother 44:647–650

Kramer NE, Smid EJ, Kok J, de Kruijff B, Kuipers OP, Breukink E (2004) Resistance of Gram-positive bacteria to nisin is not determined by lipid II levels. FEMS Microbiol Lett 239:157–161

Kramer NE, Hasper HE, van den Bogaard PT, Morath S, de Kruijff B, Hartung T, Smid EJ, Breukink E, Kok J, Kuipers OP (2008) Increased D-alanylation of lipoteichoic acid and a thickened septum are main determinants in the nisin resistance mechanism of *Lactococcus lactis*. Microbiology 154:1755–1762

Lambert MP, Neuhaus FC (1972) Mechanism of D-cycloserine action: alanine racemase from *Escherichia coli* W. J Bacteriol 110:978–987

Linnett PE, Strominger JL (1973) Additional antibiotic inhibitors of peptidoglycan synthesis. Antimicrob Agents Chemother 4:231–236

Livermore DM (2008) Defining an extended-spectrum *beta*-lactamase. Clin Microbiol Infect 14(Suppl 1):3–10

Lovering AL, de Castro LH, Lim D, Strynadka NC (2007) Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis. Science 315:1402–1405

Lugtenberg EJJ, van Schijndel-van Dam A, van Bellegem THM (1971) In vivo and *in vitro* action of new antibiotics interfering with the utilization of *N*-acetyl-glucosamine-*N*-acetyl-muramyl-pentapeptide. J Bacteriol 108:20–29

Magnet S, Arbeloa A, Mainardi JL, Hugonnet JE, Fourgeaud M, Dubost L, Marie A, Delfosse V, Mayer C, Rice LB, Arthur M (2007a) Specificity of L, D-transpeptidases from gram-positive bacteria producing different peptidoglycan chemotypes. J Biol Chem 282:13151–13159

Magnet S, Bellais S, Dubost L, Fourgeaud M, Mainardi JL, Petit-Frere S, Marie A, Mengin-Lecreulx D, Arthur M, Gutmann L (2007b) Identification of the L, D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. J Bacteriol 189:3927–3931

Mahapatra S, Scherman H, Brennan PJ, Crick DC (2005) N Glycolylation of the nucleotide precursors of peptidoglycan biosynthesis of *Mycobacterium* spp. is altered by drug treatment. J Bacteriol 187:2341–2347

Mainardi JL, Hugonnet JE, Rusconi F, Fourgeaud M, Dubost L, Moumi AN, Delfosse V, Mayer C, Gutmann L, Rice LB, Arthur M (2007) Unexpected inhibition of peptidoglycan LD-transpeptidase from *Enterococcus faecium* by the *beta*-lactam imipenem. J Biol Chem 282: 30414–30422

Mainardi JL, Villet R, Bugg TD, Mayer C, Arthur M (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS Microbiol Rev 32:386–408

Marquardt JL, Siegele DA, Kolter R, Walsh CT (1992) Cloning and sequencing of *Escherichia coli murZ* and purification of its product, a UDP-*N*-acetylglucosamine enolpyruvyl transferase. J Bacteriol 174:5748–5752

Matsuhashi M, Dietrich CP, Strominger JL (1965) Incorporation of glycine into the cell wall glycopeptide in *Staphylococcus aureus*: role of sRNA and lipid intermediates. Proc Natl Acad Sci USA 54:587–594

Matsuhashi M, Ishino F, Nakagawa J, Mitsui K, Nakajima-Iijima S, Tamaki S (1984) Functional biosynthesis of cell wall peptidoglycan by polymorphic bifunctional polypeptides. Penicillin-binding protein 1Bs of *Escherichia coli* with activities of transglycosylase and transpeptidase. J Biol Chem 259:13937–13946

Maurer P, Koch B, Zerfaß I, Krauß J, van der Linden M, Frère J-M, Contreras-Martel C, Hakenbeck R (2008) Penicillin-binding protein 2x of *Streptococcus pneumoniae*: Three new mutational pathways for remodelling an essential enzyme into a resistance determinant. J Mol Biol 376:1403–1416

Mazzotta AS, Montville TJ (1999) Characterization of fatty acid composition, spore germination, and thermal resistance in a nisin-resistant mutant of *Clostridium botulinum* 169B and in the wild-type strain. Appl Environ Microbiol 65:659–664

McCafferty DG, Cudic P, Frankel BA, Barkallah S, Kruger RG, Li W (2002) Chemistry and biology of the ramoplanin family of peptide antibiotics. Biopolymers 66:261–284

McCormick MH, McGuire JM, Pittenger GE, Pittenger RC, Stark WM (1956) Vancomycin, a new antibiotic. I. Chemical and biologic properties. Antibiot Annu 1955–1956:606–611

McCoy AJ, Maurelli AT (2005) Characterization of *Chlamydia* MurC-Ddl, a fusion protein exhibiting D-alanyl-D-alanine ligase activity involved in peptidoglycan synthesis and D-cycloserine sensitivity. Mol Microbiol 57:41–52

McDonald LA, Barbieri LR, Carter GT, Lenoy E, Lovin J, Petersen PJ, Siege MM, Singh G, Williamson RT (2002) Structures of the muraymycins, novel peptidoglycan biosynthesis inhibitors. J Am Chem Soc 124:10260–10261

Mendoza C, Garcia JM, Llaneza J, Mendez FJ, Hardisson C, Ortiz JM (1980) Plasmid-determined resistance to fosfomycin in *Serratia marcescens*. Antimicrob Agents Chemother 18:215–219

Mengin-Lecreulx D, van Heijenoort J, Park JT (1996) Identification of the mpl gene enco ding UDP-*N*-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase in *Escherichia coli* and its role in recycling of cell wall peptidoglycan. J Bacteriol 178:5347–5352

Milligan DL, Tran SL, Strych U, Cook GM, Krause KL (2007) The alanine racemase of *Mycobacterium smegmatis* is essential for growth in the absence of D-alanine. J Bacteriol 189:8381–8386

Mitsuhashi S (1981) *Beta*-Lactam Antibiotics. Japan Scientific Societies Press and Springer, Tokyo and Berlin

Molinari H, Pastore A, Lian LY, Hawkes GE, Sales K (1990) Structure of vancomycin and a vancomycin/D-Ala-D-Ala complex in solution. Biochemistry 29:2271–2277

Morin RB, Jackson BG, Flynn EH, Roeske RW (1962) Chemistry of cephalosporin antibiotics. 1. 7-Aminocephalosporanic acid. J Am Chem Soc 84:3400–3401

Müller C, Nolden S, Gebhardt P, Heinzelmann E, Lange C, Puk O, Welzel K, Wohlleben W, Schwartz D (2007) Sequencing and analysis of the biosynthetic gene cluster of the lipopeptide

antibiotic Friulimicin in *Actinoplanes friuliensis*. Antimicrob Agents Chemother 51: 1028–1037

Muramatsu Y, Ishii MM, Inukai M (2003) Studies on novel bacterial translocase I inhibitors, A-500359s. II. Biological activities of A-500359 A, C, D, and G. J Antibiot (Tokyo) 56:253-258

Muroi M, Kimura K, Osada H, Inukai M, Takatsuki A (1997) Liposidomycin B inhibits *in vitro* formation of polyprenyl (pyro)phosphate *N*-acetylglucosamine, an intermediate in glycoconjugate biosynthesis. J Antibiot (Tokyo) 50:103–104

Nakaminami H, Noguchi N, Nishijima S, Kurokawa I, Sasatsu M (2008) Characterization of the pTZ2162 encoding multidrug efflux gene *qacB* from *Staphylococcus aureus*. Plasmid 60:108–117

Neuhaus FC (1967) D-cycloserine and *O*-carbamyl-D-serine. In: Gottlieb D, Shaw PD (eds) Antibiotics: mechanism of action. Springer, New York, pp 40–83

Newton GGF, Abraham EP (1956) Isolation of cephalosporin C, a penicillin-like antibiotic containing D-a-aminoadipic acid. Biochem J 62:651–658

Nikaido H (1989) Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob Agents Chemother 33:1831–1836

Nikaido H, Rosenberg EY (1983) Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J Bacteriol 153:241–252

Noble WC, Virani Z, Cree RG (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol Lett 72:195–198

Noda Y, Yoda K, Takatsuki A, Yamasaki M (1992) TmrB protein, responsible for tunicamycin resistance of *Bacillus subtilis*, is a novel ATP-binding membrane protein. J Bacteriol 174:4302–4307

Noda Y, Takatsuki A, Yoda K, Yamasaki M (1995) TmrB protein, which confers resistance to tunicamycin on *Bacillus subtilis*, binds tunicamycin. Biosci Biotechnol Biochem 59:321–322

Nordmann P, Naas T, Fortineau N, Poirel L (2007) Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus* spp. and *Pseudomonas aeruginosa* in 2010. Curr Opin Microbiol 10:436–440

O'Hara K (1993) Two different types of fosfomycin resistance in clinical isolates of *Klebsiella pneumoniae*. FEMS Microbiol Lett 114:9–16

Ohki R, Tateno K, Okada Y, Okajima H, Asai K, Sadaie Y, Murata M, Aiso T (2003) A bacitracin-resistant *Bacillus subtilis* gene encodes a homologue of the membrane-spanning subunit of the *Bacillus licheniformis* ABC transporter. J Bacteriol 185:51–59

Ostash B, Saghatelian A, Walker S (2007) A streamlined metabolic pathway for the biosynthesis of moenomycin A. Chem Biol 14:257–267

Paik J, Kern I, Lurz R, Hakenbeck R (1999) Mutational analysis of the *Streptococcus pneumoniae* bimodular class A penicillin-binding proteins. J Bacteriol 181:3852–3856

Parenti F (1986) Structure and mechanism of action of teicoplanin. J Hosp Infect 7(Suppl A): 79–83

Parenti F, Beretta G, Berti M, Arioli V (1978) Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* Nov. Sp. I. Description of the producer strain, fermentation studies and biological properties. J Antibiot (Tokyo) 31:276–283

Parenti F, Ciabatti R, Cavalleri B, Kettenring J (1990) Ramoplanin: a review of its discovery and its chemistry. Drugs Exp Clin Res 16:451–455

Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274:8405–8410

Peteroy M, Severin A, Zhao F, Rosner D, Lopatin U, Scherman H, Belanger A, Harvey B, Hatfull GF, Brennan PJ, Connell ND (2000) Characterization of a *Mycobacterium smegmatis* mutant that is simultaneously resistant to D-cycloserine and vancomycin. Antimicrob Agents Chemother 44:1701–1704

Pitkanen M, Sirvio J, MacDonald E, Ekonsalo T, Riekkinen P Sr (1995) The effects of D-cycloserine, a partial agonist at the glycine binding site, on spatial learning and working memory in scopolamine-treated rats. J Neural Transm Park Dis Dement Sect 9:133–144

Plapp R, Strominger JL (1970) Biosynthesis of the peptidoglycan of bacterial cell walls. XVII. Biosynthesis of peptidoglycan and of interpeptide bridges in Lactobacillus viridescens. J Biol Chem 245:3667–3674

Podlesek Z, Comino A, Herzog-Velikonja B, Zgur-Bertok D, Komel R, Grabnar M (1995) *Bacillus licheniformis* bacitracin-resistance ABC transporter: relationship to mammalian multidrug resistance. Mol Microbiol 16:969–976

Queenan AM, Bush K (2007) Carbapenemases: the versatile *beta*-lactamases. Clin Microbiol Rev 20:440–458

Reynolds PE (1989) Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis 8:943–950

Rietkötter E, Hoyer D, Mascher T (2008) Bacitracin sensing in *Bacillus subtilis*. Mol Microbiol 68:768–785

Rife CL, Pharris RE, Newcomer ME, Armstrong RN (2002) Crystal structure of a genomically encoded fosfomycin resistance protein (FosA) at 1.19 A resolution by MAD phasing off the L-III edge of Tl(+). J Am Chem Soc 124:11001–11003

Rogers LA (1928) The inhibiting effect of *Streptococcus lactis* on *Lactobacillus bulgaricus*. J Bacteriol 16:321–325

Rogers HJ, Perkins HR, Ward JB (1980) Microbial cell walls and membranes. Chapman & Hall Ltd, London, United Kingdom

Rolinson GN (1998) Forty years of β-lactam research. J Antimicrob Chemother 41:589–603

Russell RR (1972) Mapping of a D-cycloserine resistance locus in *Escherichia coli* K-12. J Bacteriol 111:622–624

Ruzin A, Singh G, Severin A, Yang Y, Dushin RG, Sutherland AG, Minnick A, Greenstein M, May MK, Shlaes DM, Bradford PA (2004) Mechanism of action of the mannopeptimycins, a novel class of glycopeptide antibiotics active against vancomycin-resistant gram-positive bacteria. Antimicrob Agents Chemother 48:728–738

Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32:234–258

Schaller K, Holtje JV, Braun V (1982) Colicin M is an inhibitor of murein biosynthesis. J Bacteriol 152:994–1000

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477

Sheldrick GM, Jones PG, Kennard O, Williams DH, Smith GA (1978) Structure of vancomycin and its complex with acetyl-D-alanyl-D-alanine. Nature 271:223–225

Shinagawa S, Maki M, Kintaka K, Imada A, Asai M (1985) Isolation and characterization of bulgecins, new bacterial metabolites with bulge-inducing activity. J Antibiot (Tokyo) 38: 17–23

Siewert G, Strominger JL (1967) Bacitracin: an inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in the biosynthesis of the peptidolgycan of bacterial cell walls. Proc Natl Acad Sci USA 57:767–773

Silverman JA, Perlmutter NG, Shapiro HM (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother 47:2538–2544

Singh MP, Petersen PJ, Weiss WJ, Janso JE, Luckman SW, Lenoy EB, Bradford PA, Testa RT, Greenstein M (2003) Mannopeptimycins, new cyclic glycopeptide antibiotics produced by *Streptomyces hygroscopicus* LL-AC98: antibacterial and mechanistic activities. Antimicrob Agents Chemother 47:62–69

Spratt BG, Pardee AB (1975) Penicillin-binding proteins and cell shape in *E. coli*. Nature 254:516–517

Spratt BG, Zhou J, Taylor M, Merrick MJ (1996) Monofunctional biosynthetic peptidoglycan transglycosylases. Mol Microbiol 19:639–640

Stachyra T, Dini C, Ferrari P, Bouhss A, van Heijenoort J, Mengin-Lecreulx D, Blanot D, Biton J, Le Beller D (2004) Fluorescence detection-based functional assay for high-throughput screening for MraY. Antimicrob Agents Chemother 48:897–902

Staudenbauer W, Strominger JL (1972) Activation of D-aspartic acid for incorporation into peptidoglycan. J Biol Chem 247:5095–5102

Stone KJ, Strominger JL (1971) Mechanism of action of bacitracin: complexation with metal ion and C 55 -isoprenyl pyrophosphate. Proc Natl Acad Sci USA 68:3223–3227

Strynadka NC, Jensen SE, Johns K, Blanchard H, Page M, Matagne A, Frere JM, James MN (1994) Structural and kinetic characterization of a *beta*-lactamase-inhibitor protein. Nature 368:657–660

Strynadka NC, Jensen SE, Alzari PM, James MN (1996) A potent new mode of *beta*-lactamase inhibition revealed by the 1.7 A X-ray crystallographic structure of the TEM-1-BLIP complex. Nat Struct Biol 3:290–297

Sutton BJ, Artymiuk PJ, Cordero-Borboa AE, Little C, Phillips DC, Waley SG (1987) An X-ray-crystallographic study of *beta*-lactamase II from Bacillus cereus at 0.35 nm resolution. Biochem J 248:181–188

Suzuki H, van Heijenoort Y, Tamura T, Mizoguchi J, Hirota Y, van Heijenoort J (1980) *In vitro* peptidoglycan polymerization catalysed by penicillin-binding protein 1b of *Escherichia coli*. FEBS Lett 110:245–249

Sykes RB, Bonner DP (1985) Discovery and development of the monobactams. Rev Infect Dis 7 (Suppl 4):S579–S593

Takayama K, David HL, Wang L, Goldman DS (1970) Isolation and characterization of uridine diphosphate-*N*-glycolylmuramyl-L-alanyl-gamma-D-glutamyl-meso-alpha, alpha′- diaminopimelic acid from *Mycobacterium tuberculosis*. Biochem Biophys Res Commun 39:7–12

Tamura G (1982) Tunicamycins. Japan Scientific Press, Tokyo

Tanaka H, Oiwa R, Matsukura S, Inokoshi J, Omura S (1982) Studies on bacterial cell wall inhibitors. X. Properties of phosph-*N*-acetylmuramoyl-pentapeptide-transferase in peptidoglycan synthesis of *Bacillus megaterium* and its inhibition by amphomycin. J Antibiot (Tokyo) 35:1216–1221

Taylor JG, Li X, Zhu W, Kahne DE (2006) The total synthesis of moenomycin A. J Am Chem Soc 128:15084–15085

Templin MF, Edwards DH, Höltje JV (1992) A murein hydrolase is the specific target of bulgecin in *Escherichia coli*. J Biol Chem 267:20039–20043

Thunnissen AM, Rozeboom HJ, Kalk KH, Dijkstra BW (1995) Structure of the 70-kDa soluble lytic transglycosylase complexed with bulgecin A. Implications for the enzymatic mechanism. Biochemistry 34:12729–12737

Tipper DJ, Strominger JL (1965) Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci USA 54:1133–1141

Tsuda H, Yamashita Y, Shibata Y, Nakano Y, Koga T (2002) Genes involved in bacitracin resistance in *Streptococcus mutans*. Antimicrob Agents Chemother 46:3756–3764

van Asselt EJ, Kalk KH, Dijkstra BW (2000) Crystallographic studies of the interactions of *Escherichia coli* lytic transglycosylase Slt35 with peptidoglycan. Biochemistry 39:1924–1934

van Bambeke F, Chauvel M, Reynolds PE, Fraimow HS, Courvalin P (1999) Vancomycin-dependent *Enterococcus faecalis* clinical isolates and revertant mutants. Antimicrob Agents Chemother 43:41–47

van Heijenoort J (2007) Lipid intermediates in the biosynthesis of bacterial peptidoglycan. Microbiol Mol Biol Rev 71:620–635

van Heijenoort Y, van Heijenoort J (1980) Biosynthesis of the peptidoglycn of *Escherichia coli* K12. Properties of the in vitro polymerization by transglycosylation. FEBS Lett 110:214–244

Venkateswaran PS, Wu HC (1972) Isolation and characterization of a phosphonomycin-resistant mutant of *Escherichia coli* K-12. J Bacteriol 110:935–944

Vinella D, Albrecht C, Cashel M, D'Ari R (2005) Iron limitation induces SpoT-dependent accumulation of ppGpp in *Escherichia coli*. Mol Microbiol 56:958–970
Vollmer W, Blanot D, de Pedro MA (2008a) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32:149–167
Vollmer W, Joris B, Charlier P, Foster S (2008b) Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev 32:259–286
Vollmerhaus PJ, Breukink E, Heck AJ (2003) Getting closer to the real bacterial cell wall target: biomolecular interactions of water-soluble lipid II with glycopeptide antibiotics. Chemistry 9:1556–1565
Walker S, Chen L, Hu Y, Rew Y, Shin D, Boger DL (2005) Chemistry and biology of ramoplanin: a lipoglycodepsipeptide with potent antibiotic activity. Chem Rev 105:449–476
Wang J, Palzkill T, Chow DC (2009) Structural insight into the kinetics and DCp of interactions between TEM-1 β-lactamase and β-lactamase inhibitory protein (BLIP). J Biol Chem 284:595–609
Ward JM, Hodgson JE (1993) The biosynthetic genes for clavulanic acid and cephamycin production occur as a 'supercluster' in three *Streptomyces*. FEMS Microbiol Lett 110:239–242
Wargel RJ, Hadur CA, Neuhaus FC (1971) Mechanism of D-cycloserine action: transport mutants for D-alanine, D-cycloserine, and glycine. J Bacteriol 105:1028–1035
Weidel W, Pelzer H (1964) Bad-shaped macromolecules – a new outlook on bacterial cell walls. Adv Enzymol 26:193–232
Welzel P (2005) Syntheses around the transglycosylation step in peptidoglycan biosynthesis. Chem Rev 105:4610–4660
Welzel P (2007) A long research story culminates in the first total synthesis of moenomycin A. Angew Chem Int Ed Engl 46:4825–4829
Welzel P, Witteler FJ, Müller D, Riemer W (1981) Structure of the antibiotic moenomycin A. Angew Chem Int Ed Engl 20:121–123
Welzel P, Wietfeld B, Kunisch F, Schubert T, Hobert K, Duddeck H, Müller D, Huber G, Maggio JE, Williams DH (1983) Moenomycin A: further structural studies and preparation of simple derivatives. Tetrahedron 39:1583–1591
Werner G, Strommenger B, Witte W (2008) Acquired vancomycin resistance in clinically relevant pathogens. Future Microbiol 3:547–562
Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, Sahl HG (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem 276:1772–1779
Wiedemann I, Bottiger T, Bonelli RR, Wiese A, Hagge SO, Gutsmann T, Seydel U, Deegan L, Hill C, Ross P, Sahl HG (2006) The mode of action of the lantibiotic lacticin 3147 – a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Mol Microbiol 61:285–296
Willey JM, van der Donk WA (2007) Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol 61:477–501
Woodyer RD, Shao Z, Thomas PM, Kelleher NL, Blodgett JA, Metcalf WW, van der Donk WA, Zhao H (2006) Heterologous production of fosfomycin and identification of the minimal biosynthetic gene cluster. Chem Biol 13:1171–1182
Yuan Y, Fuse S, Ostash B, Sliz P, Kahne D, Walker S (2008) Structural analysis of the contacts anchoring moenomycin to peptidoglycan glycosyltransferases and implications for antibiotic design. ACS Chem Biol 3:429–436
Zapun A, Contreras-Martel C, Vernet T (2008) Penicillin-binding proteins and *beta*-lactam resistance. FEMS Microbiol Rev 32:361–385
Zeth K, Romer C, Patzer SI, Braun V (2008) Crystal structure of colicin M, a novel phosphatase specifically imported by *Escherichia coli*. J Biol Chem 283:25324–25331
Zhang Z, Palzkill T (2004) Dissecting the protein-protein interface between *beta*-lactamase inhibitory protein and class A *beta*-lactamases. J Biol Chem 279:42860–42866

Chapter 13
Bacterial Autolysins

Marie-Pierre Chapot-Chartier

13.1 Introduction

Bacteria produce enzymes which are capable of hydrolyzing bonds in their own protective cell wall peptidoglycan. Peptidoglycan is essential for maintaining cell shape and cell integrity. Thus, the activity of peptidoglycan hydrolases (PGHs) may lead to bacterial lysis. In this event, these enzymes are called autolysins (Shockman and Höltje 1994). Autolysis is observed under conditions which result in the cessation of peptidoglycan synthesis, such as stationary phase or exposure to antibiotics, possibly from an uncontrolled action of PGHs. Although potentially lethal, PGHs are present in bacteria throughout growth and are proposed to be involved in numerous cellular functions which require cell wall remodelling such as cell enlargement, recycling and maturation of peptidoglycan, cell division and daughter cell separation, motility, sporulation, genetic competence, protein secretion, biofilm formation and pathogenicity.

We will consider here mainly model autolysin systems from Gram-positive bacteria. The PGH system from the model Gram-negative bacterium, *Escherichia coli*, has also been studied in detail and was reviewed recently (Vollmer and Bertsche 2008).

13.2 Hydrolytic Specificity of Bacterial Autolysins

The cell wall of Gram-positive bacteria is made of a thick peptidoglycan layer decorated with other polymers: teichoic acids which are anionic polymers, proteins and polysaccharides. Peptidoglycan is the major cell wall constituent (50–80% of

M.-P. Chapot-Chartier
INRA, UMR1319 Micalis, Domaine de Vilvert, F-78352 Jouy-en-Josas, France
e-mail: Marie-Pierre.Chapot@jouy.inra.fr

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_13,

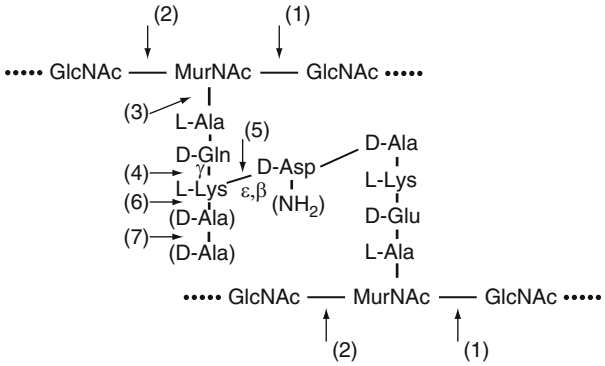

Fig. 13.1 Schematic structure of *L. lactis* peptidoglycan and sites of cleavage of PGHs. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid. D-Ala residues at positions 4 and 5 of the acceptor peptide chains may be present or not, and D-Asp may be amidated or not according to previous *L. lactis* peptidoglycan structural analysis (Courtin et al. 2006). The specific cleavage sites of the different classes of PGHs are indicated by *arrows*. (1) *N*-acetylmuramidase; (2) *N*-acetylglucosaminidase; (3) *N*-acetylmuramyl-L-Ala-amidase, (4) D,L-endopeptidase, (5) endopeptidase, (6) L,D-carboxypeptidase, (7) D,D-carboxypeptidase

the dry weight) and ensures the stability and rigidity of the cell wall. Peptidoglycan is a heteropolymer made of linear glycan chains composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), and these chains are cross-linked by short peptide chains. Four classes of PGHs can be defined on the basis of their hydrolytic bond specificity inside the peptidoglycan (Fig. 13.1):

- *N*-Acetylmuramidases (muramidases or lysozymes) which hydrolyze the β1–4 bond between MurNAc and GlcNAc.
- *N*-Acetylglucosaminidases (glucosaminidases) which hydrolyze the β1–4 bond between GlcNAc and MurNAc.
- *N*-Acetylmuramyl-L-Ala amidases (amidases) which hydrolyze the bond between the lactyl group of MurNAc and the α-amino group of L-alanine, the first amino acid of the lateral peptidic chain.
- Peptidases which hydrolyze different bonds inside the peptidoglycan.

In addition, lytic transglycosylases break the same bonds as muramidases but yield anhydromuropeptides as products. PGHs such as D-Ala-D-Ala-carboxypeptidases are unable to provoke bacterial lysis on their own, and thus they are not classified as autolysins (Shockman and Höltje 1994).

PGH activity can be detected by following the decrease of turbidity of a bacterial suspension. Also, radioactively labeled peptidoglycan was used as substrate allowing quantification of soluble radioactivity released by PGH activity. An efficient technique to detect PGHs is the zymogram technique allowing visualization of bacteriolytic activities after SDS-PAGE in a gel containing autoclaved bacteria or cell walls (Leclerc and Asselin 1989). By means of HPLC separation and

identification of the structure of peptidoglycan fragments released by PGHs with mass spectrometry, their hydrolytic specificities can be determined (Glauner et al. 1988; Horsburgh et al. 2003).

The PGH content varies between bacterial species. A given bacterial species usually produces several PGHs, with or without identical hydrolytic specificity. PGHs were initially identified and characterized after purification, which proved to be a very tough and time-consuming task, since the potentially lethal autolysins are usually present in low amounts and strongly associated to the cell wall. Subsequently, genes were cloned using oligonucleotide probes derived from the amino acid sequence of the purified proteins or alternatively after activity screening of libraries constructed in *E. coli*. The availability of complete genome sequences now allows for searching the PGH complement of a given bacteria by performing amino acid sequence similarity searches with representative sequences of all known classes of PGHs (Smith et al. 2000).

13.3 Modular Organization of Bacterial Autolysins

Most PGHs possess a modular structure and comprise most often a catalytic domain and a cell wall binding domain. This modular organization was initially proposed on the basis of sequence similarity between the major amidase from *Streptococcus pneumoniae* and the lytic enzymes, amidases or muramidases, encoded by bacteriophages which infect this bacterium (Garcia et al. 1988). This organization was confirmed by the construction of chimeric enzymes choosing, on one hand, the hydrolytic specificity and, on the other hand, the specificity of cell wall binding (Lopez et al. 1992).

Table 13.1 presents the autolysins which were characterized at the molecular level in five Gram-positive bacteria: *S. pneumoniae*, *S. aureus*, *Listeria monocytogenes*, *Lactococcus lactis* and *Bacillus subtilis*. It is worth noting that the PGH complement of each of these bacterial species most probably contains other (putative) PGHs that could be identified in their genome sequence by homology search. Such an approach was performed previously for the genome sequence of *B. subtilis* 168 leading to the identification of a total of 35 putative PGHs (Smith et al. 2000).

13.3.1 Catalytic Domains

The catalytic domains of the autolysins listed in Table 13.1 belong to nine PFAM domain families (Finn et al. 2008). These domains include one domain for glucosaminidases (PF01832), one domain for muramidases (glyco_hydro_25, PF01183), one domain for lytic transglycosylases (Transglycosylase-like, PF06737), two domains for amidases (Amidase_2, PF01510 and Amidase_3, PF01520), CHAP domain (cysteine, histidine-dependant amidohydrolase/peptidase domain)

Table 13.1 Well-characterized autolysins from several Gram-positive bacterial species

Species and name	Length (AA)	Catalytic domain		Cell wall binding domain (or other domain)[a]		Reference
		Specificity	Position	Type	Position	
Streptococcus pneumoniae						
LytA	318	Amidase[b]	N-ter	7×ChhBD	C-ter	Garcia et al. (1985)
LytB	658	Glucosaminidase[c]	C-ter	15×ChBD	N-ter	Garcia et al. (1999a)
LytC	501	Muramidase[d]	C-ter	11×ChBD	N-ter	Garcia et al. (1999b)
CbpD	448	Amidase or peptidase* (CHAP)[e]	N-ter	2×SH3 3×ChBD	N-ter	Guiral et al. (2005)
PcsB	392	Amidase or peptidase* (CHAP)[e]	C-ter	Leu zipper motif	N-ter	Ng et al. (2004)
Pmp23	204	Transglycosylase or muramidase[f]	N-ter			Pagliero et al. (2005)
Staphylococcus aureus						
AtlA	1256	Amidase[b] and glucosaminidase[c]	N-ter C-ter	2×GW 1×GW	C-ter N-ter	Oshida et al. (1995)
SleI	334	Amidase (CHAP)[e]	C-ter	3×LysM		Kajimura et al. (2005)
LytM	316	Gly-Gly-endopeptidase[g]	C-ter	Other	N-ter	Ramadurai and Jayaswal (1997)
LytN	383	Amidase or peptidase* (CHAP)[e]	C-ter	1×LysM Other	N-ter	Sugai et al. (1998)
SceD	231	Transglycosylase*[,h]	C-ter	Other	N-ter	Stapleton et al. (2007)
IsaA	233	Transglycosylase*[,h]	C-ter	Other	N-ter	Stapleton et al. (2007)
Listeria monocytogenes						
Ami	917	Amidase[b]	N-ter	4×GW	C-ter	Braun et al. (1997)
Auto	572	Glucosaminidase*[,c]	N-ter	4×GW	C-ter	Cabanes et al. (2004)
P60 (Iap)	484	DL-Endopeptidase*[,i]	C-ter	1×LysM 1×SH3 1×LysM Domain with TN repeats	N-ter Middle	Wuenscher et al. (1993)
MurA (NamA)	590	Glucosaminidase*[,c]	N-ter	4×LysM	C-ter	Carroll et al. (2003)
IspC	774	Glucosaminidase*[,c]	N-ter	7×GW	C-ter	Wang and Lin (2007)
P45	402	Endopeptidase P60*[,i]	C-ter	Other	N-ter	Schubert et al. (2000)
Lactococcus lactis						
AcmA	439	Glucosaminidase[c]	N-ter	3×LysM	C-ter	Buist et al. (1995)
AcmB	499	Glucosaminidase[c] and (amidase or peptidase) (CHAP)*[,e]	Middle C-ter	Domain rich in S, T, N, P	N-ter	Huard et al. (2003)

AcmC	194	Glucosaminidase[c]	N-ter	None		Huard et al. (2004)
AcmD	361	Glucosaminidase*,[c]	N-ter	3×LysM	C-ter	Huard et al. (2004)
YjgB	197	γ-D-Gln-L-Lys-endopeptidase[i]	N-ter	None		Redko et al. (2007)
Bacillus subtilis						
CwlA	272	Amidase[b]	N-ter	PG_binding_1	C-ter	Kuroda et al. (1991) Foster (1991)
CwlB (LytC)	496	Amidase[j]	C-ter	3× CW_binding_2	N-ter	Kuroda and Sekiguchi (1991) Margot and Karamata (1992)
CwlC	255	Amidase[j]	N-ter	SPOR	C-ter	Kuroda et al. (1993)
CwlD	236	Amidase[j]	N-ter	None		Sekiguchi et al. (1995)
LytD	879	Glucosaminidase[c]	C-ter	SPOR SH3	N-ter Middle	Margot et al. (1994)
LytE (CwlF)	334	DL-Endopeptidase*,[i]	C-ter	3×LysM	N-ter	Margot et al. (1998)
LytF (CwlE)	488	DL-Endoptidase[i]	C-ter	5×LysM	N-ter	Margot et al. (1999
CwlH	250	Amidase[b]	N-ter	PG_binding_1	C-ter	Nugroho et al. (1999)
CwlJ	142	Amidase or peptidase or lytic transglycosylase[k]	N-ter	None		Ishikawa et al. (1998)
LytG	282	Glucosaminidase[c]	N-ter	None		Horsburgh et al. (2003)
SleB	305	Amidase or peptidase or lytic transglycosylase[k]	C-ter	PG_binding_1	C-ter	Moriyama et al. (1996)
CwlO	473	DL-Endopeptidase[i]	C-ter	Domain rich in S, N	N-ter	Yamaguchi et al. (2004)

*Based on sequence homology, no experimental determination

[a]*ChBD*, choline-binding domain (PF01473); *SH3*, SH3-domain (PF08460); *GW*, GW modules; *LysM*, LysM-domain (PF01476); *PG_binding_1*, putative peptidoglycan binding domain (PF01471); *CW_binding_2*, putative cell wall binding repeats (PFAM04122); *SPOR*, sporulation-related domain (PF05036)

[b]Amidase_2 (PF01510)

[c]Glucosaminidase (PF01832)

[d]Glyco_hydro_25 (muramidase) (PF01183)

[e]CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) domain (amidase or peptidase) (PF05257)

[f]PECACE domain

[g]Peptidase_M23 (Gly-Gly-endopeptidase) (PF01551)

[h]Transglycosylase-like (PF06737)

[i]NlpC_P60 (PF00877) (including γ-glutamyl-diamino-acid endopeptidases)

[j]Amidase_3 (PF01520)

[k]Hydrolase_2 (PF07486)

(amidase or peptidase) (PF05257), two domains for peptidases (NlpC_P60, PF00877, including γ-glutamyl-diamino-acid endopeptidases and peptidase_M23 (Gly-Gly-endopeptidase, PF01551) and one domain with undetermined specificity (Hydrolase_2). Another catalytic domain (PECACE domain) was identified recently (Pagliero et al. 2005).

13.3.2 Cell Wall Binding Domains

As mentioned above, in addition to their catalytic domain, PGHs often contain a specific cell wall binding domain. The domains present in the autolysins of Table 13.1 are described below.

The LysM domain (PF01476) is a widespread protein module, present in eukaryotes and prokaryotes. In bacteria, it is found especially in PGHs. The structure of one LysM domain present in *E. coli* lytic transglycosylase MltD has been resolved by NMR studies (Bateman and Bycroft 2000), and consists of a sequence motif of about 40 residues. LysM domains are usually present as several repeats in the PGHs and their number varies (see Table 13.1). LysM domain was shown to bind peptidoglycan, and from binding studies on several peptidoglycan chemotypes it was deduced that it binds the glycan chain (Steen et al. 2003). However, the exact motif recognized by LysM is not yet identified. Recent data suggest that binding of LysM to peptidoglycan is not affected by de-*N*-acetylation of GlcNAc (Meyrand et al. 2007) or by *O*-acetylation of MurNAc (Eckert and Mesnage, unpublished data).

The choline-binding domain (CW_binding_1, PF01473) (ChBD), which consists of 20 amino acids, is usually present as several repeats, and was identified initially in the *S. pneumoniae* PGHs (Fernandez-Tornero et al. 2001). The number of ChBDs varies among the PGHs (see Table 13.1). A minimum of four ChBDs appears to be required for efficient binding to the cell wall. In *S. pneumoniae* PGHs LytA or LytB, they bind choline present in the teichoic acids of this bacterial species.

The SH3 domain initially known in eukaryotes and viruses was first identified in P60 from *Listeria grayi* (Whisstock and Lesk 1999). The SH3 bacterial domain (SH3_3, PF08460) is present in ALE-1 which is a homolog of lysostaphin, a PGH produced by *Staphylococcus simulans* that specifically lyses *S. aureus*. It was shown that the SH3-containing domain of ALE-1 binds peptidoglycan and that the length of the interpeptide cross bridge and its amino acid composition have a major impact on the binding (Lu et al. 2006).

GW modules found in *L. monocytogenes* and *S. aureus* proteins are 80-amino-acid modules starting with Gly-Trp (GW) dipeptide, and are found in variable numbers. In listerial internalin B, the C-terminal domain containing GW modules binds lipoteichoic acids (LTA) in the cell wall (Jonquieres et al. 1999). In addition, GW modules constitute a cell adhesion motif. GW-like modules were likewise

found in *S. aureus* proteins, especially in AtlA autolysin, where they are cell surface targeting signals (Baba and Schneewind 1998).

SPOR domain (sporulation-related domain, PF05036), present in *B. subtilis* PGHs, is composed of 70 residues made up of a tandem repeat of 35 amino acid residues. Its 3D structure was determined by NMR studies and evidence was obtained that this domain binds peptidoglycan (Mishima et al. 2005).

In *B. subtilis*, two other cell wall binding domains are found in the autolysins of Table 13.1: CW_binding_2 (PF04122) present in CwlB and PG_binding_1 (PF01471) present in CwlA amidase.

A domain rich in Ser/Thr/Pro/Asn residues was found at the N-terminus of *L. lactis* AcmB (Huard et al. 2003). A similar domain present in *Streptococcus salivarius* fructosyltransferase was shown to be involved in cell surface attachment of the protein (Rathsam and Jacques 1998).

13.4 Prophage-Encoded Peptidoglycan Hydrolases

Numerous bacterial strains contain prophages in their genome. Lysogenic phages usually encode PGHs, called lysins, to lyze the host cells when there are induced and subsequently liberate the phage particles after replication. Bacteriophage lytic enzymes usually possess the same modular organization as bacterial PGHs with a catalytic domain and a cell wall binding domain and these domains may present sequence homology with the one found in bacterial PGHs (Lopez and Garcia 2004).

Several lytic enzymes encoded by prophage DNA were characterized in *B. subtilis* (Smith et al. 2000). In certain lysogenic strains of lactic acid bacteria, endolysin activity was detected in cellular extracts, without any prophage-inducing factor, suggesting a base level of constitutive expression. In *L. lactis* subsp. *cremoris* AM2, a highly autolytic strain, the 46-kDa activity detected by zymogram analysis, corresponds to the endolysin of the temperate bacteriophage carried by the strain (Lepeuple et al. 1998). This was also observed for several strains of *Streptococcus thermophilus* where the major activity at 31 kDa in zymogram of bacterial cellular extracts corresponds to the endolysin of the resident temperate phage (Husson-Kao et al. 2000).

13.5 Cellular Localization

In order to reach their substrate, autolysins must be exported through the cytoplasmic membrane. Most of them are endowed with a typical signal sequence. However in a few cases, no signal sequence was detected, such as in LytA amidase of *S. pneumoniae* (Garcia et al. 1985) or in LycC muramidase of *Clostridium acetobutylicum* (Croux and Garcia 1991). Since these proteins are not encoded by

prophage DNA, these observations suggest that other unidentified export mechanisms are involved.

In contrast, although they possess a putative signal sequence, two autolytic enzymes from *Listeria monocytogenes*, the P60 endopeptidase and the NamA *N*-acetylmuramidase, were secreted through a SecA2-dependent mechanism (Lenz et al. 2003). SecA2 is a non-essential, auxiliary SecA paralog identified in several Gram-positive bacteria and proposed to promote bacterial pathogenesis.

As described above, the majority of PGHs contain a domain which anchors the proteins to the cell wall through specific binding to a cell wall component. Thus, these PGHs are found associated to the cell wall. Nevertheless, in some cases, part of the protein was also found in the culture supernatant, such as *L. lactis* AcmA major autolysin (Buist et al. 1995), Mur-2 autolysin from *Enterobacter hirae* (Kariyama and Shockman 1992) or P60 from *L. monocytogenes* (Ruhland et al. 1993). In contrast, *E. hirae* Mur-1 was found exclusively associated to the cells (Kariyama and Shockman 1992).

Inside the cell wall, the precise localization of the PGHs depends seemingly on their physiological role. Several enzymes involved in cell separation after division were found localized at specific sites inside the cell wall. *Staphylococcus aureus* amidase and glucosaminidase, obtained by maturation of Atl precursor, were both localized by immunocytochemical experiments, at the level of the equatorial ring on the cell surface that marked the future cell division sites (Yamada et al. 1996). The repeat domains (R1, R2 and R3) present in both proteins were shown to be necessary and sufficient for targeting them to the equatorial surface ring, suggesting that they bind to a localized specific receptor (Baba and Schneewind 1998). In *L. lactis*, a fusion protein made of a viral protein and the C-terminal LysM-containing domain of *L. lactis* AcmA major autolysin was found to be present only at the cell poles by immunofluorescence experiments (Steen et al. 2003). In this case, the authors proposed that although LysM binds peptidoglycan which is present on the entire cell surface of Gram-positive bacteria, its binding is hindered by other cell wall constituents, most probably (lipo)teichoic acids, and binds only those sites on the cell surface where this component is absent (Steen et al. 2003). Also, in *B. subtilis*, the PGHs LytE, LytF and CwlS, all three involved in cell separation, were found localized at cell separation sites and cell poles during vegetative growth (Yamamoto et al. 2003; Fukushima et al. 2006). This specific localization may be dependent on the LysM repeats in their N-terminal domains (Fukushima et al. 2006). In contrast, LytC which is not involved in cell separation was found uniformly distributed on the *B. subtilis* cell surface (Yamamoto et al. 2003). *S. aureus* LytM endopeptidase (Ramadurai et al. 1999) as well as *L. monocytogenes* P60 (Ruhland et al. 1993) were also found uniformly distributed over the cell surface during exponential growth phase.

Recent work using the yeast two hybrid approach identified LytE as an interacting protein of the actin homolog MreBH in *B. subtilis*, suggesting co-localization of MreB and LytE in the cells (Carballido-Lopez et al. 2006). MreB isoforms (MreB, Mbl and MreBH) were previously shown to play an important role in the control of cell shape and to be localized in helical filamentous structures which encircle the

cell (Carballido-Lopez and Formstone 2007). Thus, it appears that LytE is targeted for export by MreBH to specific sites near the membrane. The co-localization of MreB isoforms and LytE would ensure that LytE is associated with the newly synthesized peptidoglycan and play a role in hydrolyzing fully stretched peptide bonds of peptidoglycan to release the tension in the wall and allow further extension of the cell.

13.6 Regulation and Control of Autolytic Activity

PGHs which are able to hydrolyze the protective cell wall peptidoglycan are potentially lethal enzymes for the cells that produce them. Thus their expression and/or their activity inside the cell wall have to be tightly regulated and also coordinated with the activities involved in peptidoglycan biosynthesis. No general model of regulation has emerged but different mechanisms were identified in different bacterial species.

13.6.1 Regulation at the Transcriptional Level

In *Bacillus subtilis*, autolysins are regulated at the transcriptional level, by several alternative factors. Transcription of the major vegetative autolysin genes *lytC* and *lytD* as well as that of the minor vegetative endopeptidase gene *lytF* is under the control of alternative sigma factor D, which is also involved in the regulation of flagellar synthesis, motility and chemotaxis (for review see Smith et al. 2000). Gene *lytC* also belongs to the divergon *lytRABC* (Kuroda et al. 1992; Lazarevic et al. 1992). *lytR* is transcribed divergently from the three other genes and encodes a DNA-binding protein which represses its own expression and that of *lytABC*.

A specific locus involved in autolysis regulation in *S. aureus* has been identified (Brunskill and Bayles 1996). It comprises two genes *lytSR* encoding a two-component regulatory system. A *lytS* knock-out mutant exhibited a marked propensity to form aggregates and a higher ability to autolyze, associated with a modification of its PGH content. The LytSR regulatory system was shown to positively regulate expression of the *lrgAB* operon, located immediately downstream of *lytSR* (Groicher et al. 2000). The *lrgAB* operon inhibits PGH activity and decreases sensitivity to penicillin-induced killing. *lrgA* encodes a small protein with properties similar to bacteriophage holins. It is proposed to act rather as an antiholin whereas the *cidA* gene identified subsequently encodes the holin (Rice et al. 2003). Both LrgA and CidA would regulate cell lysis in a way similar to bacteriophage, based on a holin–antiholin system. Another two-component system (TCS), *arlRS,* has a negative impact on autolysis in *S. aureus* (Fournier and Hooper 2000). It may work by positively regulating *lytSR*, *lrgA* and *lrgB* (Liang et al. 2005).

More recently, several other TCSs were shown to regulate expression of certain PGHs. The YvrGH TCS negatively regulates the transcription of *B. subtilis* LytC

(CwlB) (Serizawa et al. 2005). More generally, the essential YycFG TCS was shown to be a regulator of cell wall metabolism in *B. subtilis* (Bisicchia et al. 2007). Several genes encoding PGHs were found to be positively regulated by YycF, such as *yocH* encoding a putative PGH (Howell et al. 2003), *lytE* (Margot et al. 1998) and *cwlO* (Yamaguchi et al. 2004) which both encode endopeptidase-type PGHs; in addition *ykvT* encoding a putative PGH was found to possess the consensus sequence recognized by YycF upstream of the ORF. Finally, YycFG downregulates two genes whose products can indirectly modulate autolysin activity: *yjeA* encoding a peptidoglycan deacetylase and *yoeB* which was shown to modulate autolysin activity (Salzberg and Helmann 2007).

In *S. aureus*, the YycGF-homologous TCS, renamed WalKR, has a major role in controlling cell wall metabolism and biofilm formation. WalKR positively controls autolytic activity, in particular that of the two major *S. aureus* autolysins, AtlA and LytM (Dubrac et al. 2007).

Another example of autolysin regulation by TCS is AtlA from *S. mutans*, where AtlA activity and expression of *atlA* operon, which includes a gene encoding a thiol-disulfide oxidoreductase, are subject to the control of the VicRK TCS. The VicK sensor kinase protein was reported to bear a PAS domain, which is a sensor of oxygen and redox potential (Ahn and Burne 2007).

In *S. aureus*, evidence was found for coordination between cell wall synthesis and degradation. It was recently shown that perturbation of cell wall synthesis in growing bacteria induces strong repression of the transcription of several autolysin genes (Antignac et al. 2007; Sobral et al. 2007). This regulation could involve a TCS able to sense cell envelope stress.

13.6.2 Regulation at the Post-translational Level

Proteolysis. Muramidase M1 from *E. hirae* is synthetized as a precursor (zymogen) of 130 kDa, further processed to a 87-kDa active form (Shockman 1992). In contrast, in *B. subtilis*, extracellular proteases degrade PGHs involved in cell wall turnover (Jolliffe et al. 1980). Autolysis of *L. lactis* is also affected by proteolysis (Buist et al. 1998). Strains lacking the cell wall anchored proteinase PrtP exhibited a higher level of autolysis. In addition, autolysis was influenced by the cell wall proteinase hydrolytic specificity which varies between strains, as a result of different susceptibilities of the major autolysin AcmA to the different variants of the protease. In *L. lactis*, the housekeeping cell wall protease HtrA also degrades AcmA (Poquet et al. 2000).

Interaction with a modifier protein. A modifier protein (named CwbA) of CwlB amidase activity, encoded in the same operon as the amidase, has been described (Kuroda and Sekiguchi 1992; Margot and Karamata 1992). This protein binds stochiometrically the amidase and stimulates its activity two- to threefold. In addition, it is able to change its mode of action from a random to a sequential one (Herbold and Glaser 1975).

Interaction with cell wall components. The interaction between an autolysin and certain cell wall components can also modulate its activity. For example, the amidase LytA from *S. pneumoniae* is synthesized as an inactive form (E-form) present only in the cytoplasm. Activation of this precursor happens through formation of a complex with the choline present in teichoic acids (Tomasz and Westphal 1971)).

Several studies reported the influence of the D-alanylation level of teichoic acids (TAs) on PGH activity. The alditol-phosphate groups of lipoteichoic acids (LTAs) and wall teichoic acids (WTAs) may be substituted by D-alanyl esters in variable amounts. The positively charged D-alanyl-ester residues provide counterions to the negative charges carried by the phosphate groups of TAs. The absence of D-alanine substituents on TAs, obtained by inactivation of one gene in the *dlt* operon which encodes the proteins involved in addition of D-alanine, affects autolysis in several bacterial species. However, opposite effects were described according to the bacterial species studied. In *B. subtilis* (Wecke et al. 1997), the absence of D-alanine substituents on TAs enhances autolysis. The authors proposed that D-alanine-deprived TAs are more electronegative and bind more cationic autolysins. D-Alanine depletion of teichoic acids was also shown to increase autolysis of three lactic acid bacteria. In *L. lactis*, a reduced amount of D-alanine on TAs was proposed to diminish AcmA autolysin degradation by the housekeeping cell wall protease HtrA, which results in increased autolytic activity (Steen et al. 2005). In a *dlt* mutant of *Lb. plantarum*, defects in the separation process and cell envelope perforation were also detected and linked to the activity of the Acm2 autolysin (Palumbo et al. 2006). In *Lactobacillus rhamnosus* also, a *dlt* mutant was reported to have a higher autolysis rate than the wild type (Perea Velez et al. 2007). In contrast, in *S. aureus*, reduction of D-alanine substitution in TAs obtained by interruption of a *dlt* gene (Peschel et al. 2000) or the alanine racemase gene (Kullik et al. 1998) reduces autolysis. In these cases, the authors also proposed that alanine-free, anionic TAs strongly bind positively charged autolysins, but, in this case, this would prevent their access to the peptidoglycan and thus reduce autolysis.

In a recent work (Fedtke et al. 2007), a *S. aureus* mutant was obtained by inactivation of the *ypfP* gene responsible for biosynthesis of the glycolipid found in LTA. The mutant exhibited strongly reduced LTA content and revealed strongly reduced autolytic activity although the mutant revealed no major change in patterns of cell wall proteins or autolytic enzymes. Thus this study suggests that LTAs play a role in controlling autolysin activity rather than in binding the proteins in the cell wall.

Peptidoglycan structure. Chemical modifications of the peptidoglycan substrate such as *O*-acetylation or de-*N*-acetylation can also influence PGH activity. Muramidase-1 from *Enterobacter hirae* appears to hydrolyze peptidoglycan fraction from *E. hirae* only after chemical re-*N*-acetylation (Kawamura and Shockman 1983) and a muramidase purified from *Clostridium acetobutylicum* was found to act only on non-acetylated peptidoglycan (Croux et al. 1992). The gene (*pgdA*) encoding a peptidoglycan deacetylase was identified in *S. pneumoniae* (Vollmer and Tomasz 2000). Purified *S. pneumoniae* LytA amidase has higher activity towards the native *S. pneumoniae* peptidoglycan with a high degree of deacetylation than

towards chemically acetylated peptidoglycan. A *pgdA* homologous gene was found in *Lactococcus lactis* and peptidoglycan *N*-acetylglucosamine deacetylation by PgdA was found to decrease autolysis in *L. lactis* (Meyrand et al. 2007). This peptidoglycan modification was shown to protect it from hydrolysis by the major autolysin AcmA. Another modification of peptidoglycan encountered in bacteria is MurNAc *O*-acetylation. The gene (*oatA*) encoding an *O*-acetyltransferase able to transfer an *O*-acetyl group at the C6 hydroxyl group of MurNAc was identified in *S. aureus* (Bera et al. 2005). In *Enterococcus faecalis*, PGHs were classified by a zymogram technique into enzymes that preferentially hydrolyze either *O*-acetylated or non-*O*-acetylated peptidoglycan and enzymes that show no apparent preference for either substrate type (Pfeffer et al. 2006). However, a recent study showed that peptidoglycan *O*-acetylation in *E. faecalis* had only marginal inhibitory impact on the activities of the major autolysins, the *N*-acetylglucosaminidase AtlA and the *N*-acetylmuramidase AtlB (Emirian et al. 2009). *oatA* gene is also present in *L. lactis* and was shown to be regulated positively by the SpxB regulator in response to cell envelope stress such as cell wall attack by lysozyme, an exogenous PGH. Peptidoglycan *O*-acetylation could also control endogenous PGH activity (Veiga et al. 2007).

Proton motive force. In *Bacillus subtilis*, proton motive force (PMF) generated inside the membrane seems to play a role in the regulation of PGH activity, by maintaining the cell wall pH at a relatively low value close to the cytoplasmic membrane (Kemper et al. 1993; Calamita et al. 2001). PGHs would be more active when present at the periphery of the cell where the higher pH and more stretched structure of peptidoglycan would favour their activity. This hypothesis was supported by the fact that depolarizing agents enhanced cell autolysis.

Export and localization. Controlling the access of PGHs to their substrate in the cell wall is another way to control their activity. A gene (*arpU*) controlling muramidase-2 export in *E. hirae* was identified but its mechanism of action was not studied in detail (Lleo et al. 1995). In *L. monocytogenes*, the auxilliary secretion system SecA2 was shown to play a crucial role in the secretion of two PGHs: NamA and P60 (Lenz et al. 2003). SecA2-secretion of these PGHs contributes to *L. monocytogenes* virulence. It may work by promoting coordinated hydrolysis of peptidoglycan by the two PGHs, which is predicted to generate the muropeptide GMDP, glutaminylmuramyl-dipeptide, from peptidoglycan, which is known to modify the host inflammatory response.

In *S. aureus*, the *cid* and *lrg* operons encode proteins that were shown to regulate extracellular PGH activity (Bayles 2003). As mentioned above (see Sect. 13.6.1), LrgA and CidA probably regulate cell lysis in a way similar to bacteriophages based on a holin–antiholin mechanism. They would thus make pores inside the cytoplasmic membrane that would allow release of PGHs but they could also act at the level of PMF.

Finally, differential binding of autolysins to different parts of the cell wall as a consequence of the presence of a targeting domain as already described above (see Sect. 13.5) may also constitute a control mechanism of autolysin activity (Baba and Schneewind 1998).

Formation of multienzyme complexes. It has been previously proposed that synthetic and hydrolytic reactions need to be coordinated temporally and spatially during cell wall enlargement (Shockman and Höltje 1994). This could be achieved through the formation of large protein complexes containing peptidoglycan synthases (which are the penicillin binding proteins or PBPs) and PGHs. In *E. coli*, the existence of molecular interactions between the penicillin binding protein PBP1B and the lytic transglycosylase MltA has been demonstrated (Vollmer et al. 1999).

13.7 Roles in Bacterial Physiology

The presence of autolysins in bacteria during exponential growth phase suggests that they play a role during cell multiplication. Different roles were proposed and demonstrated during bacterial growth: (1) septation and separation of daughter cells; (2) cell expansion; (3) peptidoglycan turn-over; and (4) protein secretion. They can also play a role in more specific functions such as: (1) competence for genetic transformation; (2) flagellar morphogenesis; (3) spore formation and germination; (4) biofilm formation; (5) pathogenicity; (6) autolysis and programmed cell death; and (7) waking up of dormant bacteria.

Their role was investigated after construction of mutants with one or several inactivated autolysin genes and study of their phenotypes. We give examples below of the roles established for different enzymes of the five bacterial species considered in Table 13.1.

13.7.1 Cell Division and Cell Separation

Numerous autolysin-deficient mutants exhibit defects in the cell separation process following cell division.

In *S. pneumoniae*, *lytA* mutant forms small chains of 8–10 cells (versus the diplococci morphology of wild type) whereas a *lytB* mutant forms very long chains of more than 100 cells, showing the fundamental role of the latter enzyme in cell separation (Lopez and Garcia 2004). Although the peptidoglycan-hydrolyzing activity of PcsB was has not yet been shown experimentally, PcsB contains the typical CHAP domain found in other PGHs. *pcsB* gene proved to be essential in *Streptococcus pneumoniae* (Ng et al. 2004). Reduced expression of *pcsB* resulted in formation of long chains of cells in which peptidoglycan synthesis occurred at nearly every division septum and cell equator. Severe depletion of PcsB led to abnormal, uncontrolled cell wall synthesis at misplaced septa and around large cells. Thus PcsB appears to play a role as a PGH that balances the extent of cell wall synthesis in *S. pneumoniae*.

In *Staphylococcus aureus*, inactivation of *atl*, which encodes the bifunctional autolysin with amidase and glucosaminidase specificities, does not impair growth.

However, the mutant strain forms aggregates of 10–20 cells rather than the doublets of the parental strain (Takahashi et al. 2002). These aggregates can be dissolved by the addition of amidase or muramidase to the extracellular medium (Sugai et al. 1995). These results indicate a role of Atl in cell separation after division. Inactivation of the genes encoding the amidase SleI and the putative lytic transglycosylase SceD also result in impaired cell separation and clumping of the bacterial culture (Kajimura et al. 2005; Stapleton et al. 2007).

In *L. lactis,* an *acmA* defective mutant forms long chains of cells, indicating that AcmA is involved in daughter cell separation after division (Buist et al. 1995). In contrast, inactivation of *acmB* (Huard et al. 2003) and *yjgB* (Redko et al. 2007) had no effect on cell separation.

In *L. monocytogenes*, deletion of *p60* (*iap*) gene affects cell division leading to abnormal cell forms. The *iap*-deleted mutant forms long filaments in exponential growth phase and septum formation is initiated at various points along the filaments (Pilgrim et al. 2003). Deletion of MurA has no effect on cell growth rate but it affects cell separation after division. The mutant grows as long chains of cells (3–12 cells) whereas the wild type gives mostly single cells.

In *B. subtilis,* which contain a high number of autolysins, mutants deficient in one or several enzymes were constructed by specific inactivation of the corresponding genes. At least six autolysins were found to play a role in cell separation after cell division. The major vegetative autolysins, LytC (CwlB) and LytD, were shown to have a role in cell separation. However, single inactivation of *lytC* or *lytD* leads to only slight increase in cell chain length compared to the parental strain, whereas combined inactivation of *lytC* and *lytD* results in extremely long chains of cells. These results indicate that both LytC and LytD have a role in cell separation but that each could mutually compensate for the lack of the other (Blackman et al. 1998). Endopeptidases LytE and LytF are also involved in cell separation. Inactivation of one of these genes results in cells with a two- to fourfold increase in length (Margot et al. 1998, 1999). A double *lytE lytF* mutant has a strikingly altered morphology and the cells present a fibrillar structure (Ohnishi et al. 1999). A *cwlS* mutant exhibited a cell shape similar to that of the wild type; however, a *lytE lytF cwlS* triple mutant exhibited aggregated microfiber formation. The fact that CwlS-like LytE and LytF was localized at cell separation sites and cell poles during the late vegetative phase strongly suggests that CwlS is involved in cell separation with LytF and LytE (Fukushima et al. 2006). Finally, LytG, another member of the vegetative autolysins of *B. subtilis*, is also involved in cell separation (Horsburgh et al. 2003).

13.7.2 Cell Expansion and Cell Wall Turnover

In the inside-to-outside model of cell growth for Gram-positive bacteria, peptidoglycan is synthesized close to the cytoplasmic membrane and removed from the outer surface of the cell wall (Koch and Doyle 1985). In this model, autolysins can

play a role by making cuts inside the peptidoglycan to allow newer peptidoglycan to expand and also by removing older peptidoglycan from the outer surface. However, the single or multiple autolysin mutants obtained were not affected in their growth. Only PcsB in *S. pneumoniae* was found indispensable for growth and viability. In contrast, the role of autolysins in cell wall turnover was demonstrated. In *B. subtilis*, cell wall turnover was analyzed with radiolabeled peptidoglycan precursors and it was shown that both LytC and LytD have a role in peptidoglycan turnover (Blackman et al. 1998). In *S. aureus*, the cell morphology of an *atl* mutant was altered with irregularity of the cell surface, probably indicative of abnormal recycling of peptidoglycan (Takahashi et al. 2002).

13.7.3 Spore Formation and Germination

In *B. subtilis*, several PGHs, the amidases CwlC and CwlD as well as CwlJ and SleB, have a role during sporulation. PGHs can play a role at different stages of the sporulation process such as maturation of the spore peptidoglycan (which has a unique structure), mother-cell lysis at the end of sporulation or spore germination (see Smith et al. 2000 for review).

13.7.4 Competence

In *Streptococcus pneumoniae*, three PGHs (LytA, LytB and CbpD) are induced by the competence-stimulating peptide (CSP), an exported peptide pheromone which controls competence for natural genetic transformation in *S. pneumoniae*. The combined action of the three PGHs appears to be involved in the lysis of the non-competent subpopulation of *S. pneumoniae* by the competent cell subpopulation. This lysis could provide DNA for genetic transformation (Claverys et al. 2007).

13.7.5 Biofilm Formation

In *Staphylococcus aureus*, autolysins also play a role in biofilm formation. Indeed, the *cidA* regulator, which controls the activity of PGHs in *S. aureus,* was shown to contribute to biofilm development, by release of genomic DNA, an important structural component of the biofilm matrix (Rice et al. 2007).

13.7.6 Pathogenicity

In *Listeria monocytogenes*, in contrast to P60 and MurA, Auto and Ami autolysins have no effect on cell growth, cell division and morphology. They were shown to

play a role in *L. monocytogenes* virulence. Ami contributes to adhesion of the bacteria to eukaryotic cells via its C-terminal cell wall anchoring domain (Milohanic et al. 2001) whereas Auto is required for entry into eukaryotic cells (Cabanes et al. 2004). Bacterial PGHs can also participate in the production of peptidoglycan fragments which can be recognized as PAMPs (pathogen associated molecular patterns) by the host innate immune system (Lenz et al. 2003).

In *S. aureus*, an *isaA sceD* double mutant, inactivated for the two putative lytic transglycosylases, is attenuated for virulence, while inactivation of *sceD* impaired nasal colonization in a rat model, demonstrating the importance of cell wall dynamics in host–pathogen interactions (Stapleton et al. 2007).

13.7.7 Autolysis and Programmed Cell Death

Several autolysins are involved in stationary phase autolysis or antibiotic-induced autolysis. In *S. aureus,* Atl plays a role in penicillin-induced lysis, contrary to SleI (Takahashi et al. 2002; Kajimura et al. 2005). In *Lactococcus lactis,* an *acmA* defective mutant autolyzes very slowly in stationary phase, indicating that AcmA is the major autolysin (Buist et al. 1995). Regarding AcmB, its contribution to autolysis is limited and seems to be potentialized by AcmA (Huard et al. 2003). Also, *L. monocytogenes murA* mutant is more resistant to autolysis during prolonged stationary phase incubation as well as to Triton X-100 induced autolysis (Carroll et al. 2003). In *B. subtilis*, LytC and LytD are involved in antibiotic-induced lysis (Blackman et al. 1998) and LytE and LytF in stationary phase autolysis (Ohnishi et al. 1999).

In *S. aureus*, PGHs are involved in programmed cell death (PCD) controlled by *lrgAB* and *cidAB*, which could function in bacteria to remove damaged cells from a population in response to a wide variety of stresses (Rice and Bayles 2003).

13.7.8 Others

The observed effect of autolysin mutations on motility may be partly due to their effect on cell separation leading to the production of filaments with low motility (Smith et al. 2000). Another possible role in motility is their requirement for flagellar morphogenesis by possibly playing a role in the extrusion of flagella through the cell wall (Dijkstra and Keck 1996). The cross-linked network of peptidoglycan can constitute a physical barrier to secretion of proteins, which may be lessened by the action of autolysins (Dijkstra and Keck 1996).

Finally, peptidoglycan hydrolysis was also proposed to be a mechanism for waking up bacteria from a non-growth state like the viable-but-non-culturable (VBNC) state. *Micrococcus luteus* was shown to secrete a resuscitation-promoting

factor (Rfp) able to stimulate the growth of dormant mycobacteria (Keep et al. 2006; Mukamolova et al. 2006). Rfp was shown to be a PGH with homology with lytic transglycosylase (Mukamolova et al. 2006).

13.8 Conclusions

Bacterial PGHs are remarkable for the diversity of their specificity and structure. Structural diversity results especially from modular organization. PGHs are usually exported through the cytoplasmic membrane to reach their substrate in the cell wall and their localization seems to be related to their function inside the bacterial cell.

Although they play fundamental roles in cell physiology, most PGHs are not indispensable for growth and viability, probably as a result of functional redundancy between the several enzymes found in one bacterium. Until now, only PcsB with probable PGH activity, which balances the extent of cell wall synthesis in *S. pneumoniae*, has been found indispensable for cell viability.

Due to their ability to kill the cells that produce them, their expression and their activity need to be tightly regulated. No general model of regulation has emerged up till now. In contrast, multiple mechanisms of transcriptional or post-translational regulation have been deciphered in the different bacterial species studied. Thus it is likely that different levels of regulation coexist in one bacterial species to efficiently control the activity of these lethal enzymes. Recent studies provide evidence for the regulation of their expression in response to extracellular signals and/or environmental conditions through TCSs.

In addition to their role in the processes requiring peptidoglycan remodelling for vegetative bacterial growth, it has already been shown for a long time that specific PGHs are also required for the sporulation process in sporulating bacteria such as *B. subtilis*. More recently, through their ability to lyze cells and release the intracellular bacterial content, especially DNA, certain PGHs were clearly shown to be involved in competence in *S. pneumoniae* or in biofilm formation in *S. aureus*. In pathogens such as *L. monocytogenes*, PGHs also play a crucial role in pathogenicity, where again they can play a role at different levels: adherence to eukaryotic host cells, entry in the host cells, but also, since they are able to release peptidoglycan fragments, modulation of innate immune response.

Peptidoglycan degradation activity in the bacterial cell needs to be coordinated with synthesis activity as well as with the cell division process. The spatial and temporal coordination of the activity of the enzymes involved could take place in multienzyme complexes, which would guarantee that enlargement of the cell wall occurs without lysis and with maintenance of the specific shape of the bacterium.

Acknowledgments I thank M. Yvon and V. Monnet (INRA, UR477 Biochimie Bactérienne, Jouy-en-Josas, France) for critical reading of the manuscript.

References

Ahn SJ, Burne RA (2007) Effects of oxygen on biofilm formation and the AtlA autolysin of *Streptococcus mutans*. J Bacteriol 189:6293–6302

Antignac A, Sieradzki K, Tomasz A (2007) Perturbation of cell wall synthesis suppresses autolysis in *Staphylococcus aureus*: evidence for coregulation of cell wall synthetic and hydrolytic enzymes. J Bacteriol 189:7573–7580

Baba T, Schneewind O (1998) Targeting of muralytic enzymes to the cell division site of Gram-positive bacteria: repeat domains direct autolysin to the equatorial surface ring of *Staphylococcus aureus*. EMBO J 17:4639–4646

Bateman A, Bycroft M (2000) The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). J Mol Biol 299:1113–1119

Bayles KW (2003) Are the molecular strategies that control apoptosis conserved in bacteria? Trends Microbiol 11:306–311

Bera A, Herbert S, Jakob A, Vollmer W, Gotz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan *O*-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol Microbiol 55:778–787

Bisicchia P, Noone D, Liolou E, Howell A, Quigley S, Jensen T, Jarmer H, Devine K (2007) The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. Mol Microbiol 65:180–200

Blackman SA, Smith TJ, Foster SJ (1998) The role of autolysins during vegetative growth of *Bacillus subtilis* 168. Microbiology 144:73–82

Braun L, Dramsi S, Dehoux P, Bierne H, Lindahl G, Cossart P (1997) InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. Mol Microbiol 25:285–294

Brunskill EW, Bayles KW (1996) Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*. J Bacteriol 178:611–618

Buist G, Kok J, Leenhouts KJ, Dabrowska M, Venema G, Haandrikman AJ (1995) Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J Bacteriol 177:1554–1563

Buist G, Venema G, Kok J (1998) Autolysis of *Lactococcus lactis* is influenced by proteolysis. J Bacteriol 180:5947–5953

Cabanes D, Dussurget O, Dehoux P, Cossart P (2004) Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. Mol Microbiol 51:1601–1614

Calamita HG, Ehringer WD, Koch AL, Doyle RJ (2001) Evidence that the cell wall of *Bacillus subtilis* is protonated during respiration. Proc Natl Acad Sci USA 98:15260–15263

Carballido-Lopez R, Formstone A (2007) Shape determination in *Bacillus subtilis*. Curr Opin Microbiol 10:611–616

Carballido-Lopez R, Formstone A, Li Y, Ehrlich SD, Noirot P, Errington J (2006) Actin homolog MreBH governs cell morphogenesis by localization of the cell wall hydrolase LytE. Dev Cell 11:399–409

Carroll SA, Hain T, Technow U, Darji A, Pashalidis P, Joseph SW, Chakraborty T (2003) Identification and characterization of a peptidoglycan hydrolase, MurA, of *Listeria monocytogenes*, a muramidase needed for cell separation. J Bacteriol 185:6801–6808

Claverys JP, Martin B, Havarstein LS (2007) Competence-induced fratricide in streptococci. Mol Microbiol 64:1423–1433

Courtin P, Miranda G, Guillot A, Wessner F, Mezange C, Domakova E, Kulakauskas S, Chapot-Chartier M-P (2006) Peptidoglycan structure analysis of Lactococcus lactis reveals the presence of an L,D-carboxypeptidase involved in peptidoglycan maturation. J Bacteriol 188:5293–5298

Croux C, Garcia JL (1991) Sequence of the lyc gene encoding the autolytic lysozyme of *Clostridium acetobutylicum* ATCC824: comparison with other lytic enzymes. Gene 104:25–31

Croux C, Canard B, Goma G, Soucaille P (1992) Purification and characterization of an extracellular muramidase of *Clostridium acetobutylicum* ATCC 824 that acts on non-*N*-acetylated peptidoglycan. Appl Environ Microbiol 58:1075–1081

Dijkstra AJ, Keck W (1996) Peptidoglycan as a barrier to transenvelope transport. J Bacteriol 178:5555–5562

Dubrac S, Boneca IG, Poupel O, Msadek T (2007) New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. J Bacteriol 189: 8257–8269

Emirian A, Fromentin S, Eckert C, Chau F, Dubost L, Delepierre M, Gutmann L, Arthur M, Mesnage S (2009) Impact of peptidoglycan *O*-acetylation on autolytic activities of the Enterococcus faecalis *N*-acetylglucosaminidase AtlA and *N*-acetylmuramidase AtlB. FEBS Lett 583:3033–3038

Fedtke I, Mader D, Kohler T, Moll H, Nicholson G, Biswas R, Henseler K, Götz F, Zähringer U, Peschel A (2007) A *Staphylococcus aureus ypfP* mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity. Mol Microbiol 65:1078–1091

Fernandez-Tornero C, Lopez R, Garcia E, Gimenez-Gallego G, Romero A (2001) A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. Nat Struct Biol 8:1020–1024

Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, Hotz H-R, Ceric G, Forslund K, Eddy SR, Sonnhammer ELL, Bateman A (2008) The Pfam protein families database. Nucleic Acids Res 36:D281–D288

Foster S (1991) Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*. J Gen Microbiol 137:1987–1998

Fournier B, Hooper DC (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J Bacteriol 182:3955–3964

Fukushima T, Afkham A, Kurosawa S, Tanabe T, Yamamoto H, Sekiguchi J (2006) A new D,L-endopeptidase gene product, YojL (renamed CwlS), plays a role in cell separation with LytE and LytF in *Bacillus subtilis*. J Bacteriol 188:5541–5550

Garcia E, Garcia JL, Ronda C, Garcia P, Lopez R (1985) Cloning and expression of the pneumococcal autolysin gene in *Escherichia coli*. Mol Gen Genet 201:225–230

Garcia E, Garcia JL, Garcia P, Arraras A, Sanchez-Puelles JM, Lopez R (1988) Molecular evolution of lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages. Proc Natl Acad Sci USA 85:914–918

Garcia P, Gonzalez MP, Garcia E, Lopez R, Garcia JL (1999a) LytB, a novel pneumococcal murein hydrolase essential for cell separation. Mol Microbiol 31:1275–1281

Garcia P, Paz Gonzalez M, Garcia E, Garcia JL, Lopez R (1999b) The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. Mol Microbiol 33:128–138

Glauner B, Holtje JV, Schwarz U (1988) The composition of the murein of *Escherichia coli*. J Biol Chem 263:10088–10095

Groicher KH, Firek BA, Fujimoto DF, Bayles KW (2000) The *Staphylococcus aureus* lrgAB operon modulates murein hydrolase activity and penicillin tolerance. J Bacteriol 182:1794–1801

Guiral S, Mitchell TJ, Martin B, Claverys JP (2005) Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. Proc Natl Acad Sci USA 102:8710–8715

Herbold DR, Glaser L (1975) *Bacillus subtilis N*-acetylmuramic acid L-alanine amidase. J Biol Chem 250:1676–1682

Horsburgh GJ, Atrih A, Williamson MP, Foster SJ (2003) LytG of *Bacillus subtilis* is a novel peptidoglycan hydrolase: the major active glucosaminidase. Biochemistry 42:257–264

Howell A, Dubrac S, Andersen KK, Noone D, Fert J, Msadek T, Devine K (2003) Genes controlled by the essential YycG/YycF two-component system of *Bacillus subtilis* revealed through a novel hybrid regulator approach. Mol Microbiol 49:1639–1655

Huard C, Miranda G, Wessner F, Bolotin A, Hansen J, Foster SJ, Chapot-Chartier M-P (2003) Characterization of AcmB, an *N*-acetylglucosaminidase autolysin from *Lactococcus lactis*. Microbiology 149:695–705

Huard C, Miranda G, Redko Y, Wessner F, Foster SJ, Chapot-Chartier MP (2004) Analysis of the peptidoglycan hydrolase complement of *Lactococcus lactis*: identification of a third *N*-Acetylglucosaminidase, AcmC. Appl Environ Microbiol 70:3493–3499

Husson-Kao C, Mengaud J, Cesselin B, van Sinderen D, Benbadis L, Chapot-Chartier MP (2000) The *streptococcus thermophilus* autolytic phenotype results from a leaky prophage. Appl Environ Microbiol 66:558–565

Ishikawa S, Yamane K, Sekiguchi J (1998) Regulation and characterization of a newly deduced cell wall hydrolase gene (cwlJ) which affects germination of *Bacillus subtilis* spores. J Bacteriol 180:1375–1380

Jolliffe LK, Doyle RJ, Streips UN (1980) Extracellular proteases modify cell wall turnover in *Bacillus subtilis*. J Bacteriol 141:1199–1208

Jonquieres R, Bierne H, Fiedler F, Gounon P, Cossart P (1999) Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of Gram-positive bacteria. Mol Microbiol 34:902–914

Kajimura J, Fujiwara T, Yamada S, Suzawa Y, Nishida T, Oyamada Y, Hayashi I, Yamagishi J-I, Komatsuzawa H, Sugai M (2005) Identification and molecular characterization of an *N*-acetylmuramyl-L-alanine amidase Sle1 involved in cell separation of *Staphylococcus aureus*. Mol Microbiol 58:1087–1101

Kariyama R, Shockman GD (1992) Extracellular and cellular distribution of muramidase-2 and muramidase-1 of *Enterococcus hirae* ATCC 9790. J Bacteriol 174:3236–3241

Kawamura T, Shockman GD (1983) Purification and some properties of the endogenous, autolytic *N*-acetylmuramoylhydrolase of *Streptococcus faecium*, a bacterial glycoenzyme. J Biol Chem 258:9514–9521

Keep NH, Ward JM, Cohen-Gonsaud M, Henderson B (2006) Wake up! Peptidoglycan lysis and bacterial non-growth states. Trends Microbiol 14:271–276

Kemper MA, Urrutia MM, Beveridge TJ, Koch AL, Doyle RJ (1993) Proton motive force may regulate cell wall-associated enzymes of *Bacillus subtilis*. J Bacteriol 175:5690–5696

Koch AL, Doyle RJ (1985) Inside-to-outside growth and turnover of the wall of Gram-positive rods. J Theor Biol 117:137–157

Kullik I, Jenni R, Berger-Bachi B (1998) Sequence of the putative alanine racemase operon in *Staphylococcus aureus*: insertional interruption of this operon reduces D-alanine substitution of lipoteichoic acid and autolysis. Gene 219:9–17

Kuroda A, Sekiguchi J (1991) Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. J Bacteriol 173:7304–7312

Kuroda A, Sekiguchi J (1992) Characterization of the *Bacillus subtilis* CwbA protein which stimulates cell wall lytic amidases. FEMS Microbiol Lett 74:109–113

Kuroda A, Imazeki M, Sekiguchi J (1991) Purification and characterization of a cell wall hydrolase encoded by the cwlA gene of *Bacillus subtilis*. FEMS Microbiol Lett 65:9–13

Kuroda A, Rashid MH, Sekiguchi J (1992) Molecular cloning and sequencing of the upstream region of the major *Bacillus subtilis* autolysin gene: a modifier protein exhibiting sequence homology to the major autolysin and the spoIID product. J Gen Microbiol 138(Pt 6): 1067–1076

Kuroda A, Asami Y, Sekiguchi J (1993) Molecular cloning of a sporulation-specific cell wall hydrolase gene of *Bacillus subtilis*. J Bacteriol 175:6260–6268

Lazarevic V, Margot P, Soldo B, Karamata D (1992) Sequencing and analysis of the *Bacillus subtilis* lytRABC divergon: a regulatory unit encompassing the structural genes of the *N*-acetylmuramoyl-L-alanine amidase and its modifier. J Gen Microbiol 138:1949–1961

Leclerc D, Asselin A (1989) Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. Can J Microbiol 35:749–753

Lenz LL, Mohammadi S, Geissler A, Portnoy DA (2003) SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. Proc Natl Acad Sci USA 100:12432–12437

Lepeuple AS, Van Gemert E, Chapot-Chartier MP (1998) Analysis of the bacteriolytic enzymes of the autolytic *Lactococcus lactis* subsp. *cremoris* strain AM2 by renaturing polyacrylamide gel electrophoresis: identification of a prophage-encoded enzyme. Appl Environ Microbiol 64:4142–4148

Liang X, Zheng L, Landwehr C, Lunsford D, Holmes D, Ji Y (2005) Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of *Staphylococcus aureus*. J Bacteriol 187:5486–5492

Lleo MM, Fontana R, Solioz M (1995) Identification of a gene (*arpU*) controlling muramidase-2 export in *Enterococcus hirae*. J Bacteriol 177:5912–5917

Lopez R, Garcia E (2004) Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiol Rev 28:553–580

Lopez R, Garcia JL, Garcia E, Ronda C, Garcia P (1992) Structural analysis and biological significance of the cell wall lytic enzymes of *Streptococcus pneumoniae* and its bacteriophage. FEMS Microbiol Lett 79:439–447

Lu JZ, Fujiwara T, Komatsuzawa H, Sugai M, Sakon J (2006) Cell wall-targeting domain of glycylglycine endopeptidase distinguishes among peptidoglycan cross-bridges. J Biol Chem 281:549–558

Margot P, Karamata D (1992) Identification of the structural genes for *N*-acetylmuramoyl-L-alanine amidase and its modifier in *Bacillus subtilis* 168: inactivation of these genes by insertional mutagenesis has no effect on growth or cell separation. Mol Gen Genet 232:359–366

Margot P, Mauel C, Karamata D (1994) The gene of the *N*-acetylglucosaminidase, a *Bacillus subtilis* 168 cell wall hydrolase not involved in vegetative cell autolysis. Mol Microbiol 12:535–545

Margot P, Wahlen M, Gholamhoseinian A, Piggot P, Karamata D, Gholamhuseinian A (1998) The *lytE* gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. J Bacteriol 180:749–752

Margot P, Pagni M, Karamata D (1999) *Bacillus subtilis* 168 gene *lytF* encodes a gamma-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, sigmaD. Microbiology 145:57–65

Meyrand M, Boughammoura A, Courtin P, Mezange C, Guillot A, Chapot-Chartier M-P (2007) Peptidoglycan *N*-acetylglucosamine deacetylation decreases autolysis in *Lactococcus lactis*. Microbiology 153:3275–3285

Milohanic E, Jonquieres R, Cossart P, Berche P, Gaillard JL (2001) The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. Mol Microbiol 39:1212–1224

Mishima M, Shida T, Yabuki K, Kato K, Sekiguchi J, Kojima C (2005) Solution structure of the peptidoglycan binding domain of *Bacillus subtilis* cell wall lytic enzyme CwlC: characterization of the sporulation-related repeats by NMR. Biochemistry 44:10153–10163

Moriyama R, Hattori A, Miyata S, Kudoh S, Makino S (1996) A gene (sleB) encoding a spore cortex-lytic enzyme from *Bacillus subtilis* and response of the enzyme to L-alanine-mediated germination. J Bacteriol 178:6059–6063

Mukamolova GV, Murzin AG, Salina EG, Kell DB, Kaprelyants AS, Young M (2006) Muralytic activity of *Micrococcus luteus* Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation. Mol Microbiol 59:84–98

Ng WL, Kazmierczak KM, Winkler ME (2004) Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. Mol Microbiol 53:1161–1175

Nugroho FA, Yamamoto H, Kobayashi Y, Sekiguchi J (1999) Characterization of a new sigma-K-dependent peptidoglycan hydrolase gene that plays a role in *Bacillus subtilis* mother cell lysis. J Bacteriol 181:6230–6237

Ohnishi R, Ishikawa S, Sekiguchi J (1999) Peptidoglycan hydrolase LytF plays a role in cell separation with CwlF during vegetative growth of *Bacillus subtilis*. J Bacteriol 181:3178–3184

Oshida T, Sugai M, Komatsuzawa H, Hong YM, Suginaka H, Tomasz A (1995) A *Staphylococcus aureus* autolysin that has an *N*-acetylmuramoyl-L-alanine amidase domain and an endo-beta-*N*-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. Proc Natl Acad Sci USA 92:285–289

Pagliero E, Dideberg O, Vernet T, Di Guilmi AM (2005) The PECACE domain: a new family of enzymes with potential peptidoglycan cleavage activity in Gram-positive bacteria. BMC Genomics 6:19

Palumbo E, Deghorain M, Cocconcelli PS, Kleerebezem M, Geyer A, Hartung T, Morath S, Hols P (2006) D-alanyl ester depletion of teichoic acids in *Lactobacillus plantarum* results in a major modification of lipoteichoic acid composition and cell wall perforations at the septum mediated by the Acm2 autolysin. J Bacteriol 188:3709–3715

Perea Velez M, Verhoeven TLA, Draing C, Von Aulock S, Pfitzenmaier M, Geyer A, Lambrichts I, Grangette C, Pot B, Vanderleyden J, De Keersmaecker SCJ (2007) Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG. Appl Environ Microbiol 73:3595–3604

Peschel A, Vuong C, Otto M, Gotz F (2000) The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. Antimicrob Agents Chemother 44:2845–2847

Pfeffer JM, Strating H, Weadge JT, Clarke AJ (2006) Peptidoglycan O acetylation and autolysin profile of *Enterococcus faecalis* in the viable but nonculturable state. J Bacteriol 188:902–908

Pilgrim S, Kolb-Maurer A, Gentschev I, Goebel W, Kuhn M (2003) Deletion of the gene encoding p60 in *Listeria monocytogenes* leads to abnormal cell division and loss of actin-based motility. Infect Immun 71:3473–3484

Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A (2000) HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Mol Microbiol 35:1042–1051

Ramadurai L, Jayaswal RK (1997) Molecular cloning, sequencing, and expression of lytM, a unique autolytic gene of *Staphylococcus aureus*. J Bacteriol 179:3625–3631

Ramadurai L, Lockwood KJ, Nadakavukaren MJ, Jayaswal RK (1999) Characterization of a chromosomally encoded glycylglycine endopeptidase of *Staphylococcus aureus*. Microbiology 145:801–808

Rathsam C, Jacques NA (1998) Role of C-terminal domains in surface attachment of the fructosyltransferase of *Streptococcus salivarius* ATCC 25975. J Bacteriol 180:6400–6403

Redko Y, Courtin P, Mezange C, Huard C, Chapot-Chartier MP (2007) *Lactococcus lactis* gene *yjgB* encodes a gamma-D-glutaminyl-L-lysyl-endopeptidase which hydrolyzes peptidoglycan. Appl Environ Microbiol 73:5825–5831

Rice KC, Bayles KW (2003) Death's toolbox: examining the molecular components of bacterial programmed cell death. Mol Microbiol 50:729–738

Rice KC, Firek BA, Nelson JB, Yang SJ, Patton TG, Bayles KW (2003) The *Staphylococcus aureus cidAB* operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance. J Bacteriol 185:2635–2643

Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, Bayles KW (2007) The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. Proc Natl Acad Sci USA 104:8113–8118

Ruhland GJ, Hellwig M, Wanner G, Fiedler F (1993) Cell-surface location of *Listeria*-specific protein p60-detection of *Listeria* cells by indirect immunofluorescence. J Gen Microbiol 139:609–616

Salzberg LI, Helmann JD (2007) An antibiotic-inducible cell wall-associated protein that protects *Bacillus subtilis* from autolysis. J Bacteriol 189:4671–4680

Schubert K, Bichlmaier AM, Mager E, Wolff K, Ruhland G, Fiedler F (2000) P45, an extracellular 45 kDa protein of *Listeria monocytogenes* with similarity to protein p60 and exhibiting peptidoglycan lytic activity. Arch Microbiol 173:21–28

Sekiguchi J, Akeo K, Yamamoto H, Khasanov FK, Alonso JC, Kuroda A (1995) Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis*. J Bacteriol 177:5582–5589

Serizawa M, Kodama K, Yamamoto H, Kobayashi K, Ogasawara N, Sekiguchi J (2005) Functional analysis of the YvrGHb two-component system of *Bacillus subtilis*: identification of the regulated genes by DNA microarray and northern blot analyses. Biosci Biotechnol Biochem 69:2155–2169

Shockman GD (1992) The autolytic ('suicidase') system of *Enterococcus hirae*: from lysine depletion autolysis to biochemical and molecular studies of the two muramidases of Enterococcus hirae ATCC 9790. FEMS Microbiol Lett 79:261–267

Shockman GD, Höltje JV (1994) Microbial peptidoglycan (murein) hydrolases. In: Guysen J-M, Hackenbeck R (eds) New comprehensive biochemistry, vol 27. Bacterial cell wall. Elsevier, Amsterdam, pp 131–167

Smith TJ, Blackman SA, Foster SJ (2000) Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. Microbiology 146:249–262

Sobral RG, Jones AE, Des Etages SG, Dougherty TJ, Peitzsch RM, Gaasterland T, Ludovice AM, de Lencastre H, Tomasz A (2007) Extensive and genome-wide changes in the transcription profile of *Staphylococcus aureus* induced by modulating the transcription of the cell wall synthesis gene *murF*. J Bacteriol 189:2376–2391

Stapleton MR, Horsburgh MJ, Hayhurst EJ, Wright L, Jonsson I-M, Tarkowski A, Kokai-Kun JF, Mond JJ, Foster SJ (2007) Characterization of IsaA and SceD, two putative lytic transglycosylases of *Staphylococcus aureus*. J Bacteriol 189:7316–7325

Steen A, Buist G, Leenhouts KJ, El Khattabi M, Grijpstra F, Zomer AL, Venema G, Kuipers OP, Kok J (2003) Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J Biol Chem 278:23874–23881

Steen A, Palumbo E, Deghorain M, Cocconcelli PS, Delcour J, Kuipers OP, Kok J, Buist G, Hols P (2005) Autolysis of *Lactococcus lactis* is increased upon D-alanine depletion of peptidoglycan and lipoteichoic acids. J Bacteriol 187:114–124

Sugai M, Komatsuzawa H, Akiyama T, Hong Y-M, Oshida T, Miyake Y, Yamaguchi T, Suginawa H (1995) Identification of endo-beta-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in *Staphylococcus aureus*. J Bacteriol 177:1491–1496

Sugai M, Fujiwara T, Komatsuzawa H, Suginaka H (1998) Identification and molecular characterization of a gene homologous to epr (endopeptidase resistance gene) in *Staphylococcus aureus*. Gene 224:67–75

Takahashi J, Komatsuzawa H, Yamada S, Nishida T, Labinschinski H, Fujiwara Y, Ohara M, Yamagishi J-I, Sugai M (2002) Molecular characterization of an atl null mutant of *Staphylococcus aureus*. Microbiol Immunol 46:601–612

Tomasz A, Westphal M (1971) Abnormal autolytic enzyme in a pneumococus with altered teichoic acid composition. Proc Natl Acad Sci USA 68:2627–2630

Veiga P, Bulbarela-Sampieri C, Furlan S, Maisons A, Chapot-Chartier M-P, Erkelenz M, Mervelet P, Noirot P, Frees D, Kuipers O, Kok J, Gruss A, Buist G, Kulakauskas S (2007) SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. J Biol Chem 282:19342–19354

Vollmer W, Bertsche U (2008) Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. Biochim Biophys Acta 1778:1714–1734

Vollmer W, Tomasz A (2000) The *pgdA* gene encodes for a peptidoglycan *N*-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. J Biol Chem 275:20496–20501

Vollmer W, von Rechenberg M, Holtje JV (1999) Demonstration of molecular interactions between the murein polymerase PBP1B, the lytic transglycosylase MltA, and the scaffolding protein MipA of *Escherichia coli*. J Biol Chem 274:6726–6734

Wang L, Lin M (2007) Identification of IspC, an 86-kilodalton protein target of humoral immune response to infection with *Listeria monocytogenes* serotype 4b, as a novel surface autolysin. J Bacteriol 189:2046–2054

Wecke J, Madela K, Fischer W (1997) The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*. Microbiology 143:2953–2960

Whisstock JC, Lesk AM (1999) SH3 domains in prokaryotes. Trends Biochem Sci 24:132–133

Wuenscher MD, Kohler S, Bubert A, Gerike U, Goebel W (1993) The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. J Bacteriol 175:3491–3501

Yamada S, Sugai M, Komatsuzawa H, Nakashima S, Oshida T, Matsumoto A, Suginaka H (1996) An autolysin ring associated with cell separation of *Staphylococcus aureus*. J Bacteriol 178:1565–1571

Yamaguchi H, Furuhata K, Fukushima T, Yamamoto H, Sekiguchi J (2004) Characterization of a new *Bacillus subtilis* peptidoglycan hydrolase gene, *yvcE* (named *cwlO*), and the enzymatic properties of its encoded protein. J Biosci Bioeng 98:174–181

Yamamoto H, Kurosawa S, Sekiguchi J (2003) Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall-bound or extracellular proteases. J Bacteriol 185:6666–6677

Part V
Cell Wall Interactions

Chapter 14
Prokaryotic Cell–Cell Interaction

Reinhard Wirth

14.1 Introduction

Prokaryotic cells have to interact with each other (and their abiotic surroundings) in their natural biotope to ensure survival, very often resulting in the formation of biofilms. Though biofilms are not covered in this chapter, it should be noted here that their establishment depends – as does their growth – on various factors, some of which are also used for the kinds of interactions discussed below. A typical example of such factors are cell surface appendages like flagella which are used not only for swimming but, e.g., for adhesion to various surfaces – see also below. For an overview on biofilms the reader is referred to, e.g., Kolter (2005) and the complete January 2005 issue of *Trends in Microbiology*, or Palmer and Stoodley 2007 summarizing the 2007 ASM Conference on Biofilms.

In this chapter I will concentrate on systems which are more or less well-characterized/understood with respect to the role of cell surface components of prokaryotic cells mediating interactions with prokaryotic cells of the same or another species.

14.2 Formation of Fruiting Bodies by Myxobacteria

14.2.1 Discovery and Description of the Biological System

Myxobacteria surely stand at the borderline between unicellular and multicellular microorganisms. Indeed, originally characterized as slime molds or fungi imperfecti in the early nineteenth century, it took nearly 100 years to detect their true

R. Wirth
Institute of Microbiology, University of Regensburg, 93053 Regensburg, Germany
e-mail: Reinhard.Wirth@biologie.uni-regensburg.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_14,

nature as bacteria, by Thaxter in 1892 (for an excellent overview, covering also historical aspects see Reichenbach and Dworkin 1992). Under certain conditions myxobacteria are able to aggregate into structures called fruiting bodies, consisting of up to 10^6 cells. These fruiting bodies can have very different shapes; from simple "balls" through irregularly branched forms to "mini-tree"-like structures – see Fig. 14.1a–c.

Myxobacteria grown in liquid (and solid)-rich media are long slender rods characterized by some unusual features. Their peptidoglycan seems not to consist of one huge murein sacculus, but rather of patches of this cell wall material. In addition, the rods can glide on solid surfaces (in a forwards–backward manner, with c. 0.17 reversals per min) and are flexible, able to bend over 180°. Two types of motility have been observed: single cells move in an adventurous way (A-motility), whilst cells forming aggregates move in a coordinated, social way (S-motility).

The true nature of myxobacteria becomes evident if they are grown under conditions reflecting their natural biotope, namely decaying organic matter, to whose degradation/consumption they contribute considerably. Their ability to degrade organic matter is used in a special enrichment technique, the so-called rabbit dung-pellet bait technique. For this, autoclaved rabbit dung pellets are placed in the middle of "water-agar" plates; at a distance of c. 1 cm a few soil crumbs are placed and the system is incubated for 1–2 weeks at room temperature to 37°C. Myxobacteria are able to sense the presence of organic material in the dung pellets and to reach them using their A-motility system. After growth and consumption of the organic matter they will experience starvation, which is the main signal for formation of fruiting bodies. To finally assemble into fruiting bodies, the cells use their ability to move in a concerted way (their S-motility system).

This biological system has fascinated researchers for many years and *Myxococcus xanthus* – forming yellowish balls as fruiting bodies – has been chosen as a model system, especially in the USA; *Stigmatella aurantiaca* – forming mini-trees – was and is studied mainly by German groups (see, e.g., Plaga and Schairer 1999). To date many details, especially for *M. xanthus,* are known, but the picture is far from being complete. Not the least reason for this lies in the fact that regulation is very complex – which can be understood if one considers the fact that myxobacteria have the biggest bacterial genomes known (9.1 Mbp for *M. xanthus* and 10.3 Mbp for *S. aurantiaca*). There exist many excellent reviews on this system (newer ones being those of Kaiser 2003; Sogaard-Andersen et al. 2003; Stevens and Sogaard-Andersen 2005), to which the reader is referred for further details. It has to be noted that early experiments – using mutants and complementation assays – defined various "signals" A-E, "acting" in the time-course B-A-D-E-C, of which the last one in particular is important here. These "signals" (sometimes it is argued that only the A and C components are true signals) can be characterized as follows:

B-signal. This is active in vegetative and developing cells; the *bsgA* gene product codes for a protease, being homologous to the LonA protease of *Escherichia coli*.

A-signal. This is composed of two components: the heat-labile component consists of at least two proteases and the heat-stable one is a mixture of different

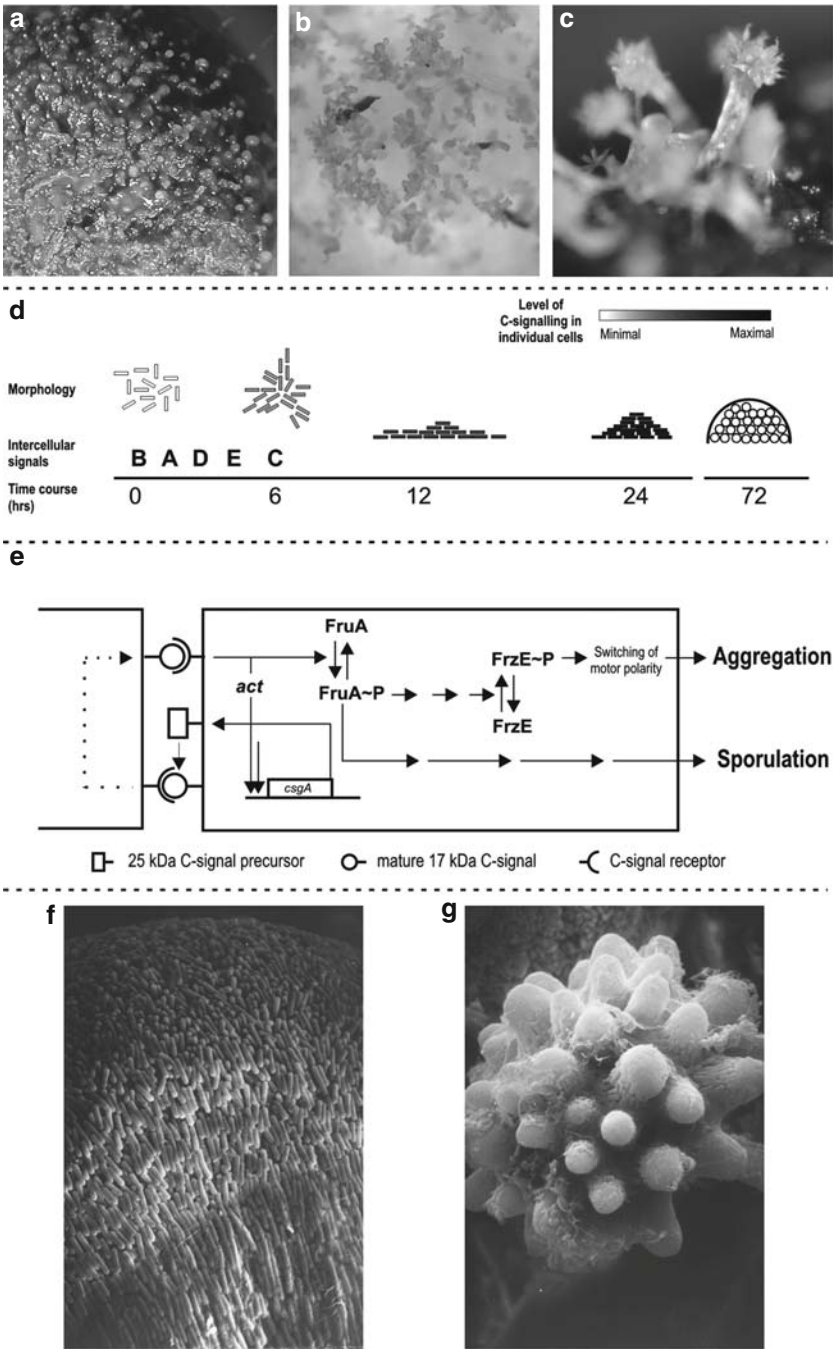

Fig. 14.1 Formation of fruiting bodies by Myxobacteria. (**a**)–(**c**): Various shapes of fruiting bodies ((**a**): ball-like; (**b**): coral-like; (**c**): tree-like) – enriched from soil samples by students participating

single amino acids. The latter ones must be present in concentrations of >10 μmol/L to act as signals; at higher concentrations (>10 mmol/L) they do not indicate the onset of starvation, but instead are used for nutrition!

D-signal. The *dsg* gene product shows >50% identity (and >80% similarity) to IF3 (the bacterial initiation factor 3 needed for translation).

E-signal. This was defined later than the other four signals; E-signal mutants are impaired in production of the fatty acid 15:1ω5c.

C-signal. This surface protein will be discussed in the next section.

Within fruiting bodies, the so-called myxospores are formed as a last step in development. These are highly refractive, differentiated round cells, which are metabolically inactive and characterized by their high content of trehalose and of special proteins (especially proteins C, U and S) in the thick spore wall. Dry myxospores can survive for >100 years; it is argued that they are contained in high numbers in sporangioles to ensure regrowth under favorable conditions. That is, a high local density of germinating myxobacteria act together to achieve concentrations of their (extracellular) lysis enzymes high enough to attack the organic matter myxobacteria feed on.

14.2.2 The Cell Surface Compounds Involved

In our context, two cell surface components in particular have to be discussed – the so-called fibrils and the C-signal. Both of these cell surface components should not be confused with two other cell "surface appendages" the bacteria use for gliding motility. These latter two players in the game – slime extrusions and type IV pili – are briefly characterized here to (it is hoped) clarify things.

A prerequisite for formation of fruiting bodies is the accumulation of many cells in so-called mounds which later will transform into fruiting bodies by accumulating more and more bacteria. The bacteria move into those mounds by gliding, which is accomplished by two different mechanisms acting at the same time. Cells are moved forward by the pulling force exerted by classical type IV pili located at their anterior cell pole; in addition the cells are pushed forward by slime excretion through nozzles at their posterior pole. Very interestingly, the forwards–backward motion mentioned above is brought about by "switching" the location of pulling pili

Fig. 14.1 (continued) in the advanced microbiology course "Organismic Microbiology I/II" at the University of Regensburg, using the rabbit dung-pellet bait technique. Size of fruiting bodies 1–2 mm. (**d**) Time-course and strength of C-signaling during *M. xanthus* fruiting body formation; redrawn with permission from Sogaard-Andersen et al. (2003). (**e**) Scheme for regulation of C-signaling; redrawn from Stevens and Sogaard-Andersen (2005). (**f**) and (**g**) Scanning electron microscopic pictures of developing *Chondromyces croacatus* fruiting bodies – cell lengths are c. 5 μm (Wirth and Wanner, unpublished). (**f**) The stem and developing sporangiole are formed by cells in extremely dense packaging. (**g**) Nearly mature fruiting body with fully developed sporangiole, but not yet differentiated myxospores

and pushing slime on the cell poles, regulated by an oscillator encoded by the *frz* system (see Fig. 14.1e). That is, both cell poles have the capacity for pulling and pushing, but do not express this ability simultaneously (see Kaiser and Yu 2005 for details). This picture was questioned by Mignot et al. (2007) who reported on the existence of a special kind of motor: according to their data, focal adhesion complexes – spaced regularly over the cell length – are used to move the cell relative to these structures. Subsequently, the Zusman laboratory presented data (Sliusarenko et al. 2007) which indicate that the posterior pole does not contribute to gliding (i.e., possesses no motor). For the formation of aggregates culminating in the formation of fruiting bodies myxobacteria have to assure that the cells move into the right direction and that cell concentrations are high enough to allow successful formation of fruiting bodies – fibrils and C-signals are used for direct cell–cell signaling to assure this.

14.2.2.1 Fibrils

Fibrils are cell surface appendages consisting of approximately equal amounts of polysaccharide and protein. Their diameter was determined by various groups to vary from 10 to 60 nm – very probably due to some kind of lateral adhesion (Behmlander and Dworkin 1994a). Since fibrils emanate from all over the cell and connect adjacent cells to form a kind of network, it is not possible to define their maximum length – some low voltage scanning electron microscopy pictures demonstrate that single fibrils can be 2 μm long (it has to be noted that fibrils are not detected by all techniques used for electron microscopy). Fibrils are formed by and connect socially gliding myxobacteria (see, e.g., Behmlander and Dworkin 1991) and are believed to be responsible for a phenomenon observed for colonies spreading on agar plates. Cells adhere very tightly to each other under these conditions making it very difficult to use an inoculation loop to remove a few cells for purification streaks – indeed, with the use of forceps colonies can be removed from such agar plates as a whole entity. The protein composition of fibrils has been determined to a certain extent – they consist of five major proteins with molecular masses ranging from 14 to 66 kDa (Behmlander and Dworkin 1994b). The fibrillar carbohydrates have been characterized even less – they contain galactose, glucose, glucoseamine, rhamnose and xylose (Behmlander and Dworkin 1994a). Li et al. (2003) reported that extracellular polysaccharides prepared from fibrils induced retraction of the type IV pili used for social motility. Since chitin could induce retraction, too, they argued that a polysaccharide containing amine sugars (present on cells [fibrils] or slime trail surfaces excreted by gliding myxobacteria) triggers pilus retraction and therefore S-motility. In their view fibrils likely form a capsule over the entire cell body.

The importance of fibrils for interactions between socially gliding myxobacteria is also supported by another line of evidence. It could be demonstrated that an ADP-ribosyl transferase activity is associated with fibrils and that its protein substrate is located on the cell surface. Dworkin (1999) therefore speculated that fibrils act as

tactile antenna to transmit a signal indicating the proximity of another cell. In this view, fibrils would act to indicate the presence of "nearby moving partners", which do not directly touch each other.

14.2.2.2 C-Signal

C-signal is first detected on cells 6 h after onset of starvation and increases in strength to finally trigger formation of fruiting bodies. The action and time course of this signal during morphogenesis (fruiting body formation) is outlined in Fig. 14.1d.

All known C-signal mutants occur in a single gene *csgA*, coding for a 25 kDa protein, for which initially a function as a short-chain alcohol dehydrogenase was postulated. Subsequently this hypothesis was abandoned, however, by the finding that the protein is processed into a 17 kDa protein located at the outside of the Gram-negative bacteria. The protein is used to sense the immediate neighboring cell; i.e., C-signal and the (as yet unknown) receptor for C-signal are present on one and the same cell. C-signaling and perception are via the poles of bacteria in direct contact, whilst fibrils are supposed to measure and assure more distant cell–cell contacts over the cell's length. Perception of C-signal influences at least 50 genes (gene products) downstream in the development process by phosphorylation of a central regulator FruA (see Fig. 14.1e). These regulatory cascades include especially: increase in C-signal density (via the *act*-operon; an Act-independent *csgA* activation exists, too); regulation of polarity switching of the two "motors" slime excretion and type IV pili (via FrzE~P); sporulation (via FruA~P). For details the reader is referred to, e.g., Stevens and Sogaard-Andersen (2005); Sogaard-Andersen et al. (2003); Kaiser (2003).

From the data outlined above it becomes clear that fibrils are used by myxobacteria to get into contact with nearby moving partners and that C-signal is used by them to measure and ensure their direct pole–pole contact. Myxobacteria, indeed, assemble during aggregation to cell densities of c. 5×10^{10} per ml (a density obtainable by centrifugation), whilst their density in maturing fruiting bodies is $>2\times10^{11}$ per ml. This extreme density of packing is very convincingly seen in Fig. 14.1f, showing the stem of a forming fruiting body with a developing sporangiole at the tip. Fig. 14.1g shows a more developed sporangiole, shortly before differentiation of the rod-shaped bacteria into round myxospores.

14.3 The Sex Pheromone System of *Enterococcus faecalis*

14.3.1 Discovery and Description of the Biological System

The sex pheromone system was discovered in D.B. Clewell's laboratory during experiments investigating the potential transfer of antibiotic resistances between Gram-positive bacteria (Dunny et al. 1978). This was at a time when it was unclear

which genetic elements (such as plasmids, transposons, etc.) , if any, exist in Gram-positives. Indeed, the technique of isolating plasmids via CsCl/ethidium bromide gradient centrifugation was developed by Clewell and Helinski (1970) just a few years previously for *E. coli*.

The original observation was that some special strains of *Enterococcus faecalis* (at that time still named *Streptococcus faecalis*), if grown together, do form huge aggregates of cells (for a certain length of time), easily visible to the naked eye. If grown alone, these strains do not form clumps. It soon became clear that formation of such aggregates involves the transfer of a plasmid from a donor strain to a recipient strain (not possessing this type of plasmid). Through a variety of experiments it was shown that the plasmid-free recipient strain induces – via the excretion of a diffusible substance – expression of an adhesin on the surface of the donor strain. The adhesin is able to interact with a receptor on the surface of *E. faecalis* cells, to allow the clumping reaction. The components involved in this special kind of interaction were named as follows:

- The adhesin is named aggregation substance.
- The receptor for aggregation substance is named binding substance.
- The diffusible inductor is named sex pheromone.
- Sex pheromones are named after the plasmids whose transfer they induce: sex pheromone cAD1 (c standing for clumping), e.g., induces the transfer of sex pheromone plasmid pAD1.

A cartoon illustrating the system is shown in Fig. 14.2; it was drawn by G. Wanner, who was especially involved in electron microscopy identifying aggregation substance as a hair-like structure on the cell surface of *E. faecalis*. The sex pheromone system was analyzed by the groups of D.B. Clewell, G. Dunny, Y. Ike, K. Waever, and R. Wirth. Since various reviews cover different aspects of this fascinating biological system, the reader is referred to those for questions such as: ideas on the evolution of the sex pheromone system (Wirth 1994); regulation of the system, which is extremely complex and seems to be different for different sex pheromone plasmids (de Freire Bastos et al. 1998; Leonard et al. 1996; Muscholl-Silberhorn 2000); general descriptions of the sex pheromone system (Clewell 1999, Wirth 1994; Dunny and Leonard 1997).

In the following, only a few further facts are mentioned.

- *E. faecalis* possesses an extremely large genetic versatility; *E. faecalis*, indeed, is the Gram-positive bacterium in which the greatest variety of antibiotic resistances has been observed. Various strains may contain transposons, conjugative transposons, cryptic plasmids, conjugative plasmids, resistance plasmids normally exhibiting an extremely broad host range, and the conjugatively transferred sex pheromone plasmids. (The existence of very many repetitive genetic elements hindered the completion of the *E. faecalis* genome sequence for 6 years!)
- The sex pheromone system is almost totally specific for *E. faecalis*; only very few sex pheromone plasmids can be transferred to and between the closest relative, *E. faecium*.

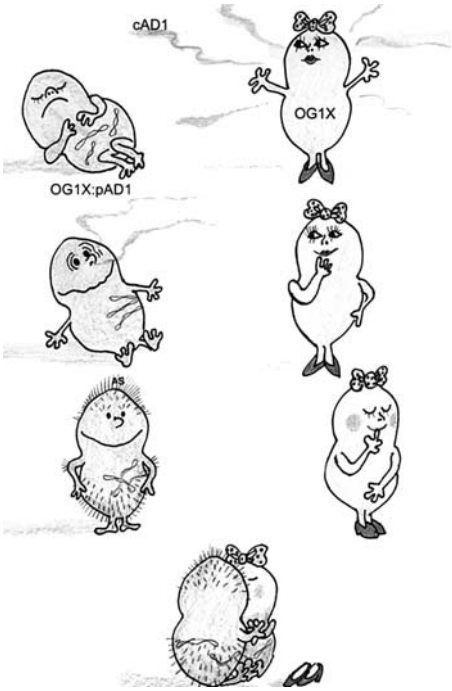

Fig. 14.2 Cartoon illustrating the sex pheromone system of *Enterococcus faecalis*. A plasmid-free recipient cell (OG1X, *right*) excretes a so-called sex pheromone cAD1, which can be sensed by a plasmid-carrying donor cell (OG1X:pAD1). In response, the donor cell expresses the adhesin aggregation substance (AS) on its surface enabling conjugative transfer of the plasmid. Courtesy of G. Wanner – see text for details

- Sex pheromones are small (7 or 8 amino acids long) peptides, which are processed from signal peptides of lipoproteins destined to be exported (Clewell et al. 2000). These peptides are almost exclusively produced by *E. faecalis*; only cAM373 is also produced by some other Gram-positive bacteria.

14.3.2 The Cell Surface Compounds Involved

14.3.2.1 Binding Substance

Very soon after the first characterization of the *E. faecalis* sex pheromone system, it was postulated that the receptor for (sex pheromone-induced) aggregation substance (= binding substance) should be unique for *E. faecalis*. Induced *E. faecalis* cells were found to bind only to *E. faecalis* cells, but not to other bacteria. Early experiments indicated that lipoteichoic acid (LTA) would represent binding substance: only extracts of cell walls from (plasmid-free) *E. faecalis* cells interfered with binding assays. These data were conclusive because LTA from *E. faecalis* and its closest relative *E. faecium* differ considerably in structure and the extracts used for the binding assay consisted mainly of LTA. Later experiments confirmed these studies (but only in part): chromosomal mutants of *E. faecalis* impaired in binding assays were found to be affected in LTA. In addition, however, in this study mutants

also had been identified, which occurred in genes obviously not directly related to LTA synthesis or integration into the cell surface (see Bensing and Dunny 1993 for details).

One has to conclude, therefore, that binding substance is composed at least in part of LTA, but that other constituents like surface proteins might also contribute to the observed high specificity of binding of sex pheromone-induced *E. faecalis* cells exclusively to partners of the same species.

14.3.2.2 Aggregation Substance

This component of the sex pheromone system is much more clearly defined than binding substance: it is a large protein encoded by sex pheromone plasmids. Indeed, sex pheromone plasmids are defined by their coding capacity for aggregation substance – quite a few of the rather large sex pheromone plasmids (>c. 50 kb) are otherwise cryptic.

Aggregation substance was studied mainly by the group of R. Wirth; *asa1* (aggregation substance encoded by pAD1) was the first aggregation substance gene being sequenced (Galli et al. 1990). In addition, the corresponding protein Asa1 was the first to be shown to form hair-like structures on the surface of (cAD1-induced) pAD1-carrying *E. faecalis* cells (Galli et al. 1989). Scanning electron microscopy revealed that aggregation substance is localized to "old cell poles"; i.e., aggregation substance is not incorporated into the cell wall region near the septum, where new murein subunits exclusively are inserted (Wanner et al. 1989).

It was important for two reasons to have available the sequence of aggregation substance. Firstly, because the sequence confirmed that aggregation substance is located on the outside of the cells, but anchored into the cell wall. This was deduced from the fact that the C-terminus of the protein contains the motif LP(X [here = Q])TGE; this motif just had been proven at that time to define extracellular proteins anchored at their most C-terminal part in the cell wall of Gram-positive bacteria. Secondly, it was recognized that aggregation substance contained two RGD motifs, namely RGDS and RGDV. Those at that time had already been shown to be responsible for binding to eukaryotic cells. This then was taken as indication that aggregation substance might be a bifunctional adhesin, allowing interaction not only with bacteria of its own species, but also with host cells (*E. faecalis* is an opportunistic pathogen, able to cause urinary tract infections, endocarditis and other serious infections). Indeed, first experiments demonstrated the binding capacity of *E. faecalis* cells expressing aggregation substance to cultured pig kidney cells (Kreft et al. 1992). Later experiments were conflicting in the sense that the results of different groups did not agree with respect to the importance of aggregation substance in various pathogenicity models. It became clear subsequently – when the *E. faecalis* genome sequence became available – that *E. faecalis* codes for >100 surface proteins, which might very well have contributed to the various results in the different experimental settings. At the time when the first aggregation substance gene was identified the deduced protein sequence was argued to be special by the

way that it did not contain internal repeats. Such repeats at that time were taken to define surface proteins functioning as adhesins of Gram-positive bacteria, a picture which changed with more (genome) sequences becoming available.

Functional analyses of aggregation substances resulted in somewhat conflicting data: for pAD1-encoded aggregation substance Asa1 it was demonstrated (using in-frame deletion mutants – see Muscholl-Silberhorn 1998) that the N-terminal region – especially region 525–617 – of the 1,024-amino-acid-long protein is responsible for binding to *E. faecalis*. Binding to eukaryotic cells was supposed to be dependent on other regions. For pCF10-encoded aggregation substance Asc10 binding to *E. faecalis* cells was found to be dependent on a region starting at position 400 (Waters and Dunny 2001). Later, the Dunny laboratory reported that position 156–358 of Asc10 is needed for internalization by epithelial cells (Waters et al. 2004). This was surprising, because both proteins Asa1 and Asc10 show a total amino acid identity of over 80%, with positions 1–280 and 600–1,024 differing by less than 5%. This discrepancy is not resolved to date; it should be noted, however, that the studies from the Dunny and Wirth laboratories used different functional detection assays.

14.4 Coaggregations of Dental (Tooth-Associated) Bacteria

14.4.1 Discovery and Description of the Biological System

It had been recognized long before the term biofilm was coined that the (human) oral cavity is dominated by prokaryotes possessing the ability to form cell–cell aggregates of different species. The main point here is that these aggregates form in a specific manner, i.e., not all bacteria involved aggregate with all others. This means that a specially structured biofilm forms in a sequential way on freshly cleaned teeth. It has to be noted that these interactions also occur on other oral surfaces, such as the tongue. For details the reader is referred to excellent reviews, e.g., Kolenbrander and London (1993), or Rickard et al. (2003).

As shown in Fig. 14.3, all oral bacteria directly adhering to teeth do this by interacting with the so-called acquired pellicle. This pellicle is laid down within seconds onto freshly cleaned teeth and is composed of glycoproteins, mucins and enzymes, present in high amounts in saliva. The so-called primary colonizers are able to use various adhesins to recognize and bind to receptors in the pellicle. The primary colonizers are more or less exclusively streptococci, namely *Streptococcus gordonii, S. oralis, S. mitis* and *S. sanguis*; only *Actinomyces naeslundii* in addition is able to interact with the acquired pellicle. This primary layer of colonizers can be used by further bacteria, like *Porphyromonas gingivalis, Haemophilus parainfluencae, Propionibacterium acnes, Veillonella atypica,* and *A. naeslundii,* within a few hours to complete the structured layer of early colonizers. It was shown that c. 4 h after cleaning of teeth only streptococci (and a few *A. naeslundii* cells) can be isolated from teeth, whilst after an additional 4 h the other species mentioned above (like *P. acnes*) are also present. *Fusobacterium nucleatum* seems to play a special

role in the establishment of plaque, because it is recognized by the late colonizers like *Actinobacillus actinomycetemcomitans* (a species which can exert dramatic effects especially in localized juvenile periodontitis), *Helicobacter pylori* and various *Treponema* species.

14.4.2 The Cell Surface Compounds Involved

The Kolenbrander laboratory, in particular, studied coaggregations in order to define – via pairwise mixing experiments – possible interactions between the most prominent of the more than 500 species of oral bacteria already recognized. It turned out that the interactions are not at all random; i.e., they are highly specific. On the other hand it is also evident that the system itself is highly complex – as an example a few data for *S. gordonii* are summarized here (see Rickard et al. 2003 for more details). As shown in Fig. 14.3, this streptococcus possess the ability for various distinct interactions involved in coaggregations:

- A 100 kDa protein is responsible for interactions with other streptococci.
- A 203 kDa protein interacts with components of saliva and, therefore, the acquired pellicle.

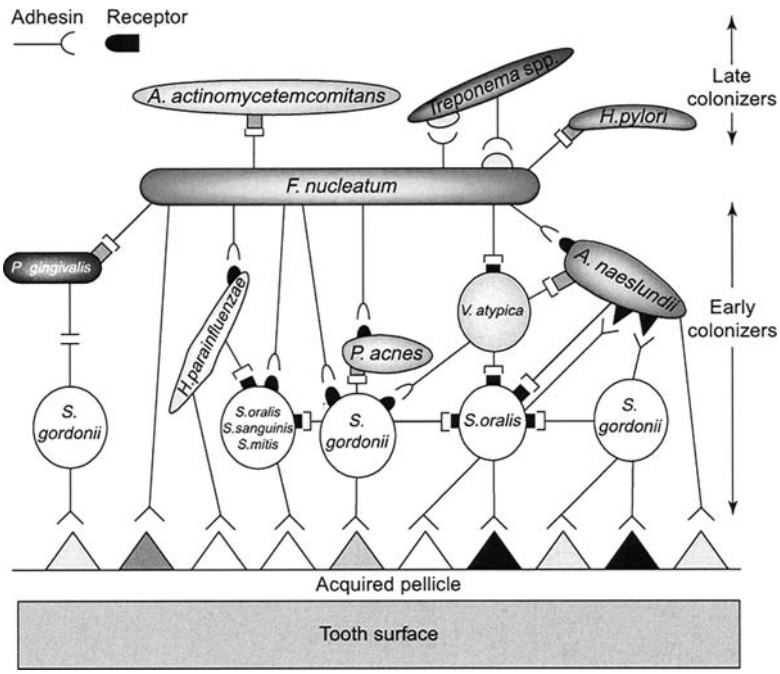

Fig. 14.3 Scheme for establishment and specificity of coaggregates on teeth. These structures lead to plaque formation and finally caries (reproduced with permission from Rickard et al. 2003)

- A 259 kDa fibrillar protein interacts with *A. naeslundii*.
- Two proteins SspA and SspB have multiple functions including interactions with salivary glycoproteins, fibronectin and collagen; in addition they allow *S. gordonii* to interact with *P. gingivalis* and *A. naeslundii*.
- In addition *S. gordonii* can express at least two different receptors for the bacteria it interacts with, namely *F. nucleatum* and *V. atypica*.

From this one example it is evident that a general picture for the various adhesins/receptors used in coaggregations cannot be expected to exist. As discussed in Palmer et al. (2003), the streptococcal receptor polysaccharides fall into two classes recognizing either GalNAcβ1→3Gal or Galβ1→3GalNac. The adhesins seem to be made from very different surface proteins, including type 2 fimbriae. The difficulty of defining the adhesins involved can be deduced from the case of *F. nucleatum*: it possesses at least three different multifunctional adhesins, enabling interactions with at least seven different genera of bacteria in plaque.

Of course, it was recognized early on that interference with coaggregations might be a way to (beneficially) influence plaque formation and thereby periodontal diseases. In this context, a high-molecular-mass constituent of cranberry juice was shown to interfere with coaggregations (Weiss et al. 1998, 2004); the use of genetically altered primary colonizers to combat plaque has also been debated.

In 2003 Palmer and colleagues showed for the first time that coaggregations are indeed very important for initial plaque accumulation in vivo. They used different antibodies, namely: (1) against the receptor polysaccharides on *S. oralis* (to investigate interactions with *S. gordonii*); (2) against *S. gordonii*; (3) against type 2 fimbriae of *A. naeslundii*. These antibodies were used on developing plaque and the results very convincingly demonstrated that in developing plaque at the first stage (4 h of development) only single cells can be detected, followed by mixed species biofilms in later stages (8 h), and that *A. naeslundii* indeed directly interacts with *S. gordonii* in plaque.

14.5 Other Bacterial Systems

Since many of the cell wall compounds involved in the cell–cell interactions listed below are long structures, the reader is referred to Eisenstein (1987) to learn more about the diverse nomenclature for fimbriae, pili etc.

14.5.1 Flagella Can Be Used for Swarming Motility

Proteus mirabilis was named for its completely remarkable ability to swarm on surfaces. For this the bacteria "differentiate" (stop division) into giant cells containing up to 50 genome equivalents and possessing up to 1000 flagella. These giant

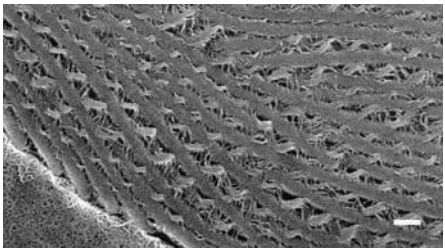

Fig. 14.4 Scanning electron micrograph of swarming *P. mirabilis* cells. The swarm edge is to the *lower left* (*size bar* = 1 μm) – note the interwoven flagella between the long cells (reproduced with permission from Jones et al. 2004)

cells swarm in a concerted way (i.e., cells at the edge of a swarm "crawl" together over the agar plate for a certain time). Thereafter consolidation is observed: i.e., cells stop movement, dedifferentiate into normal cells and after some time a new cycle of swarming is initiated. These differentiation/dedifferentiation processes result in formation of regular patterns during swarming ("terrace formation"). Jones et al. (2004) have detected that cells in migrating swarms interact in an extremely ordered way via their flagella: these are interwoven in phase, thereby forming helical connections between adjacent swarmer cells – see Fig. 14.4.

14.5.2 Type IV Pili (Bundle-Forming Pili) Can Be Used for Establishment of Microcolonies

In the case of the so-called bundle-forming pili it has been shown convincingly that these cell appendages are used not only by *E. coli* for interactions with the human intestine (binding to epithelial cell lines), but also with themselves to form microcolonies epithelial cells (see, e.g., Bieber et al. 1998). These microcolonies, however, must also be dissolved during infections to allow colonization of further intestinal regions to finally result in diarrhea; mutants not able to disperse resulted in c. 200-fold less virulent strains in trials with human volunteers. Bundle-forming pili, on the other hand, are also a prerequisite for initial colonization of human cells; therefore, formation of these type IV pili has to be highly regulated by enteropathogenic *E. coli* strains during the infection process/progress.

14.5.3 "Sex-Pili" Can Be Used for Enhanced Gene Transfer

The term sex-pilus was coined for pili encoded by the *E. coli* F-plasmid (100 kb in size), which can integrate into the genome to form Hfr-strains. The encoded pilus is used to recognize potential mating partners. When the donor strain recognizes that its pilus has bound to an *E. coli* strain not possessing a copy of the F-plasmid, the "sex-pilus" is retracted to result in two cells lying adjacent to each other. Thereafter, a mating pore is formed and a copy of the F-plasmid conjugatively transferred into the recipient strain. For further details the reader is referred to textbooks such as Firth et al. (1996).

14.5.4 *Clumping Reactions Can Be Used for Enhanced Gene Transfer*

The sex pheromone system of *E. faecalis* is not an absolutely unique system in the sense that it was also observed for other Gram-positive bacteria that clumping reactions between donor and recipient strains of one species can lead to enhanced gene transfer (see, e.g., Luo et al. 2005 and references therein). Such systems have been found in *Bacillus thuringiensis*, *Lactococcus plantarum* and *Lactococcus lactis*. The latter system is best characterized, and it was shown that a surface protein CluA (anchored in the cell wall via the canonical LPXTGE motif, but otherwise not related to *E. faecalis* aggregation substance) is responsible for the clumping reaction. CluA, however, is not the sole factor responsible for high-efficiency mating, because "super donors" could be obtained transferring plasmids/genes with frequencies of 10^2–10^7 higher than in originally CluA-dependent clumping reactions (see Luo et al. 2005 for details).

14.5.5 *Special Contact Sites in a Phototrophic Consortium*

"Chlorochromatium aggregatum" is a phototrophic consortium consisting of one central, motile, rod-shaped chemotrophic β-proteobacterium surrounded by 13–69 green sulfur bacteria. The latter has been newly isolated from this consortium by Vogl et al. (2006), and was characterized as a new species, *Chlorobium chlorochromatii*. Whilst chlorosomes are equally distributed over the cell length of *C. chlorochromatii* cells grown in pure culture, they are not found at contact sites between the green sulphur bacterium and the β-proteobacterium in the consortia. Ultrathin sections of those contact sites revealed the existence of special laminar layers, and there is evidence for a specific signal exchange between the epibionts and the central bacterium (see Vogl et al. 2006 for details).

14.6 Interactions of Archaea

It is of no surprise that archaea are able to interact with their environment and therefore also with prokaryotes – hard data in this field, however, are scarce (for a very special archaeal system involving the two species *Ignicoccus hospitalis* and *Nanoarchaeum equitans* see Chap. 12).

14.6.1 *Interactions of* Pyrococcus furiosus *via Flagella*

It has been shown quite recently in my lab that *P. furiosus* does not use its flagella only for swimming (Näther et al. 2006). This hyperthermophilic archaeon uses its

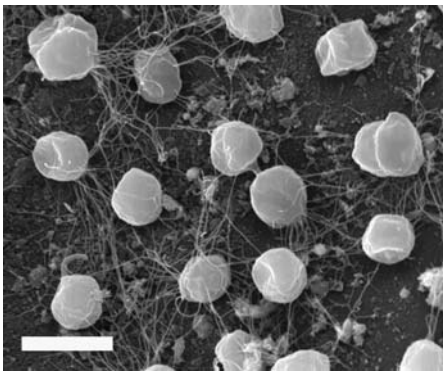

Fig. 14.5 Adherence of *Pyrococcus furiosus* cells. The cells adhere to a sand grain from their natural habitat and to each other via their flagella (note the cable connecting the central cell-pair); *size bar* = 2 μm

flagella also for adhesion to various surfaces (such as sand grains from its original biotope), including cells of its own species, allowing the formation of microcolonies and biofilms; see, e.g., Fig. 14.5. In addition *P. furiosus* can interact also with other archaea (Schopf and Wirth, unpublished). A very surprising result was that *P. furiosus* can form bundles of its flagella in a way that the up to 50 cell surface appendages form a "cable-like" structure in which all flagella run parallel. For the present, it is unclear why these cables are formed. An attractive explanation might be that via the cables (the length of the cables can vary between 2 and 0.1 μm) two cells come into close enough contact for gene transfer, which is postulated to occur in this archaeon.

14.6.2 Interactions of the "SM1" Archaeon via Hami

The SM1 archaeon cannot (yet) be cultivated in artificial medium, but can be "harvested" from its natural habitat (cold, sulfidic springs) using polyethylene nets placed in those springs (Henneberger et al. 2006). SM1 grows in nearly pure culture on these nets, exhibiting a strikingly even distribution, with cells c. 2 μm apart (Fig. 14.6a). It turned out that this even spacing is brought about by very special surface structures called hami. These are long (1–3 μm) filamentous structures with regular "spikes" ending in a hook, resembling ultrastructurally a barbed wire which ends in a grappling hook (Moissl et al. 2005) (Fig. 14.6b). Obviously this unique system allows the uncultivated euryarchaea to adhere to each other (and to structures in its biotope) to stay constantly in a niche optimal for its growth.

14.7 Conclusions

The examples presented here clearly show that interactions of microbial cells with each other very often make use of highly specific cell surface components to ensure that only the "wanted" interaction takes place. In the case of myxobacteria, the

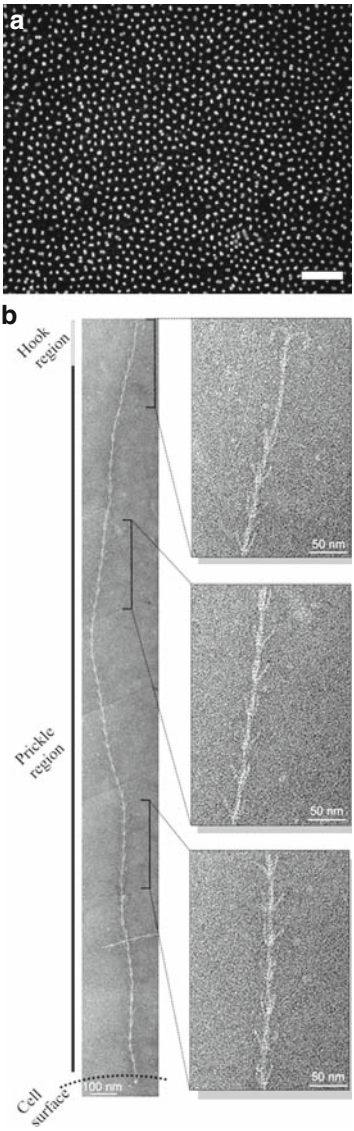

Fig. 14.6 Adherence of the SM1 archaeon via hami. (**a**) Even spacing of cells is observed for the SM1 archaeon growing on polyethylene nets; *size bar* = 10 μm. (**b**) Ultrastructure of the hami used for adhesion. Reproduced with permission from Moissl et al. (2005)

17 kDa "C-signal" protein located on the cell surface ensures a direct cell–cell contact, after cells accumulate with the help of the so-called fibrils, which consist of ca. 50% protein and carbohydrate. *Enterococcus faecalis* uses a great variety of special proteins called aggregation substance for interaction with the cell surface-associated binding substance, which consists (at least partially) of LTA. Dental-associated bacteria use a variety of cell surface components like proteins, polysaccharides, and fimbriae for highly specific interactions. In my view, the most

highly sophisticated cell surface structure used for interactions is the hami of the SM1 archaeon, functioning as a barbed wire ending in grappling hooks. Another strategy used by microorganisms for cell–cell interactions is to give one structure two functions. This has been convincingly shown for flagella, which can be used by bacteria and archaea not only as motility organelles but also for adhesion.

The examples covered here demonstrate very nicely the enormous flexibility of Nature for evolving special traits such as the ability for cell–cell interactions: on the one hand an endless variety of different structures can be invented or on the other hand existing structures can acquire new functions.

Note added in proof

Since editorial closing for this chapter was in autumn of 2008 some recent findings could not be addressed here. I refer the reader to the following references for

- A detailed investigation of the intimate/intricate surface interactions observed in *Chlorochromatium aggregatum*:

Wanner G, Vogl K, Overmann J (2008) Ultrastructural Characterization of the Prokaryotic Symbiosis in *Chlorochromatium aggregatum*. J Bacteriol 190:3721–3730

- Specific interactions between two different archaeal species:

Schopf S, Wanner G, Rachel R, Wirth R (2008) An archaeal bi-species biofilm formed by *Pyrococcus furiosus* and *Methanopyrus kandleri*. Arch Microbiol 190:371–377

- Interactions of an Archaeum via its fimbriae:

Thoma, C, Frank M, Rachel R, Schmid S, Näther D, Wanner G, Wirth R (2008) Fimbriae of *Methanothermobacter thermoautotrophicus* are encoded by *mth60*: first characterization of an archaeal fimbrium. Env Microbiol 10:2785–2795

- A new type of archaeal cell surface organelle, used for interactions:

Müller D, Meyer C, Gürster S, Küper U, Huber H, Rachel R, Wanner G, Wirth R, Bellack A (2009) The Iho670 fibers of *Ignicoccus hospitalis*: a new type of archaeal cell surface appendage. J Bacteriol 191:6465–6468

References

Behmlander RM, Dworkin M (1991) Extracellular fibrils and contact-mediated cell interactions in *Myxococcus xanthus*. J Bacteriol 173:7810–7821

Behmlander RM, Dworkin M (1994a) Biochemical and structural analyses of the extracellular matrix fibrils of *Myxococcus xanthus*. J Bacteriol 176:6295–6303

Behmlander RM, Dworkin M (1994b) Integral proteins of the extracellular matrix fibrils of *Myxococcus xanthus*. J Bacteriol 176:6304–6311

Bensing BA, Dunny GM (1993) Cloning and molecular analysis of genes affecting expression of binding substance, the recipient-encoded receptor(s) mediating mating aggregate formation in *Enterococcus faecalis*. J Bacteriol 175:7421–7429

Bieber D, Ramer SW, Wu C-Y, Murray WJ, Tobe T, Fernandez R, Schoolnik GK (1998) Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. Science 280:2114–2118

Clewell DB (1999) Sex pheromone systems in Enterococci. In: Dunny GM, Winans SC (eds) Cell-cell signalling in bacteria. ASM Press, Washington, DC, pp 47–65

Clewell DB, Helinski DR (1970) Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strans specificity of the relaxation complex. Biochemistry 9:4428–4440

Clewell DB, An FY, Flannagan SE, Antiporta M, Dunny GM (2000) Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. Mol Microbiol 35:246–247

de Freire Bastos MC, Tomita H, Tanimoto K, Clewell DB (1998) Regulation of the *Enterococcus faecalis* pAD1-related sex pheromone response: analyses of traD expression and its role in controlling conjugation functions. Mol Microbiol 30:381–392

Dunny GM, Leonard BA (1997) Cell-cell communication in Gram-positive bacteria. Annu Rev Microbiol 51:527–564

Dunny GM, Brown BL, Clewell DB (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc Natl Acad Sci USA 75:3479–3483

Dworkin M (1999) Fibrils as extracellular appendages of bacteria: their role in contact-mediated cell-cell interactions in *Myxococcus xanthus*. BioEssays 21:590–595

Eisenstein BI (1987) Fimbriae. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella typhimurium*. ASM Press, Washington, DC, pp 84–90

Firth N, Ippen-Ihler K, Skurray RA (1996) Structure and function of the F factor and mechanism of conjugation. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*. ASM Press, Washington DC, pp 2377–2401

Galli D, Wirth R, Wanner G (1989) Identification of aggregation substances of *Enterococcus faecalis* cells after induction by sex pheromones. Arch Microbiol 151:486–490

Galli D, Lottspeich F, Wirth R (1990) Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1. Mol Microbiol 4:895–904

Henneberger R, Moissl C, Amann T, Rudolph C, Huber R (2006) New insights into the lifestyle of the cold-loving SM1 euryarchaeon: natural growth as a monospecies biofilm in the subsurface. Appl Environ Microbiol 72:192–199

Jones BV, Young R, Mahenthiralingam E, Stickler DJ (2004) Ultrastructure of *Proteus mirabilis* swarmer cell rafts and role of swarming in catheter-associated urinary tract infection. Infect Immun 72:3941–3950

Kaiser D (2003) Coupling cell movement to multicellular development in Myxobacteria. Nat Rev Microbiol 1:45–54

Kaiser D, Yu R (2005) Reversing cell polarity: evidence and hypothesis. Curr Opin Microbiol 8:216–221

Kolenbrander PE, London J (1993) Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol 175:3247–3252

Kolter R (2005) Surfacing views of biofilm biology. TRENDS Microbiol 13:1–2

Kreft B, Marre R, Schramm U, Wirth R (1992) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. Infect Immun 60:25–30

Leonard BAB, Podbielski A, Hedberg PJ, Dunny GM (1996) *Enterococcus faecalis* pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. Proc Natl Acad Sci USA 93:260–264

Li Y, Sun H, Ma X, Lu A, Lux R, Zusman D, Shi W (2003) Extracellular polysaccharides mediate pilus retraction during social motility of *Myxococcus xanthus*. Proc Natl Acad Sci USA 100:5443–5448

Luo H, Wan K, Wang HH (2005) High-frequency conjugation system facilitates biofilm formation and pAMβ1 transmission by *Lactococcis lactis*. Appl Environ Microbiol 71:2970–2978

Mignot T, Shaevitz JW, Hartzell P, Zusman DR (2007) Evidence that focal adhesion complexes power bacterial gliding motility. Science 315:853–856

Moissl C, Rachel R, Briegel A, Engelhardt H, Huber R (2005) The unique structure of archaeal hami, highly complex cell appendages with nano-grappling hooks. Mol Microbiol 56:361–370

Muscholl-Silberhorn A (1998) Analysis of the clumping-mediating domain(s) of sex pheromone plasmid pAD1-encoded aggregation substance. Eur J Biochem 258:515–520

Muscholl-Silberhorn AB (2000) Pheromone-regulated expression of sex pheromone plasmid pAD1-encoded aggregation substance depends on at least six upstream genes and a cis-acting, orientation-dependent factor. J Bacteriol 182:3816–3825

Näther DJ, Rachel R, Wanner G, Wirth R (2006) Flagella of *Pyrococcus furiosus*: multifunctional organelles, made for swimming, adhesion to various surfaces, and cell-cell contacts. J Bacteriol 188:6915–6923

Palmer RJ, Stoodley P (2007) Biofilms 2007: broadened horizons and new emphases. J Bacteriol 189:7948–7960

Palmer RJ, Gordon SM, Cisar JO, Kolenbrander PE (2003) Coaggregation-mediated interactions in streptococci and actinomyces detected in initial human dental plaque. J Bacteriol 185: 3400–3409

Plaga W, Schairer HU (1999) Intercellular signalling in *Stigmatella aurantiaca*. Curr Opin Microbiol 2:593–597

Reichenbach H, Dworkin M (1992) The Myxobacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes, 2nd edn, vol IV. Springer, New York, pp 3416–3487

Rickard AH, Gilbert P, High NJ, Kolenbrandner PE, Handley PS (2003) Bacterial coaggregation: an integral process in the development of multi-species biolfims. Trends Microbiol 11:94–100

Sliusarenko O, Zusman DR, Oster G (2007) The motors powering A-motility in *Myxococcus xanthus* are distributed along the cell body. J Bacteriol 189:7920–7921

Sogaard-Andersen L, Overgaard M, Lobedanz S, Ellehauge E, Jelsbak L, Rasmussen AA (2003) Coupling gene expression and multicellular morphogenesis during fruiting body formation in *Myxococcus xanthus*. Mol Microbiol 48:1–8

Stevens A, Sogaard-Andersen L (2005) Making waves: pattern formation by a cell-surface-associated signal. Trends Microbiol 13:249–252

Vogl K, Glaeser J, Pfannes KR, Wanner G, Overmann J (2006) *Chlorobium chrlorochromatii* sp. nov., a symbiotic green sulphur bacterium isolated from the phototrophic consortium *"Chlorochromatium aggregatum"*. Arch Microbiol 185:363–372

Wanner G, Formanek H, Galli D, Wirth R (1989) Localization of aggregation substances of *Enterococcus faecalis* after induction by sex pheromones. Arch Microbiol 151:491–497

Waters CM, Dunny GM (2001) Analysis of functional domains of the *Enterococcus faecalis* pheromone-induced surface protein aggregation substance. J Bacteriol 183:5659–5667

Waters CM, Hirt H, McCormick JK, Schlievert PM, Wells CL, Dunny GM (2004) An amino-terminal domain of *Enterococcus faecalis* aggregation substance is required for aggregation, bacterial internalization by epithelial cells and binding to lipoteichoic acid. Mol Microbiol 52:1159–1171

Weiss EI, Lev-Dor R, Kashamn Y, Goldhar J, Sharon N, Ofek I (1998) Inhibiting interspecies coaggregation of plaque bacteria with a cranberry juice constituent. JADA 129:1719–1723

Weiss EI, Kozlovsky A, Steinberg D, Lev-Dor R, Greenstein RBN, Feldman M, Sharon N, Ofek I (2004) A high molecular mass cranberry constituent reduces mutans streptococci level in saliva and inhibits in vitro adhesion to hydroxyapatite. FEMS Microbiol Lett 232:89–92

Wirth R (1994) The sex pheromone system of *Enterococcus faecalis*: more than just a plasmid-collection system? Eur J Biochem 222:235–246

Chapter 15
Adhesion of Bacteria to Protists

Renate Radek

15.1 Introduction

Many unicellular eukaryotes (protists, protozoa) are associated with ecto- and/or endobiotic prokaryotes (Ball 1969; Buck and Bernhard 2001; Görtz 2006; Jeon 2006). Their relationship may be parasitic, commensalistic or mutualistic in nature; the purpose of the relationship is often unknown. Endobiotic micro-organisms can be found in virtually any compartment of their host cell, i.e., the cytoplasm (free or enclosed in a vacuole), endoplasmic reticulum, nuclear envelope, or the karyoplasm. To date, only once has a bacterium been reported to infect a mitochondrion of a protist (Fokin et al. 2003). Intracellular DNA-containing, membrane-bounded bodies or organelles are also called xenosomes (alien bodies; Corliss 1985). The present report focuses on the interaction of prokaryotes with the surface of protistan host cells, i.e., ecto- or epibionts or epixenosomes. Only stable associations typical of the respective species are included. Sometimes the whole surface of the host cell is covered, while in other cases the ectobionts are limited to certain areas of the host body. The prokaryotes are usually attached directly to the plasma membrane of their host. Occasionally, the bacteria only interact with extracellular material such as algal cell walls or gelatinous slime layers. Settlements by relatively unspecific biofilm-forming bacteria are not included, such as an increased coating of, e.g., amoeboid shells or ciliate cysts in older cultures.

I will present examples of prokaryotic associations with different protist groups. For reasons of clarity I will not follow the phylogenetic systematics of protists but instead group together the traditional assemblages of flagellated, amoeboid and ciliated protists. The spore-forming parasitic taxa (e.g., Apicomplexa, Microsporidia) are not further considered here, as they seem to be only very rarely associated with

R. Radek
Institute of Biology/Zoology, Protozoology, Free University of Berlin, Königin-Luise-Str. 1–3, Berlin 14195, Germany
e-mail: rradek@zedat.fu-berlin.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_15,

ectobionts. Reasons for this may be their mostly intracellular way of life and the physiological adaptations and exploitations of the host cell making symbiotic prokaryotes inessential. One example of an existing association is the gregarine *Porospora portunidarum* which lives in the intestine of the crab *Carcinus mediterraneus*. *Porospora* carries bacterial rods between its pellicular folds (Desportes et al. 1977).

It has not yet been possible to culture ectobiotic protistan bacteria, and therefore the identity of such ectobionts and the physiological basis of their associations are mostly unknown. However indirect approaches such as electron microscopy, the analysis of key enzymes and storage products, and the use of radioactive and stable isotopes as tracers can provide some insight. In recent years, information has been obtained with the help of PCR, using few or even a single isolated host cell with its attached partners. This enables a molecular identification of these uncultivable micro-organisms, and assumptions as to their metabolic functions can be made by comparison with sequences, e.g., coding for special enzymes. So far, mainly members of the Bacteria and, more rarely, methanogenic Archaea have been identified as ectobionts. Most effort was spent investigating the bacterial association of gut flagellates of lower termites and ciliates of micro-oxic or anoxic environments.

15.2 Ectobionts of Ciliates

The association of ciliates with ectobiotic bacteria is relatively common, especially in anoxic or micro-aerophilic environments such as guts or sediments. Recent reviews dealing with ectobionts of ciliates have been published by, e.g., Rosati (2001) and Görtz (2006).

15.2.1 Host-Associated Ciliates with Focus on Rumen Ciliates

The rumen is the most developed part of the digestive apparatus of ruminant mammals. It is a natural anaerobic fermenter in which up to 60% of the food is degraded by an abundant and diverse microbial fauna, such as bacteria, archaea, fungi and ciliates (Bohatier et al. 1990). The rumen ciliates possess hydrogenosomes instead of mitochondria and thus release H_2 and CO_2 as waste products. This is the crucial event leading to the attachment of prokaryotes.

Methanogenic bacteria (Archaea) need these substances for drawing energy from methane production. Therefore, they tend to adhere to the surface of the ciliates in order to obtain H_2 (Fig. 15.1). The free hydrogen concentration in the rumen fluid is high in a well-fed ruminant, since H_2 is also released by many hydrogenic bacteria and fungi (Bryant 1970; Stumm and Hackstein 1995; Wolin 1974). Thus the methanogens can easily acquire H_2 from the gut fluid. Under this condition, for example, only 27% of sheep ciliates were associated with ectobiotic

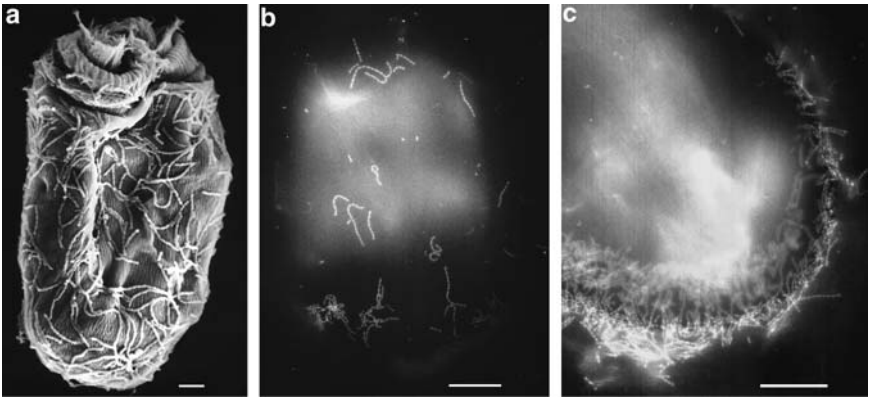

Fig. 15.1 (**a**) Rumen ciliate with attached chains of methanogenic bacteria; scanning electron microscopy (SEM). *Bar*: 10 µm. (Courtesy of Eugene B. Small, Maryland, USA). (**b**) and (**c**) Methanogenic bacteria showing autofluorescence under UV irradiation attached to a rumen ciliate cell from a well-fed sheep (**b**, few methanogens) and from a hungry sheep under unfavorable conditions (**c**, numerous methanogens), *Bars* (**b**) and (**c**): 20 µm. (Courtesy of Claudius K. Stumm, Nijmegen, Netherlands)

methanogenic bacteria (Stumm et al. 1982; Stumm and Zwart 1986). However, the free H_2 concentration is low in a hungry sheep. The ciliates still find some food and produce H_2, and therefore methanogenic bacteria approach these ciliates. Under these circumstances, nearly 70% of the ciliates are associated with ectobiotic methanogens (Stumm et al. 1982; Stumm and Zwart 1986). Apart from the frequency of association, the number of attached Archaea varies depending on the feeding status of the host animal. The number of associated methanogens is low in well-fed animals, whereas the ciliate surfaces were almost completely covered with long chains of methanogens under unfavorable conditions (Fig. 15.1b, c; Gijzen et al. 1985). Both the ciliates and the methanogenic bacteria seem to profit from the interspecies hydrogen transfer (hydrogen is inhibitory to the ciliate metabolism) (Vogels et al. 1980). The mode of the quick attachment and release of the methanogens to the ciliate surface is unknown. In vitro experiments with sheep rumen fluid demonstrated that up to 25% of methanogenesis depends on methanogens associated with rumen ciliates (Newbold et al. 1995). In addition, Tokura et al. (1997) showed the methane production of ciliates to be dependent on the number of extracellularly associated methanogens.

Methanogenic bacteria can easily be recognized using ultraviolet irradiation under the fluorescence microscope by the autofluorescence of their characteristic coenzyme F_{420} (Fig. 15.1b, c; Doddema and Vogels 1978). Analyses of the 16S rRNA sequences identified the most abundant population of attached methanogens as Methanobacteriaceae, e.g., *Methanobrevibacter ruminantium* (Sharp et al. 1998; Whitford et al. 2001). In addition to methanogens, other bacterial species have been described as adhering to rumen ciliates, e.g., *Streptococcus bovis* and *Ruminococcus albus* (Imai and Ogimoto 1978).

Two major taxa of ciliates are found in rumens, the entodiniomorphids and the isotrichids (Ogimoto and Imai 1981; Williams and Coleman 1992). Only the entodiniomorphs associate with ectobionts, e.g., the genera *Diplodinium*, *Entodinium*, *Epidinium*, *Eudiplodinium*, *Ostracodinium* and *Polyplastron* (Vogels et al. 1980). It is possible that the dense coat of cilia in the holotrichous genera of the isotrichids does not allow bacterial settlement (Stumm and Hackstein 1995).

Reports of ciliate–ectobacteria consortia from other animals are scarce. The mobiline peritrich *Trichodinopsis* from a terrestrial prosobranch mollusc is covered with attached spirochaetes (Corliss 1979) which may support locomotion of their host. *Trichodina oviducti* from the mucoid exudates from the copulatory sac of female thorny rays sometimes carry numerous nonmotile rod-like bacteria (Khan et al. 1974).

15.2.2 Anaerobic and Micro-Aerophilic Ciliates from Marine Sediments and Other Sulfide-Rich Habitats

Ectobiotic bacteria living on sand-dwelling ciliates in anoxic habitats have been known for many years (Sauerbrey 1928; Kahl 1933; Kahl 1935; Fauré-Fremiet 1950, 1951). The oxygen gradient in the sediment controls the vertical distribution of communities of aerobic, micro-aerophilic ($P_{O_2} < 2\%$) and strictly anaerobic species (Fenchel 1992). A broad diversity of anaerobic and micro-aerophilic ciliates exist, most of which have a fermentative metabolism (Esteban and Finlay 1994). Many of them harbor ecto- and/or endobiotic bacteria, the endosymbiotic ones being generally methanogens (Fenchel and Finlay 1992). Ectobiotic bacteria occur almost exclusively in marine anaerobic ciliates, and are especially conspicuous in species which do not have endobiotic methanogens (Fenchel and Ramsing 1992). The latter may suggest a competition with intracellular methanogens for common substrates (Fenchel and Finlay 1995). The diverse morphology of the ectobionts suggests that they are host-specific.

A sulfur oxidation-based autotroph energy metabolism has been confirmed for several epibionts of ciliates (Polz et al. 2000). This means that carbon is derived autotrophically, and that the necessary energy is obtained through sulfide oxidation. The problem is that in general sulfide and oxygen are mutually exclusive. They mix only at the transition zone of oxic and anoxic environments. Because this zone is variable in both time and space, free-living sulfide bacteria cannot easily find optimal conditions for growth. Ectobiotic sulfur-oxidizing bacteria have the great advantage that their agile and large host quickly brings them into favorable habitats or creates water currents which pump sulfide- and oxygen-containing water towards them. Their ability to autotrophically oxidize sulfur can be shown by different methods. The presence of stored elemental sulfur is a hint. For example, the ectobionts of *Zoothamnium niveum* (Fig. 15.2) possess elemental sulfur inclusions as an intermediary storage product (Bauer-Nebelsick et al. 1996b). The detection

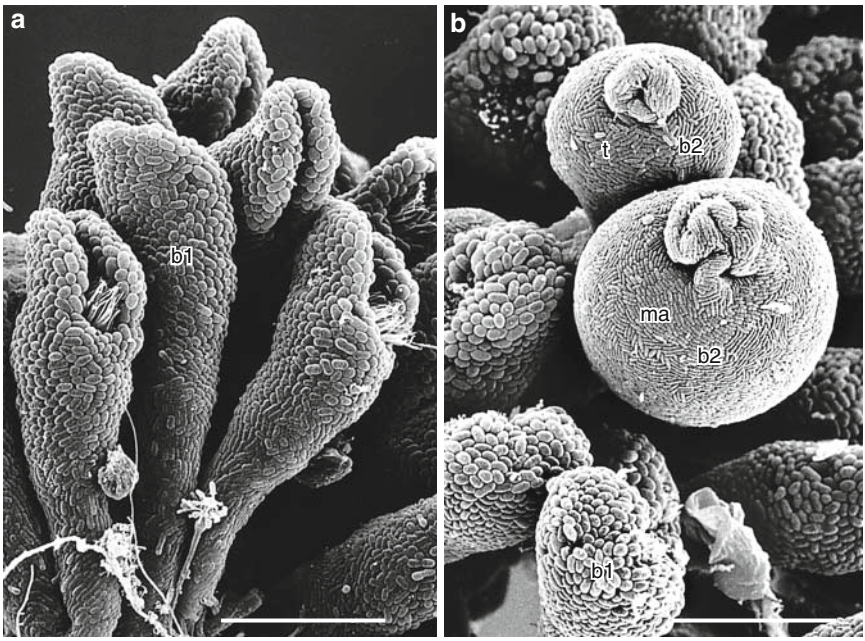

Fig. 15.2 The colonial, stalked ciliate *Zoothamnium niveum*. (**a**) Microzooids with morphotype 1 bacteria (b1); SEM. (**b**) Macrozooid (ma) and terminal zooid (t) with morphotype 2 bacteria (b2), b1 morphotype 1 bacteria; SEM. *Bars*: (**a**) and (**b**) 20 µm. (From Bauer-Nebelsick et al. 1996a)

of diagnostic enzymes is another possibility. The enzyme RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase), which is involved in the fixation of carbon dioxide for use in the Calvin Benson metabolic cycle, has been proved to be present in *Z. niveum*. In addition, the presence of the genetic code for specific enzymes such as RuBisCO, APS reductase, and the dissimilatory sulfide reductase reveals the physiological capabilities (e.g., in *Z. niveum;* Rinke 2007).

Several species of *Zoothamnium* have been reported to possess ectobiotic bacteria on their nonciliated surface areas, e.g., *Z. alternans* (Fauré-Fremiet et al. 1963), *Z. balticum* (Biernacka 1963), *Z. pelagicum* (Dragesco 1948; Hausmann and Walz 2007; Laval 1968), and *Z. penaei* (Song 1992). In sulfidic waters *Z. entzi, Z. niveum, Z. perlatum, Z. thiophilum and Z. urceolatum* carried ectobacteria (Bauer-Nebelsick et al. 1996a; Stiller 1946). The symbiotic system of *Z. niveum* has been studied in much detail. The sessile, feather-like colonies of *Zoothamnium niveum* are preferentially found in tidal channels cut into mangrove peat. At sites of high sulfide flux, they colonize areas where the microbial surface mat has been disturbed in great numbers, and disappear when the microbe mat has re-established itself after about 3 weeks (Ott et al. 1998). The surface of *Z. niveum* is densely covered by ectobionts (Fig. 15.2; Bauer-Nebelsick et al. 1996a, b). Special finely structural features of the ciliates' pellicle enabling attachment of bacteria were not recognized (Bauer-Nebelsick et al. 1996b). Bacteria on the microzooids are generally coccoid

and slightly dumbbell-shaped, whereas rod-shaped bacteria cover the macrozooids, terminal zooids and the stalk (Fig. 15.2a, b; Bauer-Nebelsick et al. 1996a). However, a gradual transition in cell shape from cocci to rods occurs, especially in the aboral parts of the microzooids. Microzooids with a mixture of both morphotypes have also been found. Recent 16S rRNA gene sequence analysis and fluorescent in situ hybridization has shown that all forms belong to one pleomorphic phylotype (Rinke et al. 2006). It is only moderately related to known symbiotic and free-living bacteria within the Gammaproteobacteria. The conspicuous white color of the colonies and inclusions seen in ultrathin sections indicates the presence of sulfur in the bacteria (Fig. 15.2c). Culture experiments demonstrated that *Zoothamnium* only survives when oxygen and sulfide are offered simultaneously, and when the ectobacteria are present (Bauer-Nebelsick et al. 1996a; Rinke et al. 2007; Vopel et al. 2001). Thus, the bacteria are obligate symbiotic sulfide oxidizers. Based on the ultrastructure, culture experiments, 16S rRNA gene, intergenic spacer region and partial 23S rRNA gene sequence, the name "*Candidatus* Thiobios zoothamnicoli" was proposed for the ectobionts (Rinke et al. 2006). A special behavior of the colonies enables acquisition of oxygen as well as sulfide: the colonies alternately expand and contract (Ott et al. 1998). They expand up to a height of 15 mm and thus extend into the oxic zone (with little or no sulfide content), but measure less than 1 mm in the contracted state. The contracted colony thus extends itself into the anoxic, sulfidic region (of about 250 µmol/L sulfide). The intervals between contractions generally range from 5 to 30 s, with a much shorter contracted phase (<1 s). During the slow expansion, sulfidic water adheres to the surface of the colony due to viscous forces, thus prolonging the time for uptake of sulfide (Ott et al. 1998). Oxic and anoxic (H_2S-containing) water are mixed by the contraction/expansion cycles and by the feeding currents, helping to supply the chemoautotrophic sulfide bacteria with both O_2 and H_2S (Vopel et al. 2001, 2005). Furthermore, a simultaneous venting of groups of *Z. niveum* colonies with sulfide-rich seawater driven by pulsating boundary-layer currents could be demonstrated (Vopel et al. 2005). The fast contraction (520 mm/s) of the colonies seems to serve another purpose as well; the shear stress caused by the high fluid velocity around the zooids, the shrinkage and bunching of the zooids leads to detachment of bacteria (Vopel et al. 2002). Once suspended, the former ectobionts may enter the feeding currents of the ciliates and are then ingested and digested. In addition, organic compounds of low molecular weight produced by the symbionts seem to be used for nutrition (Rinke et al. 2003).

Further examples of ciliates with ectobionts from anaerobic sulfide-containing sediments are *Parablepharisma pellitum, P. chlamydopherum, Metopus contortus*, *M. vestitus, Caenomorpha capucina*, *Sonderia vorax,* and *Myelostoma bipartitum* (Bernhard et al. 2000; Fenchel et al. 1977). The distribution and numbers of the bacteria differ in the host species. It seems that some of the sulfide ciliates (e.g., *Sonderia*) secrete mucus that may be involved in bacterial attachment (Fenchel et al. 1977). Using 16S rRNA binding oligonucleotide probes, Fenchel and Ramsing (1992) proved the ectobionts of *Metopus contortus* and *Caenomorpha levanderi* to be sulfate reducers belonging to the δ-group of the purple bacteria.

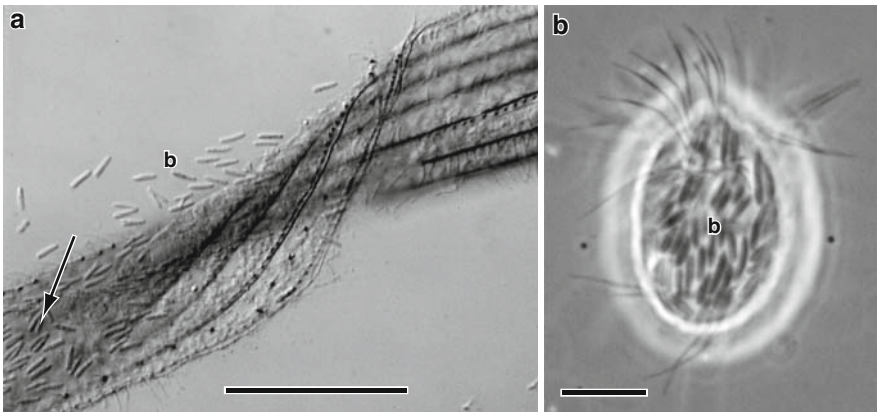

Fig. 15.3 (**a**) The flat, micro-aerophilic ciliate *Kentrophoros* with sulfide-oxidizing rod-shaped bacteria (b) on its dorsal surface; silver impregnation under differential interference contrast. *Bar*: 50 µm. (Courtesy of Genoveva Esteban, London, UK). (**b**) Ectobiotic bacteria (b) attached to the scuticociliate *Paracyclidium* sp. from a salt marsh. *Bar*: 10 µm. (Copyright by David Patterson, Linda Amaral-Zettler and Virginia Edgcomb, micro*scope internet page)

The micro-aerophile genus *Kentrophoros* is remarkable in that it has no oral apparatus. It feeds by phagocytizing part of the sulfide-oxidizing, rod-shaped bacteria that densely cover its dorsal surface (Fig. 15.3a); it has its own microbial kitchen garden (Fauré-Fremiet 1950; Fenchel and Finlay 1989; Finlay and Fenchel 1989). Pseudopodia-like cytoplasmic protrusions are involved in the uptake of the ectobionts (Raikov 1971, 1974). Newly formed food vacuoles containing single bacteria fuse later to form large digestion vacuoles (Fenchel and Finlay 1989). The bacteria are attached to the ciliate surface by a cell pole and are embedded in a thick mucous layer produced by the ciliate (Foissner 1995). They divide lengthwise, enabling both arising cells to keep contact with their host. *Kentrophoros* moves vertically in the sediment so that is it always positioned at the interface between oxidized and anaerobic sulfide-reducing layers (Fenchel and Finlay 1995). *Parablepharisma* is able to phagocytose its ectobacteria, although it has a functioning mouth (Finlay and Fenchel 1989). The bacterial rods of *Parablepharisma* are inserted in pits in the cell membrane of the ciliate.

Some species of scuticociliates from anoxic marine sediments have been reported to carry a layer of ectobiotic bacteria, i.e., *Cristigera vestita* and *C. cirrifera* (Fenchel and Finlay 1991), *Cyclidium* (Epstein et al. 1998) and *Paracyclidium* (Fig. 15.3b; micro*scope internet page). Further genera of sediment ciliates with ectobionts were described in the survey of Epstein et al. (1998): The litostomatids *Loxophyllum* and *Paraspathidium,* and the karyorelictids *Tracheloraphis* and *Geleia*. All nine of the examined species mentioned in this survey had bacteria on their surface. 2,000–10,000 bacteria per cell were arranged in or along the ciliated grooves, uniformly distributed over the entire length. In *Geleia fossata*, the ectobionts were situated in deep invaginations of the ciliate's cell surface at an angle of

about 45°. The only known folliculinid ciliate with ectobionts is *Folliculinopsis* sp., known from a deep-sea hydrothermal vent (Kouris et al. 2007). It forms dense carpets of blue colored mats adjacent to hydrothermal venting. Irregularly scattered filamentous and coccoid bacteria colonized the lorica (chitinous sheath), while greater densities of short rods and cocci settle between the ciliary rows, especially at the peristomal wings. The presence of bacteria on the lorica seems to be a case of biofouling; the regularly distributed bacteria on the cell surface, however, are symbionts (Kouris et al. 2007).

Although anoxia combined with high sulfide levels is seldom found in fresh water, this condition is found in the sulfate-rich solution lake Arcas-2 (near Arcas in central Spain), where anaerobic ciliates are found in the bottom water (Finlay et al. 1991). Two scuticociliates with a bacterial coat have been recorded, i.e., *Cristigera* sp. (Esteban et al. 1993; Finlay et al. 1991) and *Isocyclidium globosum* (Esteban and Finlay 1994). *I. globosum* was isolated from different freshwater locations from Spain and England but carried ectobionts only when the ciliates were obtained from sulfate-rich anoxic water, suggesting that they may be sulfate reducers (Esteban and Finlay 1994). *Caenomorpha medusula* from lake Arcas-2 was also shown to possess ectobionts. They are located only on its ventral side (Finlay et al. 1991).

15.2.3 Free-Living Aerobic Ciliates

Extracellular structures of free-living ciliates may be settled by random bacteria, such as the lorica of the large, trumpet-shaped ciliate *Stentor muelleri* (micro*scope internet page). The colonial peritrich ciliate *Epistylis* may attach to the skin of fish by its stalk (Hazen et al. 1978). *Epistylis* was suspected to be the aetiological agent of the deadly red-sore disease of fish, but a secondary infection by the bacterium *Aeromonas hydrophila* has since been shown to be responsible. These bacteria also colonize the extracellular sheath of the stalks (not the zooid) of *Epistylis* (Hazen et al. 1978). In an Australian population of the litonotid ciliate *Acineria uncinata*, the pellicle was thickly covered with rod-like bacteria particularly in the posterior part of the body (micro*scope internet page). Bacterial rods attach to the surface of the scuticociliate *Cyclidium* from the body of a sea urchin (Beams and Kessel 1973). The ultrastructure of the pellicle is changed at the attachment sites, e.g., a dense substance is present in the alveoli, and flattened mitochondria support the alveoli close to the attached bacteria.

15.2.4 Defensive Extrusive Ectobionts of Euplotidium

Euplotidium species are marine, sand-dwelling hypotrich ciliates. On their dorsal surface there are five rows of short cilia, and in a depression along both sides and at the anterior end lies a band of ectobiotic bacteria or epixenosomes (Fig. 15.4a;

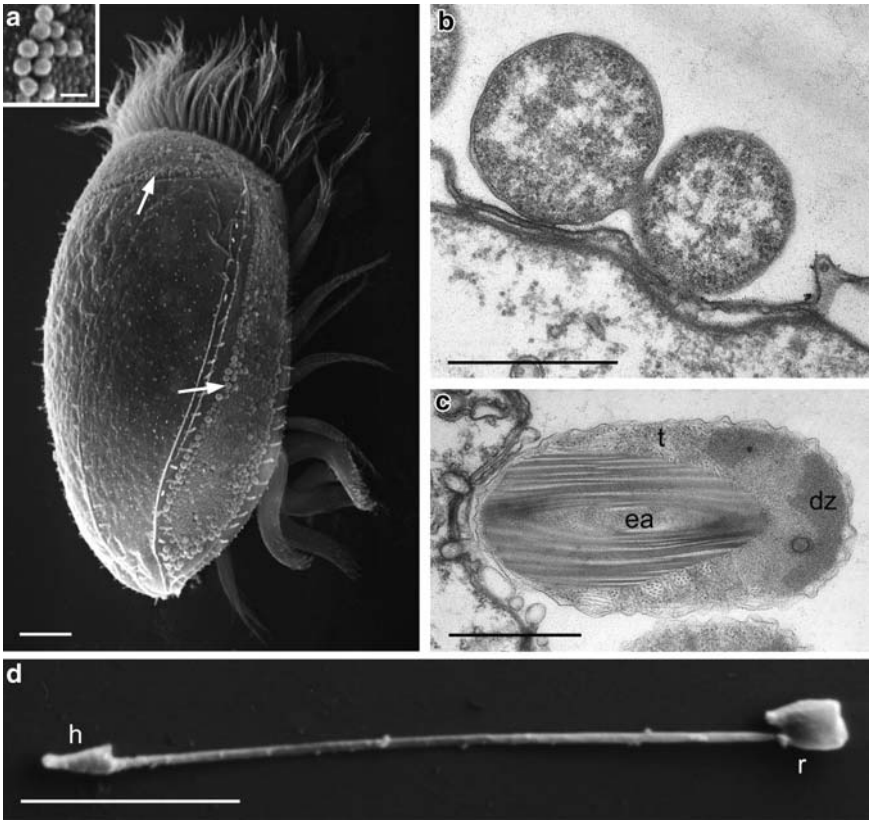

Fig. 15.4 The ciliate *Euplotidium itoi* and its epixenosomes. (**a**) SEM showing ectobacteria in the cortical band of the dorsal surface (arrows). *Inset*: Epixenosomes at higher magnification. (**b**) Stage I epixenosomes; transmission electron micrograph (TEM). (**c**) Stage II epixenosome with extrusive apparatus (ea), apical dome shaped zone (dz), and microtubule-like elements (t); TEM. (**d**) Tube at the end of injection with head (h) and rest of epixenosome (r); SEM. *Bars*: (**a**) and (**d**) 10 μm, (**a**) inset 2 μm, (**b**) and (**c**) 1 μm. ((**a**) and (**b**) from Verni and Rosati 1990, (**c**) from Rosati 2006, (**d**) from Rosati et al. 1996)

Verni and Rosati 1990; Rosati 2006). The ectobionts are located in indentations of the host cortex which exactly match their size (Verni and Rosati 1990). Giovanna Rosati et al. (1999) studied these bacteria extensively. In former descriptions of *Euplotidium* they were interpreted as being extrusomes (extrusive organelles; Ito 1958; Tuffrau 1985). Tuffrau (1985) also described such "extrusomes" from the genus *Gastrocyrrus*; they may also represent epixenosomes. Only *E. itoi* and *E. arenarium* have been studied electron microscopically, a method through which the localization and the bacterial nature of the structures could be recognized (Fig. 15.4). The epixenosomes are always on the exterior of the ciliates' cortex. DAPI staining proved them to contain DNA, i.e., showed their cellular nature (Verni and Rosati 1990). Sequence analysis of the 16S rRNA and in situ

hybridization revealed that they are bacteria phylogenetically related to *Verrumicrobia* (Petroni et al. 2000). In previous in situ hybridization experiments the bacterial probe EUB338 did not react with the ectobacteria (Rosati 1999). The explanation seems to be the exchange of two bases in *Verrumicrobia* DNA (Daims et al. 1999) that are known to prevent binding with the probe EUB338 (Rosati 2006). Two morphological forms of the epixenosomes exist: form I is spherical, able to divide and mainly localized in the central region of the epixenosomal band (Fig. 15.4b). Form II is egg-shaped, unable to divide, mainly localized in the peripheral regions of the band, and has a highly complex organization (Fig. 15.4c). In its cytoplasm are four well-defined structures: a dense apical dome-shaped zone, an inclusion body, an extrusive apparatus, and a basket of tubules. Form I transforms to form II. During this process the complex structures are gradually acquired (Rosati et al. 1993a). First, lamellar material that belongs to the extrusive apparatus appears. The concentric layers increase in number and become tightly wound around a central core. Then randomly dispersed tubule-like structures with a diameter of 20–24 nm are seen; they are later ordered in bundles. Dense material accumulates apically. Some enzymes could be localized in different regions of the cell, i.e., acid phosphatase between the two cell membranes, and endogenous peroxidase in the inclusion body and at the top of the extrusive apparatus (Rosati et al. 1996); there is thus a functional cell compartmentalization. In addition to peroxidase, the inclusion body seems to contain (presumably storage) polysaccharides. The tubules seem to be identical to microtubules, as demonstrated by antibody staining and their sensitivity to antitubulin drugs and cold (Rosati et al. 1993b). Furthermore, tubulin-like genes were found in the related free-living verrucomicrobial genus *Prosthecobacter* (Jenkins et al. 2002, Pilhofer et al. 2007). The existence of such genes in the ectobionts of *Euplotidium* is under investigation. The extrusive apparatus resembles R-bodies described in several genera of eubacteria. R-bodies are cylindrical structures of coiled, highly insoluble protein ribbons. The extrusive apparatus of epixenosomes has an even more complex structure (Rosati et al 1993a, 1996). During the ejection, a tube is formed by unrolling of the wound layers of the extrusive apparatus. The elongating tube penetrates through a hole in the dome-shaped zone and carries away DNA and proteins of this zone. The extended tube is about 40 μm long and has a spear-shaped head consisting mainly of the genetic material (Fig. 15.4d). Natural discharge occurs during disturbances in the vicinity of the cells. The signals for activation were investigated by Rosati et al. 1997. Membrane receptors at the top of the ectobiont detect an external signal. This leads to an activation of the adenylate–cyclase–cAMP system triggering the discharge. The functions of the ejection are still under discussion, but at least a defense against predators such as *Litonotus lamella* could be shown (Rosati et al. 1999; Rosati 2006). *Litonotus* never ingests *Euplotidium* with intact epixenosomes but feeds well on stocks without epixenosomes. Several facts indicate that the association of *Euplotidium itoi* with its peculiar ectobionts is obligate: all ciliates in nature and in well-fed lab cultures have ectobionts, and the host cell cycle is coordinated with their multiplication and differentiation from form I to II (Giambelluca and Rosati 1996). However, experimentally depleted ciliates can

survive, i.e., the ectobionts are not vital (at least for survival in the lab). In contrast, isolated epibionts could not be cultured in artificial media. An obligate symbiosis for the bacteria is also indicated by the presence of only one ribosomal operon with unlinked 16S and 23S rRNA genes, which is typical of a reduced efficiency of ribosome synthesis (Rosati 2006).

15.3 Ectobionts of Amoeboid Protozoa

Little information exists on interactions of amoeboid species with extracellular bacteria. Generally, the bacterial symbionts of amoeba are intracellular (Jeon 2006). Possibly the flexible, rapidly changing surface areas and the ability of phagocytosis nearly everywhere on the surface are responsible for this. Cyst walls and tests may be accidentally colonized by bacteria, as was described for, e.g., the cysts of the heliozoon *Echinosphaerium nucleofilum* (Patterson and Thompson 1981). Light and electron microscopic images of the cochliopodid amoeba *Gocevia fonbrunei* show numerous bacteria enclosed in a mucus layer (in French, "cuticle") (Pussard 1965; Pussard et al. 1977). Also the trophic stages of *Endostelium amerosporum* and *E. zonatum* always possess bacteria associated with the cell coat (Bennett 1986). A direct interaction with the cell surface occurs, for example, in the pelobiont *Mastigamoeba aspersa* (syn. *Dinamoeba mirabilis*) (Fig. 15.5; Simpson et al. 1997). In addition to numerous pseudopods, *Mastigamoeba* cells mostly possess a single, slow-beating anterior flagellum. The conical, hyaline pseudopods of *M. aspersa* are covered with many thin rods, giving them a somewhat hairy appearance (Page 1970). The rods are embedded in the thin, multilayered glycocalyx of their host (Frolov et al. 2005). When a pseudopod is formed, some of the adherent rods are carried along its surface; only the tip is usually free of them (Page 1970). Adherent bacteria were also found surrounding the cell body of the filose amoeba *Nuclearia delicatula* (Cann 1986). They are embedded in a mucilaginous glycocalyx. The only foraminiferan with ectobionts known to date is the

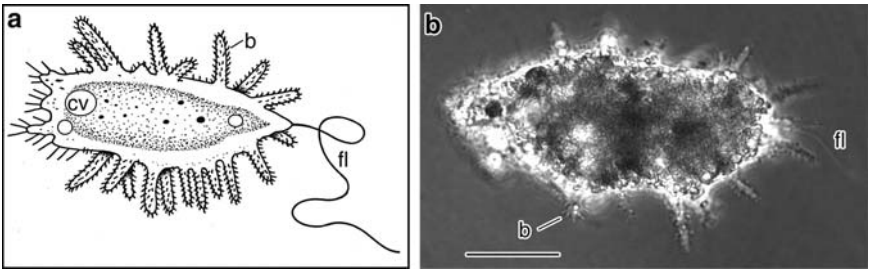

Fig. 15.5 *Mastigamoeba aspersa*. Drawing and phase contrast image depicting the anterior flagellum (fl) and pseudopodia with attached bacterial rods (b); cv, contractile vacuole. *Bar*: 20 μm. ((**a**) after micro*scope internet page; (**b**) courtesy of Josef Brief, Frankfurt, Germany)

benthic *Uvigerina peregrine* from a Monterey Bay cold seep (Bernhard et al. 2001). It harbors rod-shaped bacteria in its pores, typically just above the pore plug.

15.4 Ectobionts of Flagellated Protozoa

Many bacteria/flagellate associations had already been found by light microscopy early in the twentieth century (Jeon 2006). Most of them were ectobionts of termite flagellates. The bacterial nature of presumed endobionts was not confirmed until new techniques such as electron microscopy and advanced biochemical tests arose in the late 1950s (Jeon 2006). In the following, free-living and host-associated flagellates (particularly termite flagellates) associated with ectobiotic bacteria are presented.

15.4.1 Free-Living Flagellated Protists

There do not seem to be any major taxa of free-living flagellates regularly associated with attached bacteria. Instead there are single reports of different flagellate species. Often the bacteria are not directly attached to the plasma membrane of their host cell but to extracellular substances covering the surface; the specificity of the interactions is thus questionable. For example, there are some photoautotrophic flagellates bearing ectobionts. The chlorophycean *Metapolytoma bactiferum* living in freshwater habitats rich in sulfur generally possesses numerous bacteria on its surface (Skuja 1958). Resting cells are especially densely covered. The colonial chlorophycean *Volvox aureus* needs rod-like *Pseudomonas fluorescens* cells associated with the mucilage of the mother colony for continuous growth (Hamburger 1957). Bacteria-free daughter colonies removed from the mother cell under sterile conditions stopped developing after only a few generations; their bacteria seem to be obligate symbionts. The mucilaginous sheath of the colonial chrysomonad *Chrysostephanosphaera globulifera* regularly contains bacteria (Geitler 1948). However, since these globular bacteria are also phagocytosed and digested by the chrysomonads, their symbiotic role is unclear. Bacteria on loricas are found, e.g., in the chrysomonad *Lepochromulina* and the euglenoid *Trachelomonas* (Geitler 1948; Rosowski and Langenberg 1994).

The prokaryotes described in the following are in direct contact with the host's cell membrane. Crescent-shaped bacteria were found on the surface of the colorless soil-dwelling chrysomonad *Spumella elongata* (Belcher and Swale 1976). A free-living photosynthetic cryptomonad of the genus *Cryptomonas* was described as having a dense carpet of *Caulobacter* bacteria (Klaveness 1982). *Caulobacter* cells are attached to a transversal groove of their host cell by the polar appendage. It was possible to culture bacteria-free *Cryptomonas* clones without any negative effect on the flagellate. The only visible change was a loss of the transversal constriction so

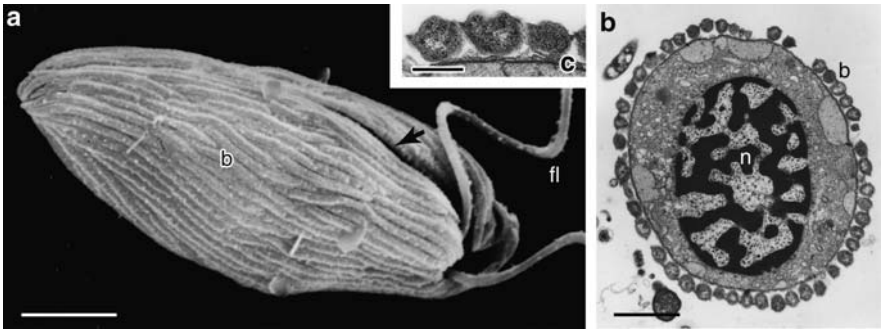

Fig. 15.6 Numerous rod-like epibiotic bacteria are laterally attached to the surface of the euglenozoan *Postgaardi mariagerensis*. (**a**) SEM; *arrow* points to lipped depression including flagellar pocket; b, bacteria; fl, flagellum. (**b**) and (**c**) Ultra-thin cross-sections; n, nucleus. The ectobionts possess wing-like projections. *Bars*: (**a**) and (**b**) 2 μm, (**c**) 0.5 μm. (From Simpson et al. 1996/1997)

that the cryptomonad assumed the characteristic shape of *Cryptomonas curvata*. This change of cell form brought about by bacterial ectobionts may challenge the descriptions of new species based on their anomalous cell shape.

Some habitats seem to favor the establishment of symbioses. The *Beggiatoa* mats of the dysoxic and sulfidic sediments of the Santa Barbara Basin (California, USA) contain abundant protists with prokaryotic ecto- and endosymbionts (Bernhard et al. 2000). For example, anaerobic euglenozoans such as *Postgaardi mariagerensis* (Fig. 15.6) and *Calkinsia aureus* and further undescribed species carry numerous ectobiotic bacteria (Bernhard et al. 2000; Simpson et al. 1996/1997). Diverse metabolic relationships of the bacteria/protist association are suggested, for example sulfate reduction or sulfide-oxidizing capabilities of the bacteria. Three morphotypes of euglenozoans associated with ectobacteria were reported to be found in sulfidic cold seeps in Monterey Bay (Buck et al. 2000). In all of them (one seems to be *Postgaardi*) the surface was completely covered by bacterial rods attached by their sides. As many as 68% of the euglenozoans reported from this site had ectobionts. One of the euglenozoan morphotypes possessed ectobacteria that somehow approached their neighboring bacterium with short processes, as is also the case for the ectobionts of *Postgaardi mariagerensis* (Fig. 15.6b, c; Simpson et al. 1996/1997). These bacteria contained electron-lucent vacuoles with a multilayered membrane reminiscent of sulfur vesicles, suggesting that the bacteria are sulfide oxidizers (Buck et al. 2000). The ectobionts of the euglenozoans do not seem to be ingested by their host. Two species of the colorless euglenid genus *Dylakosoma* are known to have ectobionts. *Dylakosoma symbionticum* (syn. *Petalomonas symbiontica*) is densely covered by rod-like bacteria (Tschermak-Woess 1950). Phagocytosed bacteria are only seldomly seen. *D. pelophilum* is covered by coccoid bacteria (Wołowski 1995). Phototrophic euglenids normally lack ectobionts, however the photoautotrophic freshwater species *Euglena helicoideus* possesses long, rod-shaped bacteria (Leander and Farmer 2000). These bacteria fit tightly within the minor grooves of the pellicle. The minor grooves lie

within a proteinaceous strip and are thus not distorted during euglenoid movement, i.e., the attachment sites lie in a stable region. Furthermore, the areas between the large keels of the pellicle are predestined for any excretion of the host cell such as muciferous secretions, possibly giving the ectobionts easy access to nutrients (Leander and Farmer 2000). Some non-photosynthetic genera of marine dinoflagellates were reported to contain symbiotic ecto- or endobiotic cyanobacteria (Foster et al. 2006).

15.4.2 Further Host-Associated Flagellated Protists

An example for a parasitic flagellate that carries ectobiotic bacteria is the kinetoplastid *Cryptobia vaginalis*, which infects the vagina of leeches (Vickerman 1977), but only some of the individuals were shown to have elongate bacteria here. They are attached to the body in parallel with the cortical microtubules and are most abundant at the flagellates' posterior end. The opalinid flagellate *Protoopalina pomacantha* lives in the hindgut chamber of angelfishes (Grim et al. 2000). Ectosymbionts have been observed in numerous specimens of *P. pomacantha* from different specimens of two angelfish species. They are attached between the flagella of a kinety and lie within shallow pits on the surface.

15.4.3 Gut Flagellates of Termites and Wood-Feeding Cockroaches

Flagellate genera with ectobiotic associations: The numerous oxymonad and parabasalid flagellates inhabiting the hindgut of lower termites and wood-feeding cockroaches (*Cryptocercus*) are involved in the their host's cellulose digestion (Brune and Stingl 2006; O'Brien and Slaytor 1982; Radek 1999). Generally, these flagellates are associated with ecto- and/or endobiotic bacteria (Ball 1969; Bloodgood and Fitzharris 1976; Dolan 2001; Radek et al. 1992; Smith and Arnott 1974). Oxymonad genera carrying ectobionts include *Barroella, Dinenympha*, *Microrhopalodina*, *Oxymonas*, *Pyrsonympha*, *Sauromonas* and *Streblomastix* (see references in Yamin 1979). Parabasalid genera with ectobacteria include *Astronympha*, *Barbulanympha*, *Bullanympha*, *Caduceia*, *Calonympha*, *Deltotrichonympha*, *Devescovina*, *Eucomonympha*, *Evemonia*, *Foaina*, *Hexamastix*, *Holomastigotoides*, *Hyperdevescovina*, *Joenia*, *Kirbynia*, *Lophomonas*, *Macrotrichomonas*, *Metadevescovina*, *Microspironympha*, *Mixotricha*, *Parajoenia*, *Projoenia*, *Pseudodevescovina*, *Rostronympha*, *Snyderella*, *Spirotrichonympha*, *Spirotrichonymphella*, *Staurojoenina*, *Stephanonympha*, *Trichonympha* and *Urinympha* (see references in Yamin 1979). The bacteria occupy typical positions on their host cell so that these stable associations may be even part of the diagnosis and species descriptions of the flagellates (Dolan 2001). For example, of 177

trichomonad species, bacterial symbionts are included in the diagnosis of 56 species, and bacteria are mentioned in the descriptions of 18 further species.

The ectobiotic micro-organisms are either rod-like bacteria attached by a cell pole or by a side, or spirochaetes which are always attached by a pole (Bloodgood and Fitzharris 1976; Bloodgood et al. 1974; Hollande and Carruette-Valentin 1970; Kirby 1945; Smith and Arnott 1974; Tamm 1980; Maaß and Radek 2006). Long rods attached by a tip may be undulated, resembling spirochaetes (Hongoh et al. 2007a, b). Normally, only the nonflagellated regions of the surface are partly or totally colonized species-specifically. To date, spirochaetes have been found in a typical localization between the flagella only in the multiflagellate trichonymphids *Trichonympha* (Hongoh et al. 2007b) and *Eucomonympha imla* (Carpenter and Keeling 2007). Carpenter and Keeling (2007) showed that the spirochaetes are not directly attached to the cell body of *E. imla*.

Morphology of attachment sites and adhesion mechanisms: Mostly, the attachment sites at the flagellates' surface have special structures that are developed by the protist and/or the prokaryote. The junctional zones may be located in special indentations or on elevations (Bloodgood and Fitzharris 1976; Cleveland and Grimstone 1964; Grassé 1938; Tamm 1980). For example, rods which are attached to *Joenia annectens* via a pole lie in a circular indentation of the plasma membrane (Fig. 15.7a–c; Radek et al. 1992). The indentation is supported by electron-dense material. In other cases, rods lying parallel to the surface are situated on top of elongate ridges, e.g., in *Devescovina glabra* (Radek et al. 1996), *Hoplonympha natator* (Brugerolle and Bordereau 2004), *Staurojoenina* sp. (Fig. 15.7d–f; Dolan and Margulis 1997; Stingl et al. 2004) or *Streblomastix strix* (Leander and Keeling 2004). A certain amount of electron-dense material is often found supporting the flagellates' plasma membrane at the contact site, but sometimes there is no visible specialization at the attachment site (Radek et al. 1996). For example, in the trichomonad *Stephanonympha nelumbium,* bacterial rods are laterally attached only to the glycocalyx (which has a constant thickness of 0.2 μm) (Fig. 15.7g). In the oxymonad *Microrhopalodina multinucleata,* the ectobiotic rods do not even have direct access to the plasma membrane but are in contact with extracellular surface structures (Fig. 15.7h; Radek et al. 1996). Often, filamentous extracellular material of the glycocalyx bridges the small space between the partners. Sometimes the bacteria possess special attachment structures, such as the nose-like, teat-like or more complexly structured appendages of spirochaetes (Fig. 15.7i; Bloodgood and Fitzharris 1976; Radek et al. 1992; Wenzel et al. 2003). The spirochaetes attached to the oxymonad *Pyrsonympha vertens* have special internal structures in their attached tip, and a rootlet extends into the cytoplasm of the flagellate (Smith and Arnott 1974; Smith et al. 1975). It has been suggested that the particular partner which benefits from the association tends to form specialized attachment structures (Bloodgood and Fitzharris 1976). The association of the ectobionts to the surface is generally tight and specific, and even persists on disrupted fragments of the host membrane (Tamm 1980; Radek and Tischendorf 1999).

The nature of the interactions on the molecular level is poorly understood; only a few studies exist (Radek et al. 1992, 1996; Radek and Tischendorf 1999; Tamm

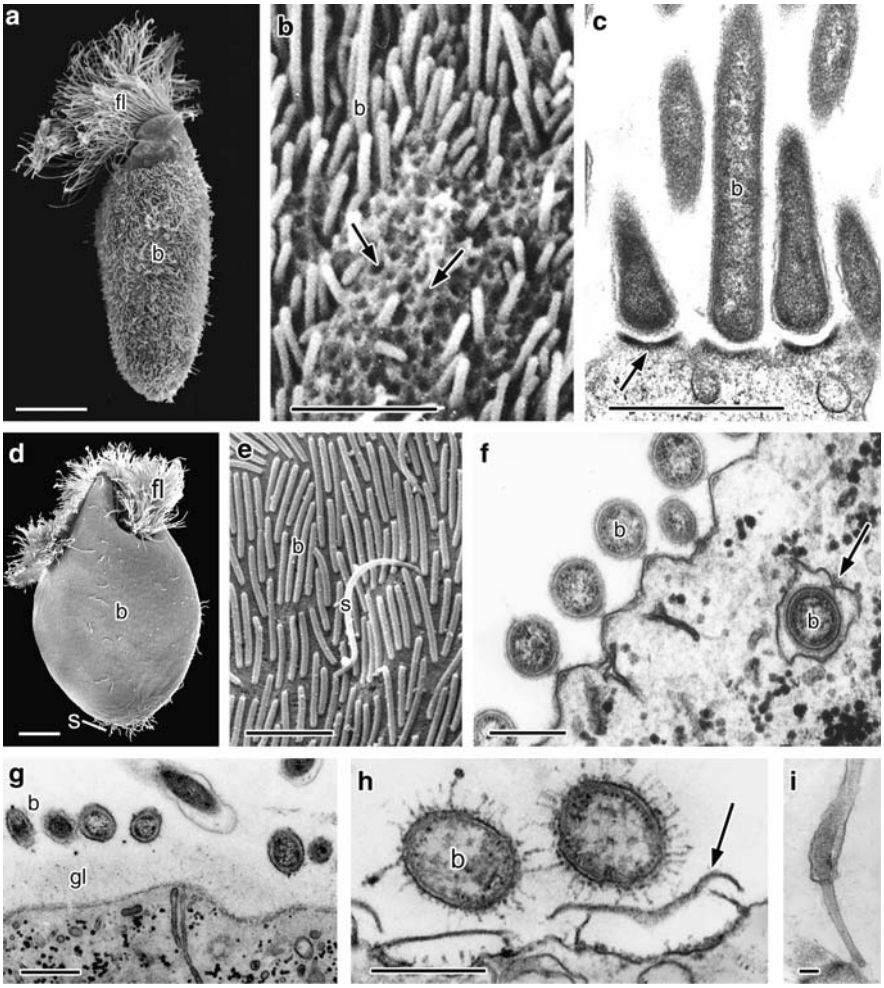

Fig. 15.7 Termite flagellates associated with rod-like bacteria (b) and spirochetes (s). (**a**), (**b**), (**d**) and (**e**) SEM, (**c**), (**f**), (**g**)–(**i**) TEM. fl, flagella. ((**a**)–(**c**)) *Joenia annectens*. (**a**) and (**b**) Rod-like bacteria attached by a cell pole cover most of the body surface. Circular indentations are seen where the ectobionts were accidentally removed (*arrows*). *Bars*: (**a**) 20 μm, (**b**) 5 μm. (**c**) Electron-dense material (*arrow*) supports the attachment sites of the rods. *Bar*: 1 μm. (**d**)–(**f**) The bacteria are laterally attached to elongated ridges on the surface of *Staurojoenina* sp. *Arrow* in (**f**): phagocytosed ectobiont with its attachment site. *Bars*: (**d**) 20 μm, (**e**) and (**f**) 0.5 μm. (**g**) Ectobionts on the thick glycocalyx (gl) of *Stephanonympha nelumbium*. *Bar*: 0.5 μm. (**h**) Bacteria adhering to extracellular surface structures (*arrow*) of *Microrhopalodina multinucleata*, lanthanum nitrate impregnation. *Bar*: 0.5 μm. (**i**) Nose-like end of attached spirochete on *J. annectens*. *Bar*: 0.1 μm. ((**a**) and (**i**) from Radek et al. 1992; (**d**) courtesy of Annelie Maaß, London, UK; (**e**) and (**f**) from Stingl et al. 2004, (**g**) and (**h**) from Radek et al. 1996)

1980, 1982). The cells must be able to discriminate between potential partners, bind specifically and establish a firm final adhesion. Interaction of sugars of the glycocalyx with lectins seems to be involved in the adhesion, e.g., in *Joenia annectens* (Radek et al. 1996). However in other flagellates, e.g., *Devescovina glabra*, a role for sugars in mediating the attachment could not be demonstrated. Basically, either ionic or hydrophobic interactions may be involved in such associations. Experiments to remove the bacteria of *D. glabra, J. annectens, Mixotricha paradoxa* and *Deltotrichonympha* with chemicals disturbing ionic or hydrophobic binding revealed that no ionic binding is involved (Radek and Tischendorf 1999; Radek and Nitsch 2007). Instead, results indicated hydrophobic interactions between proteins, except for *J. annectens*. Freeze–fracture studies showed aggregations of integral membrane proteins at the cell junctions of some species (Radek and Tischendorf 1999; Radek and Nitsch 2007; Tamm 1980). For *D. glabra*, the membrane proteins seem to be in contact with an additional outer, probably proteinaceous S-layer (surface layer) of the ectobiotic rods (Radek and Tischendorf 1999).

Apparently, there is no single, general mechanism for bacterial attachment to termite flagellates. Differences in the morphology of the binding partners, the fine structure of the cell contacts, the sugar compositions of the glycocalyces and chemicals evoking detachment rather indicate that adhesion follows different strategies.

Suggested roles of ectobionts; motility symbioses: The roles that the pro- and eukaryotic cells play in the partnership are mostly unknown; they possibly often profit from an exchange of nutrients. Former ectobacteria are also found in digestion vacuoles of their flagellate host with the attachment structures still present, and may supplement the nitrogen-poor diet of their host (Lavette 1969; Noda et al. 2006a; Radek et al. 1992). An involvement of flagellate-associated bacteria in cellulose digestion has also been proposed but remains unproved (Bloodgood and Fitzharris 1976). Instead, the flagellates and termites themselves were shown to produce sets of enzymes involved in cellulose hydrolysation (e.g., Breznak and Brune 1994; Todaka et al. 2007; Yoshimura 1995). *Streblomastix strix* harbors long rod-like ectobionts that are presumed to have a chemosensory function which directs the flagellate toward food sources (Dyer and Khalsa 1993). Furthermore, being laterally attached to the 6–7 longitudinal vanes of their host cell, they stabilize the flagellate's typical cell form. Antibiotic treatment leads to a loss of most bacteria and to a transformation of the slender cell to a teardrop-shaped cell (Leander and Keeling 2004). Thus the ectobionts seem to function as an extracellular skeleton structurally or osmotically supporting the vane morphology (Dyer and Khalsa 1993; Leander and Keeling 2004). Similarly, *Mixotricha paradoxa* drastically changes its cell form (from pear-shaped to rounded) when the system of ectobionts and attachment structures is destroyed (Radek and Nitsch 2007). In the micro-oxic gut environment, ectosymbionts may also participate in protecting their host against oxygen by consuming it in the surrounding area (Noda et al. 2006a).

The motility symbioses of *Mixotricha paradoxa* (Fig. 15.8a–d) and *Caduceia versatilis* (Fig. 15.8e–g) are remarkable. Here, the associated prokaryotes are responsible for the locomotion of their host. The large trichomonad *Mixotricha paradoxa* is propelled by the action of regularly attached spirochaetes (Brugerolle

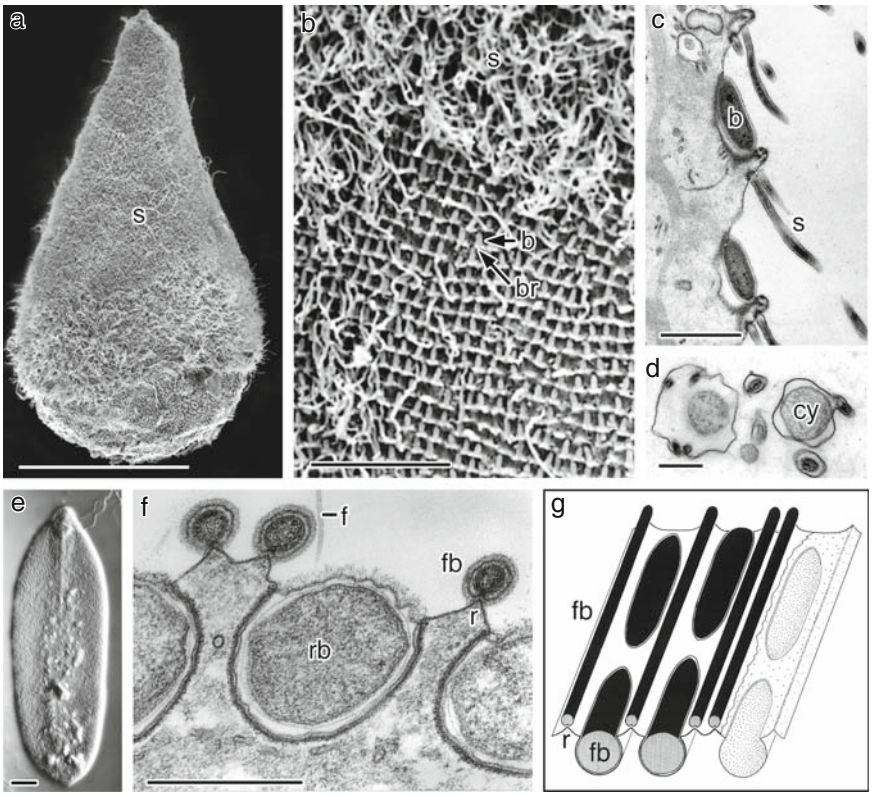

Fig. 15.8 (**a**)–(**d**) *Mixotricha paradoxa*. (**a**)–(**c**) Spirochetes (s) and rod-like bacteria (b) densely cover the cell surface; they are attached to brackets (br). Only the posterior cell pole is free of ectobionts. (**d**) Spirochetes transformed to cystic stages (cy) in starved termites. (**a**) and (**b**) SEM, (**c**) and (**d**) TEM. *Bars*: (**a**) 100 μm, (**b**) 10 μm, (**c**) 1 μm, (**d**) 0.5 μm. (**e**)–(**g**) *Caduceia versatilis* with attached bacteria. (**e**) Differential interference contrast image focused on the ectobionts. (**f**) Cortical cytoplasm depicting cross-sectioned flagellated bacterial rods (rb) (*Candidatus* Tammella caduceiae) within deep pockets and fusiform bacteria (fb) on surface ridges (r); f, bacterial flagella; TEM. (**g**) Diagram of the junctional complexes with rod-like bacteria (flagella not shown) and fusiform bacteria. *At right side* distribution of intramembrane particles of P face. *Bars*: (**e**) 10 μm, (**f**) 0.5 μm. ((**a**) from Wenzel et al. 2003, (**d**) From Radek and Nitsch 2007, (**e**)–(**g**) from Tamm 1980)

2004; Cleveland and Grimstone 1964; König et al. 2007) that were first taken for cilia (Sutherland 1933). Their movement is synchronized so that metachronal waves resembling ciliary beating travel across the cell body. The thousands of spirochaetes are attached to small brackets of complex internal structures which arise in rows from the cell surface (Fig. 15.8a–c). A cortical microfibrillar network of strands is found subjacent to the cell contact structures (Brugerolle 2004). While up to four spirochaetes are in contact with the posterior faces of the brackets, a single short rod is found at the anterior faces. At least three different spirochete clones of the *Treponema* cluster could be identified on the surface of *Mixotricha*

(Wenzel et al. 2003). They have a special distribution. Starvation and the application of antibiotics lead to a transformation of the treponemes into rounded cystic stages with enclosed protoplasmic cylinders (Fig. 15.8d; Radek and Nitsch 2007). This may be a survival mechanism when faced with hostile conditions. In addition to the treponemes and short rods, large spirochaetes resembling *Canaleparolina* and long rods may contact the surface of *Mixotricha* (Brugerolle 2004; Wier et al. 2000).

The devescovinid *Caduceia versatilis* (informally "Rubberneckia") exhibits a rapid gliding movement when it is in contact with a substratum, powered by the flagella of thousands of rod-like ectobionts (Fig. 15.8e–g; d'Ambrosio et al. 1999; Tamm 1980, 1982; Hongoh et al. 2007a). The laterally attached flagellated rods are embedded in specialized invaginations (Fig. 15.8f, g). These pockets are coated by dense material and contain high densities of intramembrane particles (Tamm 1980). The motility symbiont bears a thick glycocalyx and flagella on the area of the surface which is exposed to the external medium. Slender, fusiform bacteria are attached to surface ridges between the flagellated rods. Dense material underlies their attachment sites, and intramembrane particles are integrated into the ridge membrane. Generally, however, the ectobionts of termite flagellates are not responsible for the movement of their host. They are either nonmotile rods or motile spirochaetes which do not move in a coordinated manner due to an irregular attachment pattern (Beams et al. 1960; Bloodgood and Fitzharris 1976; Kirby 1941, 1945). In the latter case they disturb rather than support the normal movement of the flagellates.

Ectobiotic phylotypes and coevolution with flagellate and termite hosts: None of the flagellates' ectobionts have been successfully cultured to date. Most of them are only described morphologically, e.g., as Gram-negative rods of a certain size. The recognition of phylotypes has been possible since the introduction of PCR and sequence analyses. Meanwhile, a number of bacterial ectobionts have been characterized by comparison of their 16S rRNA sequences. All ectobiotic spirochaetes known to date belong to the *Treponema* branch of the Spirochaetes which is divided into two clusters, i.e., *Treponema* clusters I and II (II = *Treponema bryantii* subgroup). For example, spirochaetes of *Devescovina*, *Stephanonympha* and *Oxymonas* from *Neotermes koshunensis* (Noda et al. 2003), *Dinenympha* and *Pyrsonympha* from *Hodotermopsis sjoestedti* and *Dinenympha porteri* from *Reticulitermes speratus* (Iida et al. 2000), and *Mixotricha paradoxa* from *Mastotermes darwiniensis* (Wenzel et al. 2003) are definitely treponemes. *Treponema* cluster II spirochaetes seem to be restricted to the oxymonad group of the pyrsonymphids (Noda et al. 2003). Often the flagellates harbor several spirochete species on their surface, and common phylotypes may be shared among different protists. Thus, there seem to be multiple independent origins of ectobiotic spirochaetes (Noda et al. 2003). However, at least some specificity to the host protist is present; e.g., in the termite *Neotermes koshunensis* two bacterial phylotypes (NkS-Dev1, NkS-Dev14) from *Devescovina* and *Stephanonympha* never occur on *Oxymonas* and the *Oxymonas* phylotype (NkS-Oxy8) is never attached to these devescovinids. Results suggest that there is a co-evolution with the host flagellate rather than with the host termite (Noda et al. 2003).

Members of the Bacteroidales are also widely distributed ectosymbionts of gut flagellates. For example, Bacteroidales were described from *Mixotricha paradoxa* (Wenzel et al. 2003), *Staurojoenina* (candidate taxon "Vestibaculum illigatum", Stingl et al. 2004), *Barbulanympha* (Merritt et al. 1996, Noda et al. 2006a), *Hoplonympha, Urinympha, Devescovina* and *Streblomastix* (Noda et al. 2006a), *Caduceia* (Goss and Gunderson 2000, Hongoh et al. 2007a) and *Dinenympha* ("*Candidatus* Symbiothrix dinenymphae", Hongoh et al. 2007b). The ectobiotic *Bacteroidales* are divided into three distinct lineages, and their protist hosts also belong to diverse phylogenetic groups (Noda et al. 2006a). This implies that the flagellates acquired their ectobionts independently several times. Freely swimming lineages of *Bacteroidales* are abundant and common in the guts. Some co-evolution with the protist host definitely exists; e.g., the ectobionts of the families Hoplonymphidae and Staurojoeninidae of the order Trichonymphida form a monophyletic group within the *Bacteroidales* Cluster IV (Noda et al. 2006a). However, one of the two ectobiotic phylotypes of the oxymonad *Oxymonas* was grouped with the sequences of ectobionts from the parabasalid *Devescovina* (Noda et al. 2006b). Thus in this case the bacteria are unrelated to their hosts' phylogeny. The authors conclude that the host-ectobiotic evolutionary relationships are not simple (Noda et al. 2006b). One flagellate species can be associated with more than one lineage of *Bacteroidales*; e.g., two phylotypes were found on *Oxymonas* (Noda et al. 2006b) and three on *Streblomastix strix* (Noda et al. 2006a). Generally, however, each host cell of one species carries a single *Bacteroidales* phylotype, suggesting a competitive behavior (e.g., on *Oxymonas*, Noda et al. 2006b). Members of *Bacteroidales* are also found as endobionts (Noda et al. 2007).

It has recently been discovered also that a member of the phylum Synergistes can be an ectobiont of termite flagellates (Hongoh et al. 2007a). It is present on the surface of *Caduceia versatilis,* in addition to a fusiform *Bacteroidales* bacterium. It is a flagellated motility symbiont and was named "*Candidatus* Tammella caduceiae" (Hongoh et al. 2007a). Other members of the "*Synergistes*" group, including one sharing 95% sequence identity with *Tammella*, are free-living in the gut suggesting that the motility symbiont evolved from such a free-living bacterium. Methanoarchaea of termite guts are also occasionally attached to the surface of flagellates, such as on a small trichomonad from *Schedorhinotermes lamanianus* (Brune and Stingl 2006).

15.5 Conclusions

Multiple and complex associations of bacterial ectobionts and their protist hosts exist. This review does not mention all known examples, nor are all relationships yet understood. Bacterial–eukaryotic symbiotic communities seem to be widespread in nature, especially in low oxygen habitats. The degree of adaptation of the partners varies widely, ranging from accidental to facultative and obligatory interactions. In some of the cases, a co-evolution of the ectobiont and its host cell

could be demonstrated. Successful systems seem to have an ecological advantage, for example by enabling the exploitation of further nutrients, survival in new ecological niches or better defense against hostile environmental conditions or predators. Furthermore, a stable ectosymbiosis may be an important precondition for establishing endosymbiotic associations (Smith 1979).

Many questions regarding the relationship of host and ectobiont still remain unanswered. For example, the recognition and binding mechanisms are mostly unknown, as well as the control mechanism for growth of the prokaryotes. Also the roles the partners play in the association are mostly obscure. Who benefits and who suffers, or do both profit from the union?

References

Ball GH (1969) Organisms living on and in protozoa. In: Chen TT (ed) Research in protozoology, vol 3. Pergamon Press, New York, pp 566–718

Bauer-Nebelsick M, Bardele CF, Ott JA (1996a) Redescription of *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg, 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria. Eur J Protistol 32:18–30

Bauer-Nebelsick M, Bardele CF, Ott JA (1996b) Electron microscopic studies on *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg, 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria. Eur J Protistol 32:202–215

Beams HW, Kessel RG (1973) Studies on the fine structure of the protozoan *Cyclidium*, with special reference to the mitochondria, pellicle and surface-associated bacteria. Z Zellforsch 139:303–310

Beams HW, King RL, Tahmisian TN, Devine R (1960) Electron microscope studies on *Lophomonas striata* with special reference to the nature and position of the striations. J Protozool 7:91–101

Belcher JH, Swale EMF (1976) *Spumella elongata* (Stokes) nov. comb., a colourless flagellate from soil. Arch Protistenkd 118:215–220

Bennett WE (1986) Fine structure of the trophic stage of *Endostelium* Olive, Bennett & Deasey, 1984 (Eumycetozoea, Protostelia). Protistologica 22:205–212

Bernhard JM, Buck KR, Farmer MA, Bowser SS (2000) The Santa Barbara basin is a symbiosis oasis. Nature 403:77–80

Bernhard JM, Buck KR, Barry JP (2001) Monterey Bay cold-seep biota: assemblages, abundance, and ultrastructure of living foraminifera. Deep-Sea Res Part I 48:2233–2249

Biernacka I (1963) Die Protozoenfauna in der Danziger Bucht. II. Die Charakteristik der Protozoen in untersuchten Biotopen der Seeküste. Polsk Arch Hydrobiol 11:17–75

Bloodgood RA, Fitzharris TP (1976) Specific association of prokaryotes with symbiotic flagellate protozoa from the hindgut of the termite *Reticulitermes* and the wood-eating roach *Cryptocercus*. Cytobios 17:103–122

Bloodgood RA, Miller KR, Fitzharris TP, McIntosh R (1974) The ultrastructure of *Pyrsonympha* and its associated microorganisms. J Morphol 143:77–106

Bohatier J, Sénaud J, Benyahya M (1990) In situ degradation of cellulose fibres by the entodiniomorph rumen ciliate *Polyplastron multivesiculatum*. Protoplasma 154:122–131

Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose by termites. Annu Rev Entomol 39:453–487

Brugerolle G (2004) Devescovinid features, a remarkable surface cytoskeleton, and epibiotic bacteria revisited in *Mixotricha paradoxa*, a parabasalid flagellate. Protoplasma 224:49–59

Brugerolle G, Bordereau C (2004) The flagellates of the termite *Hodotermopsis sjoestedti* with special reference to *Hoplonympha, Holomastigotes* and *Trichomonoides trypanoides* n. comb. Eur J Protistol 40:163–174

Brune A, Stingl U (2006) Prokaryotic symbionts of termite gut flagellates: Phylogenetic and metabolic implications of a tripartite symbiosis. In: Overmann J (ed) Progress in molecular and subcellular biology. Molecular basis of symbiosis. Springer, Berlin, pp 39–60

Bryant MP (1970) Normal fauna – rumen bacteria. Am J Clin Nutr 23:1440–1450

Buck KR, Bernhard JM (2001) Protistan-prokaryotic symbioses in deep-sea sulfidic sediments. In: Seckbach J (ed) Symbiosis. Kluwer, Netherlands, pp 509–517

Buck KR, Barry JP, Simpson GB (2000) Monterey Bay cold seep biota: Euglenozoa with chemoautotrophic bacterial epibionts. Eur J Protistol 36:117–126

Cann JP (1986) The feeding behaviour and structure of *Nuclearia delicatula* (Filosea: Aconchulinida). J Eukaryot Microbiol 33:392–396

Carpenter KJ, Keeling P (2007) Morphology and phylogenetic position of *Eucomonympha imla* (Parabasalia: Hypermastigida). J Eukaryot Microbiol 54:325–332

Cleveland LR, Grimstone AV (1964) The fine structure of the flagellate *Mixotricha paradoxa* and its associated microorganisms. Proc R Soc Lond B 159:668–686

Corliss JO (1979) The ciliated protozoa: Characterization, classification, and guide to the literature, 2nd edn. Pergamon Press, London

Corliss JO (1985) Concept, definition, prevalence, and host-interactions of xenosomes (cytoplasmic and nuclear endosymbionts). J Protozool 32:373–376

Daims H, Brühl A, Amman R, Schleifer K-H, Wagner M (1999) The domain-specific probe EUB338 is insufficient for detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 23:434–444

Desportes I, Théodorides J, Vivarés Ch (1977) Association entre la Grégarine *Porospora portunidarum* et des Bactéries intestinales de son hôte: *Carcinus mediterraneus* Czer. (Crustacé Décapode). Protistologica 13:611–619

D'Ambrosio U, Dolan M, Wier AM, Margulis L (1999) Devescovinid trichomonad with axostyle-based rotary motor ("Rubberneckia"): taxonomic assignment as *Caduceia versatilis* sp. nov. Eur J Protistol 35:327–337

Doddema HJ, Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36:752–754

Dolan MF (2001) Speciation of termite gut protists: the role of bacterial symbionts. Int Microbiol 4:203–208

Dolan M, Margulis L (1997) *Staurojoenina* and other symbionts in *Neotermes* from San Salvador Island, Bahamas. Symbiosis 22:229–239

Dragesco J (1948) Sur la biologie du *Zoothamnium pelagicum* (du Plessis). Bull Soc Zool de France 73:130–134

Dyer BD, Khalsa O (1993) Surface bacteria of *Streblomastix strix* are sensory symbionts. BioSystems 31:169–180

Epstein SS, Bazylinski DA, Fowle WH (1998) Epibiotic bacteria on several ciliates form marine sediments. J Eukaryot Microbiol 45:64–70

Esteban G, Finlay BJ (1994) A new genus of anaerobic scuticociliate with endosymbiotic methanogens and ectobiotic bacteria. Arch Protistenkd 144:350–356

Esteban G, Finlay BJ, Embley TM (1993) New species double the diversity of anaerobic ciliates in a Spanish lake. FEMS Microbiol Lett 109:93–100

Fauré-Fremiet E (1950) Caulobactéries épizoiques associées aux *Centrophorella* (Cilies holotriches). Bull Soc Zool France 75:134–137

Fauré-Fremiet E (1951) The marine sand dwelling ciliates of Cape Cod. Biol Bull 100:59–70

Fauré-Fremiet E, Favard P, Carasso N (1963) Images électroniques d'une microbiocénose marine. Cah Biol Mar 4:61–64

Fenchel T (1992) What can ecologists learn from microbes: life beneath a square centimetre of sediment surface. Funct Ecol 6:499–507

Fenchel T, Finlay BJ (1989) *Kentrophoros*: a mouthless ciliate with a symbiotic kitchen garden. Ophelia 30:75–93
Fenchel T, Finlay BJ (1991) The biology of free-living anaerobic ciliates. Eur J Protistol 26:201–215
Fenchel T, Finlay BJ (1992) Production of methane and hydrogen by anaerobic ciliates containing symbiotic methanogens. Arch Microbiol 157:475–480
Fenchel T, Finlay BJ (1995) Ecology and evolution in anoxic worlds, Oxford series in ecology and evolution. Oxford University Press, Oxford
Fenchel T, Ramsing NB (1992) Identification of sulphate-reducing ectosymbiotic bacteria from anaerobic ciliates using 16S rRNA binding oligonucleotide probes. Arch Microbiol 158:394–397
Fenchel T, Perry T, Thane A (1977) Anaerobiosis and symbiosis with bacteria in free-living ciliates. J Eukaryot Microbiol 24:154–163
Finlay BJ, Fenchel T (1989) Everlasting picnic for protozoa. New Scientist 123:66–69
Finlay BJ, Clarke KJ, Vincente E, Miracle MR (1991) Anaerobic ciliates from a sulphide-rich solution lake in Spain. Eur J Protistol 27:148–159
Foissner W (1995) *Kentrophoros* (Ciliophora, Karyorelictea) has oral vestiges: a reinvestigation of K. *fistulosus* (Faurét-Fremiet 1950) using protargol impregnation. Arch Protistenkd 146:165–179
Fokin SI, Schweikert M, Görtz H-D, Fujishima M (2003) Bacterial endocytobionts of Ciliophora. Diversity and some interactions with the host. Eur J Protistol 39:475–480
Foster RA, Carpenter EJ, Bergman B (2006) Unicellular cyanobionts in open ocean dinoflagellates, radiolarians, and tintinnids: ultrastructural and immunolocalization of phycoerythrin and nitrogenase. J Phycol 42:453–463
Frolov AO, Chystjakova LV, Goodkov AV (2005) Light- and electron-microscopic study of *Pelomyxa binucleata* (Gruber, 1884) (Peloflagellatea, Pelobiontida). Protistology 4:57–73
Geitler L (1948) Symbiosen zwischen Chrysomonaden und knospenden Bakterien-artigen Organismen sowie Beobachtungen über Organisationseigentümlichkeiten der Chrysomonaden. Öst Bot Z 95:300–324
Giambelluca MA, Rosati G (1996) Behavior of epixenosomes and epixenosomal band during divisional morphogenesis in *Euplotidium itoi* (Ciliata, Hypotrichida). Eur J Protistol 32:77–80
Gijzen HJ, Zwart KB, Stumm CK (1985) Episymbiotic association of methanogenic bacteria and rumen ciliates. Antonie von Leeuwenhoek 51:453–454
Görtz H-D (2006) Symbiotic associations between ciliates and prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes. A handbook on the biology of bacteria. 3rd ed, vol 1: Symbiotic associations, biotechnology, applied microbiology. Springer, New York, pp 364–402
Goss H, Gunderson JH (2000) Identity of an ectosymbiont of a devescovinid. Abstr 100th Gen Meet Am Soc Microbiol 2000, abstr N-149
Grassé P-P (1938) La vêture schizophytique des Flagellés termiticole: *Parajoenia, Caduceia* et *Pseudodevescovina*. Bull Soc Zool France 63:110–122
Grim JN, Perez-Espana H, Martinez-Diaz SF (2000) The morphology of *Protoopalina pomacantha*, n. sp., symbiont in the rectum of the angelfishes, *Pomacanthus zonipectus* and *Holacanthus passer*. A light, scanning electron and transmission electron microscopic study. Eur J Protistol 36:343–350
Hamburger B (1957) Bakteriensymbiose bei *Volvox aureus* Ehrenberg. Arch Mikrobiol 29: 291–310
Hausmann K, Walz B (2007) *Zoothamnium pelagicum*: Ein sessiler, kolonialer Ciliat lebt vereint zu Gruppen. Mikrokosmos 96:217–222
Hazen TC, Raker ML, Gerald WE, Fliermans CB (1978) Ultrastructure of red-sore lesions on largemouth bass (*Micropterus salmoides*): Association of the ciliate *Epistylis* sp. and the bacterium *Aeromonas hydrophila*. J Protozool 25:351–355
Hollande A, Carruette-Valentin J (1970) La lignée des Pyrsonymphines et les caractéres infrastructuraux communs aux genres *Opisthomitus, Oxymonas, Saccinobacculus, Pyrsonympha* et *Streblomastix*. C R Acad Sci, Paris, Ser D 270:1587–1590

Hongoh Y, Sato T, Dolan MF, Noda S, Ui S, Kudo T, Ohkuma M (2007a) The motility symbiont of the termite gut flagellate *Caduceia versatilis* is a member of the "*Synergistes*" group. Appl Environ Microbiol 73:6270–6276

Hongoh Y, Sato T, Noda S, Ui S, Kudo T, Ohkuma M (2007b) *Candidatus* Symbiothrix dinenymphae: bristle-like *Bacteroidales* ectosymbionts of termite gut protists. Environ Microbiol 9:2631–2635

Iida T, Ohkuma M, Ohtoko K, Kudo T (2000) Symbiotic spirochetes in the termite hindgut: phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol Ecol 34:17–26

Imai S, Ogimoto K (1978) Scanning electron and fluorescent microscopic studies on the attachment of spherical bacteria to ciliate protozoa in the ovine rumen. Jpn J Vet Sci 40:9–19

Ito S (1958) Two new species of marine ciliates. *Euplotidium itoi* sp. nov. and *Gastrocirrhus* sp. nov. Zool Mag Tokyo 67:184–187

Jenkins C, Samudrala R, Anderson I, Hedlund BP, Petroni G, Michailova N, Pinel N, Overbeek R, Rosati G, Staley JT (2002) Genes for the cytoskeletal protein tubulin in the bacterial genus *Prosthecobacter*. PNAS 99:17049–17054

Jeon KW (2006) Prokaryotic symbionts of amoeba and flagellates. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes. A handbook on the biology of bacteria. 3rd ed, vol 7: Proteobacteria: Delta and epsilon subclasses. Deeply rooting bacteria. Springer, New York, pp 1028–1037

Kahl A (1933) Ciliata libera et ectocommensalia. In: Grimpe G, Wagler E (Hrsg) Die Tierwelt der Nord- und Ostsee, Teil II. Akademische Verlagsgesellschaft Becker & Erler, Lepzig, pp 29–146

Kahl A (1935) Urtiere oder Protozoa I: Wimpertiere oder Ciliata (Infusoria). 4: Peritricha und Chonotricha. Nachtrag. In: Dahl F (Hrsg) Die Tierwelt Deutschlands. Gustav Fischer, Jena, pp 806–842

Khan RA, Barber VC, McCann S (1974) A scanning electron microscopical study of the surface topography of a trichodinid ciliate. Trans Amer Microsc Soc 93:131–134

Kirby H (1941) Devescovinid flagellates of termites. I. The genus *Devescovina*. Univ Calif Publ Zool 45:1–92

Kirby H (1945) Devescovinid flagellates of termites. IV. The genera *Metadevescovina* and *Pseudodevescovina*. Univ Calif Publ Zool 45:247–318

Klaveness D (1982) The *Cryptomonas-Caulobacter* consortium: Facultative ectocommensalism with possible taxonomic consequences? Nord J Bot 2:183–188

König H, Fröhlich J, Li L, Wenzel M, Berchtold M, Dröge S, Breunig A, Pfeiffer P, Radek R, Brugerolle G (2007) The flagellates of the Australian termite *Mastotermes darwiniensis*: Identification of their symbiotic bacteria and cellulases. Symbiosis 44:51–65

Kouris A, Juniper SK, Frébourg G, Gaill F (2007) Protozoan-bacterial symbiosis in a deep-sea hydrothermal vent folliculinid ciliate (*Folliculinopsis* sp.) from the Juan de Fuca Ridge. Mar Ecol 28:63–71

Laval M (1968) *Zoothamnium pelagicum* du Plessis. Cilié péritriche planctonique: morphologie, croissance et comportement. Protistologica 4:333–363

Lavette A (1969) Sur la vêture schizophytique des Flagellés symbiotes de Termites. C R Acad Sci Paris Ser D 268:2585–2587

Leander BS, Farmer MA (2000) Epibiotic bacteria and a novel pattern of strip reduction on the pellicle of *Euglena helicoideus* (Bernard) Lemmermann. Eur J Protistol 36:405–413

Leander BS, Keeling PJ (2004) Symbiotic innovation in the oxymonad *Streblomastix strix*. J Eukaryot Microbiol 51:291–300

Maaß A, Radek R (2006) The gut flagellate community of the termite *Neotermes cubanus* with special reference to *Staurojoenina* and *Trichocovina hrdyi* nov. gen. nov. sp. Eur J Protistol 42:125–141

Merritt P, Goss S, Gunderson J (1996) Identification of the ectosymbiotic eubacteria of *Barbulanympha* spp. from the wood-eating roach *Cryptocercus punctulatus*. Abstr 96th Gen Meet Am Soc Microbiol 1996, abstr N-180

Micro*scope Internet page: http://starcentral.mbl.edu/microscope/portal.php
Newbold CJ, Lassalas B, Jouany JP (1995) The importance of methanogens associated with ciliate protozoa in ruminal production in vitro. Lett Appl Microbiol 21:230–234
Noda S, Ohkuma M, Yamada A, Hongoh Y, Kudo T (2003) Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl Environ Microbiol 69:625–633
Noda S, Inoue T, Hongoh Y, Kawai M, Nalepa CA, Vongkaluang C, Kudo T, Ohkuma M (2006a) Identification and characterization of ectobionts of distinct lineages in *Bacteroidales* attached to flagellated protists in the gut of termites and a wood-feeding cockroach. Environ Microbiol 8:11–20
Noda S, Kawai M, Nakajima H, Kudo T, Ohkuma M (2006b) Identification and in situ detection of two lineages of *Bacteroidales* ectosymbionts associated with a termite gut protist, *Oxymonas* sp. Microbes Environ 21:16–22
Noda S, Kitade O, Inoue T, Kawai M, Kanuka M, Hiroshima K, Hongoh Y, Constantino R, Uys V, Zhong J, Kudo T, Ohkuma M (2007) Cospeciation in the triplex symbiosis of termite gut protists (*Pseudotrichonympha* spp.), their hosts, and their bacterial endosymbionts. Mol Ecol 16:1257–1266
O'Brien RW, Slaytor M (1982) Role of microorganisms in the metabolism of termites. Aust J Biol Sci 35:239–262
Ogimoto K, Imai S (1981) Atlas of rumen microbiology. Japan Scientific Societies Press, Tokyo
Ott JA, Bright M, Schiemer F (1998) The ecology of a novel symbiosis between a marine peritrich ciliate and chemoautotrophic bacteria. Mar Ecol 19:229–243
Page FC (1970) *Mastigamoeba aspersa* from estuarine tidal pools in Maine. Trans Amer Microsc Soc 89:197–200
Patterson DJ, Thompson DW (1981) Structure and elemental composition of the cyst wall of *Echinosphaerium nucleofilum* Barrett (Heliozoea, Actinophryida). J Protozool 28: 188–192
Petroni G, Spring S, Schleifer K-H, Verni F, Rosati G (2000) Defensive extrusive ectosymbionts of *Euplotidium* (Ciliophora) that contain microtubule-like structures are bacteria related to *Verrumicrobia*. Proc Natl Acad Sci USA 97:1813–1817
Pilhofer M, Rosati G, Ludwig W, Schleifer K-H, Petroni G (2007) Coexistence of tubulins and *FtsZ* in different *Prosthecobacter* species. Mol Biol Evol 24:1439–1442
Polz MF, Ott JA, Bright M, Cavanaugh CM (2000) When bacteria hitch a ride. Association between sulphur-oxidizing bacteria and eukaryotes represent spectacular adaptations to environmental gradients. ASM News 66:531–539
Pussard M (1965) Cuticule et caryocinese de *Gocevia fonbrunei*, n. sp. (Cochliopodiidae – Testacealobosa). Arch Zool Exp Gen 105:101–107
Pussard M, Senaud J, Pons R (1977) Observations ultrastructurales sur *Gocevia fonbrunei* Pussard 1965 (Protozoa, Rhizopodea). Protistologica 13:265–285
Radek (1999) Flagellates, bacteria, and fungi associated with termites: diversity and function in nutrition – a review. Ecotropica 5:183–196
Radek R, Nitsch G (2007) Ectobiotic spirochetes of flagellates from the termite *Mastotermes darwiniensis*: Attachment and cyst formation. Eur J Protistol 43:281–294
Radek R, Tischendorf G (1999) Bacterial adhesion to different termite flagellates: ultrastructural and functional evidence for distinct molecular attachment modes. Protoplasma 207:43–53
Radek R, Hausmann K, Breunig A (1992) Ectobiotic and endobiotic bacteria associated with the termite flagellate *Joenia annectens*. Acta Protozool 31:93–107
Radek R, Rösel J, Hausmann K (1996) Light and electron microscopic study of the bacterial adhesion to termite flagellates applying lectin cytochemistry. Protoplasma 193:105–122
Raikov IB (1971) Bactéries épizoiques et mode du nutrition du Cilié psammophile *Kentrophoros fistulosum* Fauré-Fremiet (étude au microscope electronique). Protistologica 7:365–378
Raikov IB (1974) Étude ultrastructurale des Bactéries épizoiques et éndozoiques de *Kentrophoros latum* Raikov, Cilié holotriche mésopsammique. Cah Biol Mar 15:379–393

Rinke C (2007) Interactions in the chemoautotrophic *Zoothamnium niveum* symbiosis. Dissertation, University of Vienna, Austria

Rinke C, Ott JA, Bright M (2003) Fatal picnic on a high speed elevator: Nutrition in the chemautotrophic *Zoothamnium niveum* symbiosis. IV Int Congress of ISS Halifax, 83

Rinke C, Schmitz-Esser S, Stoecker K, Nussbaumer AD, Molnár DA, Vanura K, Wagner M, Horn M, Ott JA, Bright M (2006) "*Candidatus* Thiobios zoothamnicoli", an ectosymbiotic bacterium covering the giant marine ciliate *Zoothamnium niveum*. Appl Environ Microbiol 72:2014–2021

Rinke C, Lee R, Katz S, Bright M (2007) The effects of sulphide on growth and behaviour of the thiotrophic *Zoothamnium niveum* symbiosis. Proc R Soc B 274:2259–2269

Rosati G (1999) Epixenosomes: symbionts of the hypotrich ciliate *Euplotidium itoi*. Symbiosis 26:1–23

Rosati G (2001) Ectosymbiosis in ciliated protozoa. Seckbach J (ed) Symbiosis. Mechanisms and model systems. Kluwer, Netherlands, pp 475–488

Rosati G (2006) Extrusive bacterial ectosymbiosis of ciliates. In: Overmann J (ed) Molecular basis of symbiosis. Springer, Berlin, pp 97–115

Rosati G, Verni F, Lenzi P (1993a) "Epixenosomes": Peculiar epibionts of the ciliate *Euplotidium itoi*. The formation of the extrusive apparatus and the ejecting mechanism. Eur J Protistol 29:238–245

Rosati G, Lenzi P, Verni F (1993b) "Epixenosomes": peculiar epibionts of the ciliate *Euplotidium itoi*: do their cytoplasmic tubules consist of tubulin? Micron 24:465–471

Rosati G, Giambelluca MA, Taiti E (1996) Epixenosomes: peculiar epibionts of the ciliate *Euplotidium itoi*: morphological and functional cell compartmentalization. Tissue Cell 28:313–320

Rosati G, Giambelluca MA, Grossi M, Morelli A (1997) Epixenosomes: peculiar epibionts of the ciliate *Euplotidium itoi*: involvement of membrane receptors and the adenylate-cyclase cyclic-AMP system in the ejecting process. Protoplasma 147:57–63

Rosati G, Petroni G, Quochi S, Modeo L, Verni F (1999) Epixenosomes: peculiar epibionts of the hypotrich ciliate *Euplotidium itoi* defend their host against predators. J Eukaryot Microbiol 46:278–282

Rosowski JR, Langenberg WG (1994) The near-spineless *Trachelomonas grandis* (Euglenophyceae) superficially appears spiny by attracting bacteria to its surface. J Phycol 30:1012–1022

Sauerbrey E (1928) Beobachtungen über einige neue oder wenig bekannte marine Ciliaten. Arch Protistenkd 61:355–407

Sharp R, Ziemer CJ, Stern MD, Stahl DA (1998) Taxon-specific association between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiol Ecol 26:71–78

Simpson AGB, van den Hoff J, Bernhard C, Burton HR, Patterson DJ (1996/1997) The ultrastructural and systematic position of the euglenozoon *Postgaardi mariagerensis*, Fenchel et al. Arch Protistenkd 147:213–225

Simpson AGB, Bernard C, Fenchel T, Patterson DJ (1997) The organization of *Mastigamoeba schizophrenia* n. sp.: more evidence of ultrastructural idiosyncrasy and simplicity in pelobiont protists. Eur J Protistol 33:87–98

Skuja H (1958) Eine neue vorwiegend sessil oder rhizopodial auftretende synbakteriotische Polytomee aus einem Schwefelgewässer. Svensk Bot Tidskr 52:379–390

Smith DC (1979) From extracellular to intracellular: the establishment of a symbiosis. Proc R Soc Lond B Biol Sci 204:115–130

Smith HE, Arnott HJ (1974) Epi- and endobiotic bacteria associated with *Pyrsonympha vertens*, a symbiotic protozoon of the termite *Reticulitermes flavipes*. Trans Am Microsc Soc 94: 401–410

Smith HE, Jun BHE, Stamler SJ (1975) Possible formation and development of spirochaete attachment sites found on the surface of symbiotic polymastigote flagellates of the termite *Reticulitermes flavipes*. BioSystems 7:374–379

Song W (1992) A new marine ciliate, *Zoothamnium penaei* sp. nov. (Ciliophora, Peritrichida). Oceanol Limnol Sinica 23:90–94

Stiller J (1946) Beitrag zur Kenntnis der Peritrichenfauna der Schwefelthermen von Split. Ann Hist Nat Mus Nat Hung 39:19–57

Stingl U, Maass A, Radek R, Brune A (2004) Symbionts of the gut flagellate *Staurojoenina* sp. from *Neotermes cubanus* represent a novel, termite-associated lineage of Bacteriodales: description of '*Candidatus* Vestibaculum illigatum'. Microbiology 150:2229–2235

Stumm CK, Hackstein JHP (1995) Methanbakterien und Protisten in den Gärkammern von Wiederkäuern und Insekten. In: Hausmann K, Kremer BP (Hrsg) Extremophile – Mikroorganismen in ausgefallenen Lebensräumen. 2. Auflage, VCH, Weinheim, pp 299–324

Stumm CK, Zwart KB (1986) Symbioses of protozoa with hydrogen-utilizing methanogens. Microbiol Sci 3:100–105

Stumm CK, Gijzen HJ, Vogels GD (1982) Association of methanogenic bacteria with ovine rumen ciliates. Br J Nutr 47:95–99

Sutherland JL (1933) Protozoa from Australian termites. Q J Microsc Sci 76:145–163

Tamm SL (1980) The ultrastructure of prokaryotic-eukaryotic cell junctions. J Cell Sci 44:335–352

Tamm SL (1982) Flagellated ectosymbiotic bacteria propel a eukaryotic cell. J Cell Biol 94:697–709

Todaka N, Moriya S, Saita K, Hondo T, Kiuchi I, Takasu H, Ohkuma M, Piero C, Hayashizaki Y, Kudo T (2007) Environmental cDNA analysis of the genes involved in lignocellulose digestion in the symbiotic protist community of *Reticulitermes speratus*. FEMS Microbiol Ecol 59:592–599

Tokura M, Ushida K, Miyazaki K, Kojima Y (1997) Methanogens associated with rumen ciliates. FEMS Microbiol Ecol 22:137–143

Tschermak-Woess E (1950) Über eine Synbacteriose und andere ähnliche Symbiosen. Plant Syst Evol 97:188–206

Tuffrau M (1985) Une nouvelle espèce du genre *Euplotidium* (Noland 1937: *Euplotidium prosaltans* n. sp. (Cilié hypotriche). Cah Biol Mar 26:53–62

Verni F, Rosati G (1990) Peculiar epibionts in *Euplotidium itoi* (Ciliata, Hypotrichida). J Protozool 37:337–343

Vickerman K (1977) DNA throughout the single mitochondrium of a kinetoplastid flagellate: observations on the ultrastructure of *Cryptobia vaginalis* (Hesse, 1910). J Eukaryot Microbiol 24:221–233

Vogels GD, Hoppe WF, Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. Appl Environ Microbiol 40:608–612

Vopel K, Pohn M, Sorgo A, Ott J (2001) Ciliate-generated advective seawater transport supplies chemoautotrophic ectosymbionts. Mar Ecol Prog Ser 210:93–99

Vopel K, Reick CH, Arlt G, Pöhn M, Ott JA (2002) Flow microenvironment of two marine peritrich ciliates with ectobiotic chemoautotrophic bacteria. Aquat Microb Ecol 29:19–28

Vopel K, Thistle D, Ott J, Bright M, Røy H (2005) Wave-induced H_2S flux sustains a chemoautotrophic symbiosis. Limnol Oceanogr 50:128–133

Wenzel M, Radek R, Brugerolle G, König H (2003) Identification of the ectosymbiotic bacteria of *Mixotricha paradoxa* in movement symbiosis. Eur J Protistol 39:11–23

Wier A, Ashen J, Margulis L (2000) *Canaleparolina darwiniensis*, gen. nov., sp. nov., and other pillotinaceous spirochetes from insects. Int Microbiol 3:213–223

Whitford MF, Teather RM, Forster RJ (2001) Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol 1:5

Williams AG, Coleman GS (1992) The rumen protozoa. Brock/Springer Series in Contemporary Bioscience, New York

Wolin MJ (1974) Metabolic interaction among intestinal microorganisms. Am J Clin Nutr 27:1320–1328

Wołowski K (1995) *Dylakosoma pelophilum* Skuja, a rare and colourless euglenophyte found in Poland. Algol Studies 76:75–78

Yamin MA (1979) Flagellates of the orders Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà reported from lower termites (Isoptera families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, and Serritermitidae) and from the wood-feeding roach *Cryptocercus* (Dictyoptera: Cryptocercidae). Sociobiology 4:3–117

Yoshimura T (1995) Contribution of the protozoan fauna to nutritional physiology of the lower termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Wood Res 82:68–129

Part VI
Application of Cell Wall Components

Chapter 16
Prokaryotic Cell Wall Components: Structure and Biochemistry

Nanobiotechnological Applications of S-Layers

Uwe B. Sleytr, Eva-Maria Egelseer, Nicola Ilk, Paul Messner, Christina Schäffer, Dietmar Pum, and Bernhard Schuster

16.1 Introduction

In nano(bio)technology one of the key challenges is the technological utilization of self-assembly systems, wherein molecules spontaneously associate under equilibrium conditions into reproducible supramolecular aggregates ("bottom-up strategy"). The attractiveness of "bottom-up" processes lies in their capability to build uniform, ultra-small functional units and in the possibility of exploiting such structures at meso- and macroscopic scale for life- and nonlife-science applications (Sleytr et al. 2004a).

In this chapter, we describe the application potential of crystalline bacterial cell surface layers (S-layers), which are one of the most commonly observed cell envelope structures in prokaryotic micro-organisms (see Chap. 3).

Studies of their structure, chemistry, genetics, morphogenesis, and function clearly demonstrated that S-layers represent the simplest type of proteinaceous membrane developed during evolution (for review see Sleytr et al. 2005; Sára and Sleytr 2000). They are composed of a single protein or glycoprotein species endowed with the intrinsic ability to assemble into monomolecular lattices representing a structure of low free energy during cell growth. The broad application potential of S-layers depends on the capability of isolated subunits to assemble in defined orientation into crystalline arrays in suspension or on suitable surfaces or interfaces (Fig. 16.1). Since S-layers are periodic structures, they exhibit identical physicochemical properties on each molecular unit down to the subnanometer scale and possess pores identical in size and morphology. Very important for most nanobiotechnological applications is the fact that on S-layer lattices functional groups are aligned on the surface and within the pore areas in well-defined spacings

U.B. Sleytr (✉)
Department for NanoBiotechnology, University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 11, 1190 Vienna, Austria
e-mail: uwe.sleytr@boku.ac.at

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_16,

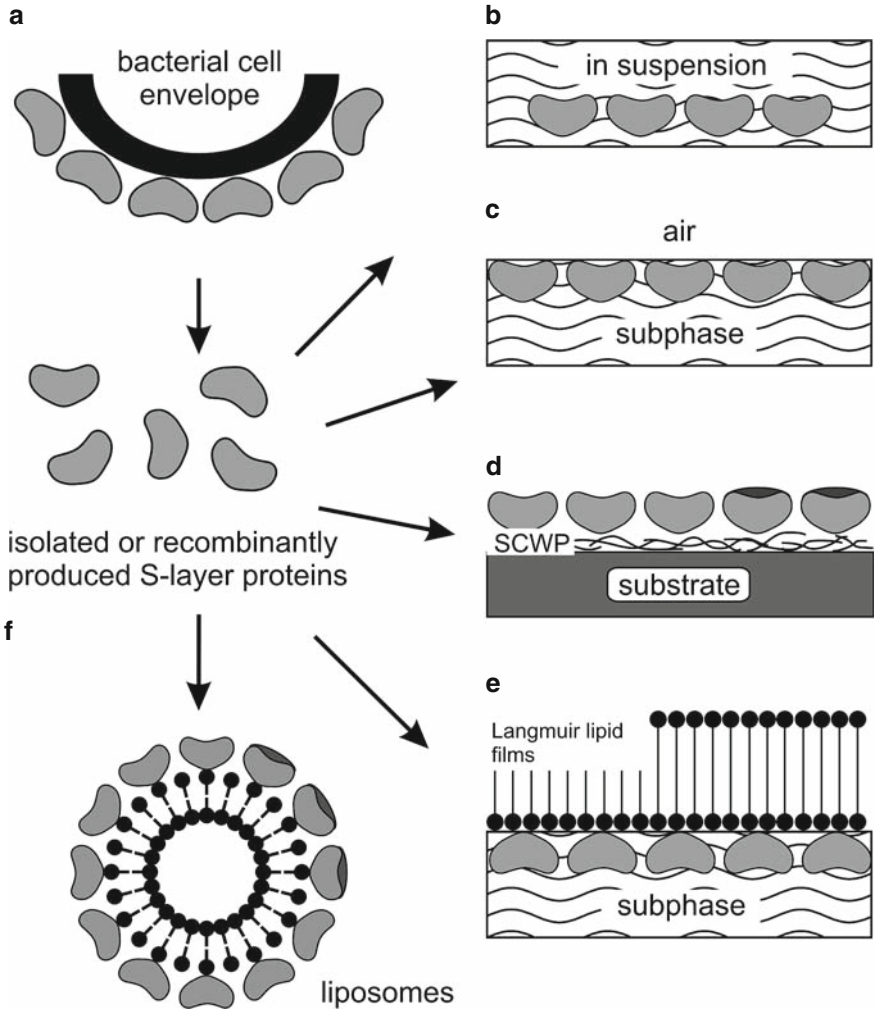

Fig. 16.1 Schematic illustration of the reassembly of isolated native S-layer proteins (**a**) or recombinant S-layer fusion proteins into crystalline arrays. Formation of self-assembly products in suspension (**b**). The self-assembly process can occur in defined orientation at the air/water interface (**c**), on solid supports which may be coated with secondary cell wall polymer (SCWP) (**d**), on Langmuir lipid films (**e**), and on liposomes or nanocapsules (**f**). (Modified after Sára et al. 2005)

and orientation. Moreover, the possibility of changing the properties of S-layer proteins by chemical modifications and genetic engineering breaks new ground for tuning their structural and functional features. S-layers also represent a unique structural basis for generating complex supramolecular assemblies, involving all relevant "building blocks" for life, such as proteins, lipids, glycans, nucleic acids, and combinations of them (for reviews see Egelseer et al. 2008; Sleytr et al. 2007a, b; Sára et al. 2006; Sleytr et al. 2001, 2005).

16.2 Self-Assembly of S-Layer Proteins

Important information on the morphogenesis of S-layer lattices on intact cells has been derived from self-assembly studies of isolated S-layer subunits in vitro (Sleytr and Messner 1989; Sleytr and Plohberger 1980; Sleytr et al. 2005). Up to now, a great variety of methods has been described for the detachment and disintegration of S-layers from bacteria and archaea (Messner and Sleytr 1988; Sleytr et al. 1996; Schuster et al. 2005). Generally, in Gram-positive bacteria, a complete disintegration of S-layers into monomers can be obtained by treatment of intact cells or cell walls with high concentrations of hydrogen bond-breaking agents (e.g., urea, guanidine hydrochloride), because the S-layer proteins are not covalently linked to each other or to the supporting cell wall component.

From various extraction and disintegration experiments it can be concluded that the intersubunit bonds in the S-layer lattice are stronger than those binding the crystalline array to the supporting envelope layer (Sleytr 1975). Isolated S-layer proteins reassemble into two-dimensional arrays on removal of the disrupting agent used in the dissolution procedure (Fig. 16.1). The self-assembly products generated in suspension may have the form of flat sheets, open-ended cylinders, or closed vesicles (Sleytr 1978, 1981; Sleytr et al. 2003, 2005). Shape and size of the self-assembly products depend on several environmental parameters, such as temperature, pH, ion composition, and/or ionic strength (Sleytr et al. 1993; Schuster et al. 2005). Particularly recrystallization of S-layer subunits on technologically relevant substrates, such as silicon wavers, noble metals, or synthetic polymers, revealed a broad range of applications in nano(bio)technology (Pum and Sleytr 1995; Györvary et al. 2003; Sleytr et al. 2005).

Adsorption and recrystallization studies have shown that S-layer lattices are highly anisotropic structures with regard to their inner and outer surfaces. Most detailed studies on the recrystallization have been performed with S-layer lattices from *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) and *Geobacillus stearothermophilus* strains. It has been shown that the formation of coherent crystalline arrays on solid supports, lipid films, liposomes, and the air/water interface is initiated simultaneously at many randomly distributed nucleation points and proceeds in-plane until the crystalline domains meet, thus leading to a closed coherent mosaic of individual S-layer patches several micrometers in size (Pum and Sleytr 1995; Györvary et al. 2003; Sleytr et al. 2005).

Recrystallization of isolated S-layer subunits at the air/water interface and on lipid films has been shown to be an easy and reproducible way of producing coherent S-layer protein meshworks at large scale (Pum et al. 1993a; Pum and Sleytr 1994). As on solid supports, the defined orientation of S-layer lattices at liquid interfaces and lipid films is determined by the anisotropy in the physicochemical surface properties of the protein lattice. In *Bacillaceae* it has been shown that in vivo the N-terminal part of the S-layer proteins interacts via a lectin-type binding with heteropolysaccharides, so-called secondary cell wall polymers (SCWPs) which are covalently linked to the peptidoglycan-containing sacculi (see Chap. 3; Sára 2001).

For their exploitation as biomimetic linkers (Sleytr et al. 2004b), SCWPs are extracted from the peptidoglycan and the reducing end of the polymer chains is chemically modified to introduce an amino or thiol group (Mader et al. 2004; Györvary et al. 2003; Völlenkle et al. 2004). Solid supports (e.g., gold, polymers) coated with these modified SCWPs enable recrystallization of S-layer lattices with an orientation resembling the situation in vivo (Sára et al. 2005). This is particularly important for the use of recombinant S-layer proteins with functional groups exposed on the C-terminal part (for reviews see Sleytr et al. 2007b; Egelseer et al. 2008).

16.3 S-Layers as Matrix for the Immobilization of Functional Macromolecules and Nanoparticles

The bioinspired synthesis of metallic and semiconducting nanoparticles has attracted much attention over the last two decades. Nanoparticle arrays on S-layers may be either formed by wet chemical synthesis or, alternatively, by binding of preformed nanoparticles. In the wet chemical approach, self-assembled S-layer structures have been exposed to metal-salt solutions, such as tetrachloroauric(III) acid ($HAuCl_4$) solution, followed by slow reaction with a reducing agent such as hydrogen sulfide (H_2S) or by electron irradiation in an electron microscope (Shenton et al. 1997; Dieluweit et al. 1998; Mertig et al. 1999). Since the precipitation of the metals was confined to the pores of the S-layer lattice, the nanoparticles also resembled the distribution and morphology of the pores. In a more controlled and specific way of making highly ordered nanoparticle arrays, genetic approaches have been used for the construction of chimeric S-layer fusion proteins with polypeptides responsible for precipitation and biomineralization processes (Naik et al. 2002). Based on work on binding biomolecules, such as enzymes or antibodies, it has already been demonstrated that preformed metallic and semiconducting nanoparticles can be bound in regular arrangements on S-layers (Hall et al. 2001; Györvary et al. 2004). The pattern of bound molecules frequently reflects the lattice symmetry, the size of the morphological units, and the physicochemical properties of the array. Specific binding of molecules on S-layer lattices may be induced by different noncovalent forces or by genetically introduced functional domains (Moll et al. 2002). This approach offers the advantage that particle size and hence the contact distances of neighboring particle surfaces may be precisely controlled (Fig. 16.2). Both parameters are important for studying and exploiting quantum phenomena. In fact, the controlled binding of biomolecules, such as glucose oxidase, was developed more than two decades ago for amperometric and optical biosensors (for reviews see Pum et al. 1993b; Neubauer et al. 1993). Subsequently, a layer-by-layer technique was established allowing the fabrication of multi-enzyme biosensors (Neubauer et al. 1994).

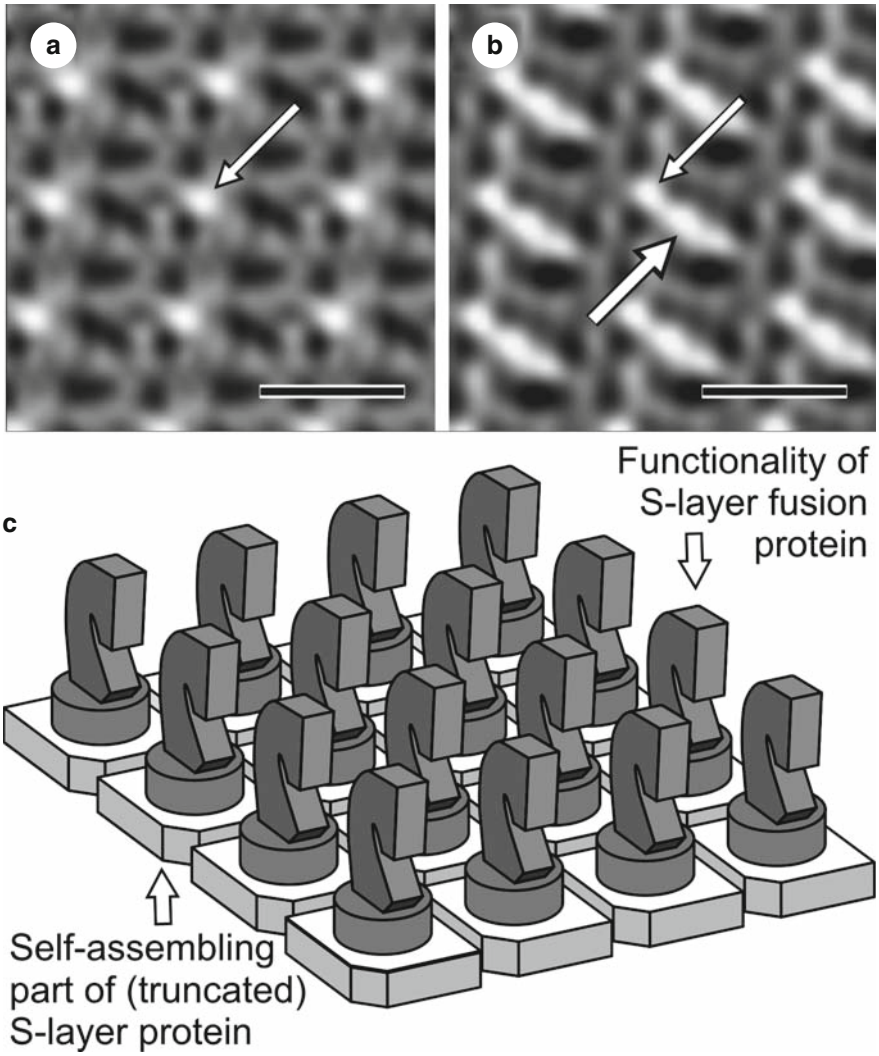

Fig. 16.2 Digital image reconstructions of transmission electron micrographs of negatively stained preparations of (**a**) the native S-layer protein SbsB from *Geobacillus stearothermophilus* PV72/p2 and (**b**) the SbsB streptavidin fusion protein (*bars* = 10 nm). In the lattice of the fusion protein (b), the streptavidin heterotetramers show up as additional mass (*arrows*). The schematic illustration shows the self-assembling part of S-layer fusion proteins and their well-oriented functional domains (**c**). Such arrays provide, theoretically, the highest possible order (spatial control and orientation) of functional domains at the nanometer scale. The knights resemble the functional domains (antigens, enzymes, antibodies, ligands, etc.) and the cut squares represent the S-layer. (Modified after Fig. 3 from Ref. Pum et al. 2006)

16.4 S-layer Fusion Proteins

One of the most relevant areas in nanobiotechnology concerns the technological utilization of self-assembly systems. Thus, S-layer technology has been advanced by the construction of recombinant functional S-layer fusion proteins comprising (1) the N-terminal cell wall anchoring domain, (2) the self-assembly domain, and (3) a functional sequence introduced by genetic engineering (Sleytr et al. 2007a, b; Egelseer et al. 2008). A great variety of functional S-layer fusion proteins has been cloned and heterologously expressed in *Escherichia coli* (Table 16.1). Based on the high density and regular display of the introduced functions when self-assembled in suspension or on various supports, a broad range of applications of S-layer fusion proteins is envisaged, particularly in the fields of biotechnology, molecular nanotechnology, and biomimetics (Sára et al. 2006; Sleytr et al. 2007a, b).

Table 16.1 Properties of S-layer fusion proteins

S-layer fusion protein	Length of function	Functionality	Reference
$rSbsB_{1–889}$/core streptavidin $rSbpA_{31–1068}$/core streptavidin	118 amino acids (aa)	Binding of biotinylated molecules	Moll et al. (2002); Huber et al. (2006)
$rSbpA_{31–1068}$/Bet v 1 $rSbsC_{31–920}$/Bet v 1	116 aa	Birch pollen allergen for immunomodulation	Ilk et al. (2002); Breitwieser et al. (2002)
rSbpA31–1268/ Strep-tag $rSbpA_{31–1068}$/ Strep-tag	9 aa	Affinity tag for streptavidin	Ilk et al. 2002
$rSbpA_{31–1068}$/ZZ	116 aa	Binding of IgG	Völlenkle et al. (2004)
$rSbpA_{31–1068}$/EGFP	238 aa	Intrinsic fluorescence	Ilk et al. (2004)
$rSbpA_{31–1068}$/cAb	117 aa	Heavy chain camel antibody recognizing lysozyme	Pleschberger et al. (2003)
$rSbpA_{31–1068}$/cAb	117 aa	Heavy chain camel antibody recognizing PSA	Pleschberger et al. (2004)
$rSbpA_{31–1068}$/AG4 and AGP35 $rSbpA_{31–1068}$/CO2P2	12 aa 12 aa	Binding of silver Binding of cobalt	Naik et al. 2002; Personal communication
$rSbpA_{31–1068}$/LamA	263 aa	(Hyper)thermophilic enzyme activity	Tschiggerl et al. (2008a)
$rSgsE_{331–903}$/RmlA	299 aa	Enzyme activity (biocatalyst)	Schäffer et al. (2007)
SbsA/Omp26	200 aa	Vaccine against *Haemophilus influenzae* infection	Riedmann et al. (2003)
$rSbpA_{31–1068}$/F1 $rSbsB_{1–889}$/F1	20 aa	Antibody binding for EBV diagnostics	Tschiggerl et al. (2008b)

Mature S-layer proteins:
SbpA of *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) CCM 2177 (1,238 aa)
SbsB of *Geobacillus stearothermophilus* PV72/p2 (889 aa)
SbsC of *G. stearothermophilus* ATCC 12980 (1,099 aa)
SgsE of *G. stearothermophilus* NRS 2004/3a (903 aa)
SbsA of *G. stearothermophilus* PV72/p6 (1,228 aa)

To generate an universal affinity matrix for binding biotinylated molecules, S-layer streptavidin fusion proteins have been constructed (Huber et al. 2006; Moll et al. 2002). Minimum-sized core streptavidin has been fused either to the N-terminus of the S-layer protein SbsB from *G. stearothermophilus* PV72/p2 or to the C-terminus of a truncated form of SbpA from *L. sphaericus* CCM 2177 (see Table 16.1). Functional heterotetramers (HT) have been obtained by mixing excess of core streptavidin with the fusion proteins, followed by a protein refolding procedure. Analysis of negatively stained preparations of self-assembly products formed by these S-layer fusion proteins revealed that neither the oblique S-layer lattice of SbsB (Fig. 16.2), nor the square lattice of SbpA, was changed due to the presence of streptavidin. Hybridization experiments with biotinylated and fluorescence-labeled oligonucleotides have been performed by means of surface-plasmon-field-enhanced fluorescence spectroscopy and surface plasmon resonance (SPR) spectroscopy, and demonstrated that a functional sensor surface can be generated by the recrystallization of HT on gold chips (Huber et al. 2006).

To generate the S-layer fusion protein rSbpA/ZZ, two copies of the Fc-binding domain (ZZ), a synthetic analog of the IgG binding domain of protein A from *Staphylococcus aureus*, were incorporated (Völlenkle et al. 2004). On average, approximately 66% of the theoretical saturation capacity of a planar surface was covered by IgG with Fab regions in the condensed state. By recrystallization of rSbpA/ZZ on microbeads, a biocompatible matrix for the microsphere-based detoxification system used for extracorporeal blood purification of patients suffering from autoimmune disease has been generated (Völlenkle et al. 2004).

The two chimeric S-layer proteins $rSbpA_{31–1068}$/Bet v 1 and $rSbsC_{31–920}$/Bet v 1 have been generated, each carrying the major birch pollen allergen Bet v 1 at the C-terminus (Table 16.1), maintaining not only the ability to self-assemble but also the functionality of the fused allergen to bind the Bet v 1-specific monoclonal mouse antibody (Breitwieser et al. 2002; Ilk et al. 2002). In cells of birch pollen-allergic individuals, the histamine-releasing capacity induced by the S-layer fusion protein was significantly reduced compared to stimulation with free Bet v 1 and no Th2-like immune response was observed (Bohle et al. 2004; Gerstmayr et al. 2007). Due to its immunomodulating capacity, this fusion protein is generally considered to be a promising approach for specific treatment of allergic diseases (Sleytr et al. 2007b).

The S-layer fusion protein comprising the C-terminally truncated form of SbpA, $rSbpA_{31–1068}$, and the hypervariable region of heavy chain camel antibodies recognizing a prostate-specific antigen (PSA), has been constructed. At least three of four possible PSA molecules can be bound per morphological unit of the square lattice (Pleschberger et al. 2003, 2004). The fused ligands on the S-layer lattice show a well-defined spatial distribution down to the sub-nanometer scale, which might reduce diffusion limited reactions. These S-layer fusion proteins can be considered as sensing layers for label-free detection systems such as SPR, surface acoustic wave, or quartz crystal microbalance, where the specific binding event can be directly measured by the increase in mass (Sleytr et al. 2007a).

A completely new field of applications may emerge from the recent evaluation of S-layers as a combined carrier/patterning element for the conceptualization of

novel types of biocatalysts, aiming at controllable display of biocatalytic epitopes, storage stability, and reuse (Schäffer et al. 2007; Tschiggerl et al. 2008a). Currently used immobilization methods are based on the adsorption or covalent binding of enzymes to water-insoluble carriers, the incorporation of enzymes into semi-permeable gels, and the enclosure of enzymes in polymer membranes (Angenendt 2005; Zhu and Snyder 2003). Due to the diversity of (nano)biotechnological processes, the development of novel carriers is still a focus of recent attention, with the major goals of improving enzyme performance and reducing process costs. Based on the demonstrated suitability of the S-layer protein self-assembly system for covalent enzyme immobilization (Neubauer et al. 1994; Pum et al. 1993b; Sára et al. 1993), a genetic engineering, bottom-up approach has been chosen to construct multi-domain proteins from a selected, self-assembling portion of an S-layer protein and an enzyme. This concept has been exemplified on two different S-layer/enzyme fusion proteins: the enzymes glucose-1-phosphate thymidylyltransferase RmlA from *G. stearothermophilus* NRS 2004/3a and the extremophilic β-1, 3-endogluanase LamA from *Pyrococcus furiosus* have been translationally fused to the C-terminus of the S-layer proteins $SgsE_{31–773}$ or $SgsE_{31–573}$ from *G. stearothermophilus* NRS 2004/3a (Schäffer et al. 2007) and $SbpA_{31–1068}$ from *L. sphaericus* CCM 2177 (Tschiggerl et al. 2008a), respectively. Triggered by the intrinsic self-assembly property of the chimeric S-layer monomers into an oblique (SgsE) or square (SbpA) 2D crystalline array with a periodicity in the nanometer scale, two types of S-layer biocatalysts have been constructed: (1) self-assembled biocatalysts in solution, and (2) self-assembled biocatalysts on diverse supports, such as liposomes, planar glass slides, or porous polymer membranes. It is noteworthy that the measured enzyme activity of the recrystallized S-layer/enzyme fusion proteins can reach 100% of that of the native enzyme. The S-layer protein part of the biocatalysts confers significantly improved shelf-life of the fused enzyme without loss of activity over more than three months, and also enables biocatalyst recycling. Significant advantages for enzyme immobilization by the S-layer self-assembly system include (1) the requirement of only a simple, one-step incubation process for site-directed immobilization without preceding surface activation of the support, (2) the provision of a cushion for the enzyme through the S-layer moiety of the fusion protein preventing denaturation and consequently loss of enzyme activity upon immobilization, (3) the principal applicability of the "S-layer tag" to any enzyme, (4) the high flexibility for variation of enzymatic groups within a single S-layer array by co-crystallization of different enzyme/S-layer fusion proteins to construct multifunctional, nanopatterned biocatalysts, and (5) the possibility for deposition of the biocatalysts on different supports with the additional option of cross-linking of individual monomers to improve robustness. Liposome-type biocatalysts in particular could make valuable contributions to the fields of nanomedicine, pharmacy, and also nutrition, where engineering of multifunctional nanocarriers combining properties such as targetability, longevity, and loading is in great demand (Koo et al. 2005). To produce essentially endotoxin-free biocatalysts for such applications, recombinant S-layer protein production has been set up in *Lactococcus lactis* (Novotny et al. 2005). Enzyme immobilization on membranes

also constitutes an interesting area of applied research, because such microporous composites favor easy flow of substrates and products (Bora et al. 2006), and may be integrated in more complex processes, where combination of a catalytic function with a conventional filtration function is required (Hilal et al. 2006; Pum et al. 1993b; Sára et al. 1993). Despite none of the constructed nanopatterned biocatalysts representing a fully developed product, it is expected that these novel, S-layer-based architectures can be optimized by directed evolution and will open up new functional concepts for biocatalytic applications.

Current studies focus on the production of S-layer fusion proteins consisting of the S-layer proteins *L. sphaericus* CCM 2177 and *G. stearothermophilus* PV72/p2 and the peptide mimotope F1 which mimics an immunodominant epitope of Epstein–Barr virus (EBV) (Tschiggerl et al. 2008b). Diagnostic studies have been performed by screening 83 individual EBV IgM-positive, EBV-negative, and potential cross-reactive sera and resulted in 98.2% specificity and 89.3% sensitivity as well as no cross-reactivity with related viral diseases. This result demonstrates the potential of these S-layer fusion proteins to act as a matrix for site-directed immobilization of small ligands in solid-phase immunoassays (Tschiggerl et al. 2008b)

Finally, the uptake of vesicular structures into eukaryotic cells has been visualized by means of liposomes which have previously been coated with the fluorescent S-layer fusion protein $rSbpA_{31–1068}$/EGFP (enhanced green fluorescent protein) (Ilk et al. 2004).

16.5 S-Layers as Carrier/Adjuvant for Immunotherapy

Basic research on bacterial S-layers as attenuated pathogens, antigen/hapten carrier, adjuvants, or as part of vaccination vesicles has progressed in three areas of application: (1) antibacterial vaccines, (2) immunotherapy of cancers, and (3) anti-allergic immunotherapy (Table 16.2) (for review see Messner et al. 1996; Sleytr et al. 2003).

For the development of fish vaccines to fight *Aeromonas* infections which can cause furunculosis in fish, freshwater, and marine environments, the S-layer protein of the fish-pathogenic bacteria itself is essential for virulence and has been considered to constitute a good vaccine candidate (Ford and Thune 1992) (Table 16.3).

Table 16.2 S-layers in vaccine development

S-layer properties	Preparations
Attenuated pathogen	S-layers on whole cells, cell sonicates, crude cellular preparations
Antigen/hapten carrier	Chemical coupling of antigen/hapten to S-layers, S-layer antigen/hapten fusion proteins
Adjuvant	Exploitation of the immunomodulating properties of the S-layer moiety in S-layer conjugates and fusion proteins
Part of vaccination vehicles	liposomes coated with S-layer (fusion) proteins and bacterial cells, self-assembly products in bacterial ghosts (vaccine delivery system)

Table 16.3 Immunogenic S-layer (fusion) proteins

Antigen/hapten	Conjugation	S-layer source (S-layer protein)	References
None	Sole S-layer	*Aeromonas salmonicida* A449, *A. hydrophila* TF7	Ford and Thune (1992)
Hematopoietic necrosis virus glycoprotein segment	S-layer fusion protein[a]	*Caulobacter vibrioides*[c] JS 4011 (RsaA)	Simon et al. (2001)
Streptococcus pneumoniae serotype 8 poly- and oligosaccharides	Chemically coupled hapten	*Paenibacillus alvei*[d] CCM 2051	Malcolm et al. (1993a, b)
Tumor marker T-disaccharide	Chemically coupled hapten	*Thermoanaerobacter thermohydrosulfuricus*[e] L111-69, *Geobacillus stearothermophilus* NRS 2004/3a, *P. alvei*[d] CCM 2051	Messner et al. (1992)
Tumor-associated Lewis Y (Le^y) tetrasaccharides	Chemically coupled hapten	*G. stearothermophilus* PV72/p6	Smith et al. (1993)
Major birch pollen allergen, Bet v 1	Chemically coupled antigen	*L. sphaericus*[f] CCM 2177, *T. thermohydrosulfuricus*[e] L111-69 and L110-69,	Jahn-Schmid et al. (1996a, b, 1997)
Major birch pollen allergen, Bet v 1	S-layer fusion protein[b]	*L. sphaericus*[f] CCM 2177, *G. stearothermophilus* ATCC 12980	Breitwieser et al. (2002); Ilk et al. (2002); Bohle et al. (2004)
H. influenzae antigen, Omp26	S-layer fusion protein[b]	*G. stearothermophilus* PV72/p6	Riedmann et al. (2003)
Adhesintope of the *Pseudomonas aeruginosa* pilin	S-layer fusion protein[a]	*C. vibrioides*[c] JS 4011 (RsaA)	Umelo-Njaka et al. (2001)
Human *c-myc* protooncogene	S-layer fusion protein[a]	*Lactobacillus brevis* ATCC 8287 (GRL1) (SlpA)	Åvall-Jääskeläinen et al. (2002)
Tetanus toxin fragment, ToxC	S-layer fusion protein[a]	*Bacillus anthracis* RPL686 (EA1)	Mesnage et al. (1999)
Mycobacterium tuberculosis protein, mpt64	S-layer fusion protein[b]	*L. sphaericus*[f] CCM 2177, *G. stearothermophilus* PV72/p2	Tschiggerl et al. (2008b)

[a]surface display of S-layer fusion proteins
[b]in vitro formation of immunogenic S-layers
[c]formerly *Caulobacter crescentus*
[d]formerly *Bacillus alvei*
[e]formerly *Clostridium thermohydrosulfuricum*
[f]formerly *B. sphaericus*

S-layers may be deployed as a carrier for chemically coupled immunogenic antigens and haptens (Sleytr et al. 1991, 1993). Since common carriers for peptide epitopes are used as monomers in solution (e.g., tetanus or diphtheria toxoids) or as dispersions of unstructured aggregates on aluminium salts, reproducible immobilization of ligands to the carrier protein cannot be achieved (Brown et al. 1993; Powell and Newman 1995). Consequently, the applicability of regularly structured S-layer self-assembly products as immobilization matrices represents a highly promising approach. One project has been directed at immunotherapy of cancer, where conjugates of S-layer with small, tumor-associated oligosaccharides have been found to elicit hapten-specific delay-type hypersensitivity (DTH) responses (Smith et al. 1993). Further investigations focused on the development of several model conjugate vaccines with S-layer (glyco)proteins of thermophilic bacilli and clostridia, and weekly immunogenic carbohydrate antigens, e.g., *Streptococcus pneumoniae* serotype 8 poly- and oligosaccharides, haptens, or recombinant birch pollen allergen have given promising results at vaccination trials (Jahn-Schmid et al. 1997; Malcolm et al. 1993a, b; Messner and Sleytr 1992; Messner et al. 1996; Smith et al. 1993) (Table 16.3). Furthermore, there is an urgent need for new vaccines allowing mucosal administration instead of intramuscular injections to achieve desired specific effects, such as adjuvant targeting, site-specific delivery, and controlled immune responses. Significant vaccination responses have been induced even after oral/nasal administration of S-layer/hapten conjugates. To overcome the disadvantage of using potentially toxic chemicals during the coupling procedure, a challenging approach can be seen in the production of recombinant S-layer fusion proteins comprising immunogenic sequences (Table 16.3). Allergen/S-layer conjugates and fusion proteins have been prepared with the intention of suppressing the Th2-directed, IgE-mediated allergic responses to Bet v 1, the major allergen of birch pollen. Studies have been performed resulting in the production of S-layer protein conjugate induced interferon-γ (IFN-γ), thus activating the phagocytotic cells and confirming the Th1-enhancing properties of the S-layer protein conjugate (Jahn-Schmid et al. 1996a, b). Furthermore, the recombinant allergen–S-layer fusion protein rSbsC/Bet v 1 altered an established Th2-dominated phenotype as well as the de novo cytokine secretion profile toward a more balanced Th1/Th0-like phenotype (Bohle et al. 2004; Gerstmayr et al. 2007). These studies have clearly demonstrated the immunomodulating properties of the S-layer moiety in S-layer allergen conjugates.

Recombinant S-layer fusion proteins and empty bacterial cell envelopes (ghosts) have been investigated for possible antigens (Omp26) acting as vaccines against nontypeable *Haemophilus influenzae* (NTHi) infection. The bacterial ghost system inducing Omp26-specific antibody response in mice is a vaccine delivery system endowed with intrinsic adjuvant properties (Riedmann et al. 2003).

For the development of vaccination vehicles with enhanced immune response to an antigen, S-layers have been explored with the intrinsic property of forming highly ordered displays of foreign peptides like the pilus tip epitope (adhesintope) of the *Pseudomonas aeruginosa* pilin on the *Caulobacter vibrioides* (formerly *Caulobacter crescentus*) cell surface (Bingle et al. 1997; Umelo-Njaka et al. 2001).

This presentation system may have many potential applications, such as whole-cell vaccines, tumor suppressors, cellular adsorbents, and peptide display libraries (Bhatnagar et al. 2006; Georgiou et al. 1997; Nomellini et al. 2004; Simon et al. 2001). Furthermore, the eleven amino acids epitope c-myc from the human c-*myc* proto-oncogene has been successfully expressed in every S-layer subunit of the *Lactobacillus brevis* S-layer (SlpA) (Åvall-Jääskeläinen et al. 2002). Delivery of antigens to mucosal surfaces by lactic acid bacteria can be considered a safe alternative to live attenuated pathogens because of their GRAS status. In another study, a recombinant *Bacillus anthracis* strain has been constructed by integrating a translational fusion harboring DNA fragments encoding the cell-wall targeting domain of the S-layer protein EA1 and tetanus toxin fragment C (ToxC). The humoral immune response has been studied and was sufficient to protect mice against tetanus toxin challenge (Mesnage et al. 1999). Current vaccination studies using immunogenic self-assembly products of S-layer fusion proteins comprising the antigen mpt64, a *Mycobacterium tuberculosis* protein, are investigating the ability of S-layer proteins to serve as a carrier and adjuvant for vaccination against tuberculosis (Tschiggerl et al. 2008b).

16.6 S-Layers as Naturally Tailored Ultrafiltration Membranes

Studies on the permeability properties of isolated S-layers from *Bacillaceae* have shown that the protein lattices function as isoporous molecular sieves in the ultrafiltration range (Sára and Sleytr 1987a). This observation has led to the development of a completely new type of ultrafiltration membrane (Sára and Sleytr 1987b). Unlike S-layer lattices which show a very sharp exclusion limit, conventional ultrafiltration membranes produced of synthetic polymers reveal a wide pore size distribution with pores differing as much as one order of magnitude. The heteroporous structure of conventional ultrafiltration membranes is also responsible for their less accurate separation characteristics, low resolution of protein mixtures, uneven pore clogging and inhomogeneous flux distribution (Sára et al. 1996).

For the production of S-layer ultrafiltration membranes (SUMs), S-layers or S-layer coated cell wall fragments from *Bacillaceae* were deposited on commercial microfiltration membranes with either a spongy open cell structure and a variable pore size or a smooth surface and a well-defined pore size, such as radiation track membranes. Subsequently, the S-layer protein was inter- and intramolecularly cross-linked with glutaraldehyde, and Schiff bases were reduced with sodium borohydrate to increase the mechanical and thermal resistance of the active filtration layer. The nominal molecular weight cut-off of SUMs from S-layers of a variety of mesophilic and thermophilic *Lysinibacillus* and *Geobacillus* strains has been determined to be in the range of 30,000–40,000 Da (Sára and Sleytr 1987b, 1988; Sára et al. 1988, 1996). The porosity of S-layers lies between 30% and

50% and, therefore, is significantly higher than that measured for polymeric ultrafiltration membranes with a maximum in the region of 10%.

Since S-layers are periodic structures composed of identical subunits, functional groups on the polypeptide chains (e.g., carboxyl, amino, hydroxyl groups) are located on each constituent subunit at an identical position and identical orientation. This uniformity of reactive groups on the surface and the pores of the S-layer lattice provide possibilities for a broad range of chemical modifications for obtaining differently charged or hydrophilic and hydrophobic membranes (Küpcü et al. 1991, 1993; Sára et al. 1996). Different surface properties of ultrafiltration membranes are of particular interest for minimizing membrane adsorption (fouling), and thus leading to a increase in membrane performance (Weigert and Sára 1995). By immobilizing molecules of different size in the pore areas, SUMs with different molecular sieving properties and accurate cut-off values can be obtained (Sára et al. 1993). In this context it is interesting to note that native S-layers reveal a remarkable antifouling characteristic which is seen as an essential requirement to prevent pore plugging and to maintain a free exchange of molecules up to a defined molecular weight in living cells.

16.7 S-Layer-Stabilized Liposomes and Lipid Membranes

One promising approach for the generation of biomimetic membrane systems includes the stabilization of lipid membranes with S-layer lattices. These composite structures mimic the supramolecular assembly of archaeal cell envelopes which are composed of a cytoplasmic membrane and a closely associated S-layer as exclusive wall component (König 1988; Schuster and Sleytr 2005, 2006). In this biomimetic architecture, artificial or isolated purified lipids replace the cytoplasmic membrane, and isolated or recombinantly produced S-layer proteins are attached on either one or both sides of the lipid membrane. By a combination of microscopic, spectroscopic and surface sensitive (scattering) techniques, at least two or three contact points have been proposed between the adjacent lipid monolayer and the attached S-layer protein (Weygand et al. 2000; Wetzer et al. 1998). Thus, S-layer-supported lipid membranes, also referred to as "semifluid lipid membranes" (Pum and Sleytr 1994), possess nanopatterned properties where less than 5% of the lipid molecules are anchored to protein domains on the S-layer lattice while the remaining $\geq$95% lipid molecules may diffuse freely in the membrane between pillars consisting of anchored lipid molecules (Schuster and Sleytr 2006; Schuster 2005).

S-layer-coated liposomes (Fig. 16.1f) are biomimetic structures resembling the building principle of archaeal cells or virus envelopes. S-layer proteins or fusion proteins, once crystallized on liposomes, can be cross-linked and exploited as matrix for the covalent attachment of functional molecules as required for drug-targeting or immunodiagnostic assays (Küpcü et al. 1995, 1996; Nomellini et al. 1997; Mader et al. 2000). Furthermore, a general stabilization of the whole

composite structure has been achieved by coating liposomes with S-layer proteins (Mader et al. 1999).

The high mechanical and thermal stability of S-layer-coated liposomes and the possibility of immobilizing or entrapping biologically active molecules (Mader et al. 1999, 2000; Moll et al. 2002) reveal a broad application potential, particularly as carrier and/or drug delivery systems, as artificial viruses, and for medicinal applications such as drug targeting systems or in gene therapy (Sára et al. 2005, Schuster and Sleytr 2006; Sleytr et al. 2003, 2004a, b, c, 2007a).

Planar-supported lipid membranes have been generated across an aperture, on S-layer ultrafiltration membranes (SUMs; see section 19.6), and on S-layer coated electrodes or structured silicon chips, with the S-layer functioning as a stabilizing and biomimetic layer (Schuster et al. 2001; Gufler et al. 2004). S-layer-coated silicon chips with attached lipid membranes are also referred to as lipid chips and, combined with microfluidics, these platforms constitute a prerequisite for the lab-on-a-chip technology (Bayley and Cremer 2001). The electrochemical properties of S-layer-supported lipid membranes on porous and solid supports (Fig. 16.3) are comparable with those of free-standing lipid membranes (Schuster et al. 2001, 2003; Gufler et al. 2004). In addition, membranes on S-layer-covered gold electrodes exhibit a remarkable long-term robustness of up to 1 week, which is not feasible with any other stabilization technique. The functionality of lipid membranes resting on SUMs and S-layer-covered gold electrodes has been demonstrated by the reconstitution of the pore-forming protein α-hemolysin and membrane-active peptides like alamethicin, gramicidin, or valinomycin (Schuster et al. 1998, 2001, 2003; Gufler et al. 2004). Recently, even recordings on single pores have been performed with α-hemolysin

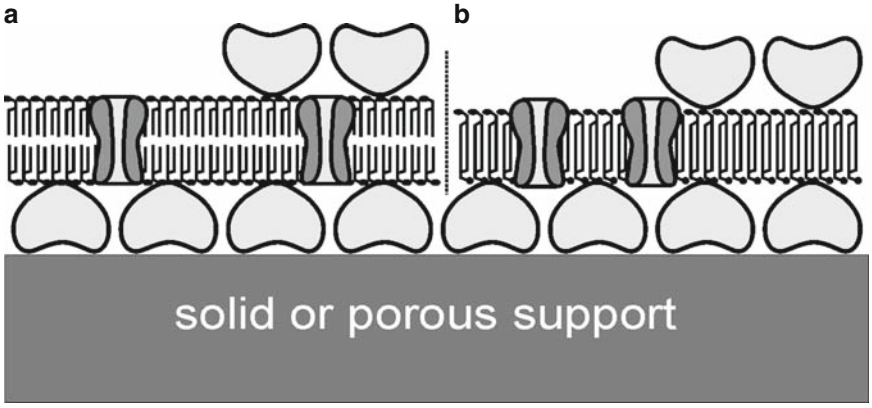

Fig. 16.3 Schematic drawing of an S-layer-coated solid (e.g., gold electrode, sensor surface) or porous support onto which a phospholipid bilayer (**a**) or a tetraetherlipid monolayer (**b**) has been generated. Integral membrane proteins can be reconstituted into this S-layer supported lipid membrane. Furthermore, a second S-layer lattice can be recrystallized on the top of this biomimetic structure to provide enhanced long-term stability and to act as a protective coat with pores in the nanometer range. (Modified after Sleytr et al. 2003)

and gramicidin reconstituted in S-layer-supported lipid membranes (Schuster et al. 2001, 2003; Schuster and Sleytr 2002).

These results have demonstrated that the biomimetic approach of copying the supramolecular architecture of archaeal cell envelopes opens new possibilities for exploiting functional lipid membranes at the meso- and macroscopic scale. Moreover, this technology has the potential to initiate a broad range of developments in many areas such as diagnostics, high-throughput screening for drug discovery, membrane protein-based sensor technology, electronic and optical devices, and might even find application in DNA sequencing (Sleytr et al. 2003, 2004c; Schuster 2005; Schuster and Sleytr 2005, 2006; Kasianowicz et al. 1996; Meller et al. 2000).

16.8 Functional S-Layer Neoglycoproteins

Since the 1970s it has been known that S-layer proteins can be modified with covalently attached glycans (see Chap. 3). S-layer glycoproteins have been identified in all major lineages of Archaea (Mescher and Strominger 1976; Wieland et al. 1982) and in a few Gram-positive organisms of the domain Bacteria, affiliated to the *Bacillaceae* family (Sleytr and Thorne 1976, Messner and Schäffer 2003; Schäffer and Messner 2004; Messner et al. 2008). Only very recently, the first studies have been reported on the occurrence of S-layer glycoproteins in the Gram-negative species *Tannerella forsythia* (Lee et al. 2006) and *Bacteroides distasonis* (Fletcher et al. 2007). In contrast to the S-layer glycoproteins of *Bacillaceae* (Messner et al. 2008) investigated to date, these glycoproteins originate from potential pathogens and, therefore, exploitation of their S-layer nanoglycobiology might be of medical relevance (Messner 2004). Generally, S-layer glycosylation might be a relevant virulence factor in pathogenic organisms.

S-layer nanoglycobiology (Messner et al. 2008) accounts for the nanometer-scale cell surface display feature of S-layer protein glycosylation and includes structural and biochemical aspects of S-layer glycans as well as molecular data on the machinery underlying the glycosylation event. The concept of S-layer protein modification with glycans was developed when the first attempts to construct model vaccines based on the use of S-layer proteins as immobilization matrix for glycans were successful (Sleytr et al. 1991; Messner et al. 1992; see also Sect. 19.5). A key aspect for the full potency of S-layer nanoglycobiology for applied research is the unique self-assembly feature of the S-layer protein matrix (Sleytr et al. 2005; Schäffer and Messner 2004; see also Chap. 3), because molecular self-assembly systems that exploit the molecular scale manufacturing precision of biological systems are prime candidates in nanobiotechnology. Knowing that in many cases the glycan structures associated with a protein are the key to protein function (Spiro 2002), tailor-made ("functional") S-layer protein glycosylation will add a new and very valuable component to an "S-layer based molecular construction kit" (Sleytr et al. 2007a, b). It is conceivable that this technology, as was the case for DNA and protein engineering, will become an important tool, both in basic

and applied S-layer research, for analyzing the S-layer protein glycosylation process and for artificially equipping S-layer proteins with "functional" glycosylation motifs for (nano)biotechnological or biomedical purposes. Engineering of tailor-made self-assembly S-layer neoglycoproteins will decisively change our capabilities to influence and control complex biological systems and to conceive novel self-assembly nanomaterials.

As a necessary prerequisite for the design of S-layer neoglycoproteins, based on the considerable body of S-layer glycan structures from organisms of the *Bacillaceae* family (see Chap. 3), investigation of the S-layer protein glycosylation process at the molecular level was initiated about 10 years ago. This endeavor has been lagging behind the structural work due to the lack of suitable molecular tools. In 2002, the first S-layer gene sequence of the S-layer glycoprotein-carrying bacterium *G. stearothermophilus* NRS 2004/3a became available (Schäffer et al. 2002), and shortly afterwards it was evident that S-layer protein glycosylation of the investigated bacterium is encoded by an S-layer glycosylation (*slg*) gene cluster (Novotny et al. 2004). Only recently, the initiation enzyme as a first key module for S-layer protein glycosylation and, consequently, also for glyco-engineering of S-layer proteins was functionally characterized (Steiner et al. 2007).

Our approach for "functional" S-layer protein glycosylation is the utilization of the native glycosylation sites on the protein, because these sites can be expected to be located in surface-exposed loops within the bulk of the S-layer protein, allowing the display of attached glycans. The research strategy follows two principal lines of development. The first one is the in vivo display of glycoproteins with specific functions on the surface of bacteria enabled by means of recombinant DNA technology. This has become an increasingly used strategy in various applications in microbiology, nanobiotechnology, and vaccinology (Samuelson et al. 2002, Campbell et al. 2007; Thibodeaux et al. 2007), with the S-layer display system being a very attractive and promising alternative. The second strategy concerns the in vitro line of development utilizing the recrystallization capability of the S-layer protein matrix on various supports. In both lines, the S-layer "anchor" offers the unique advantage of providing a crystalline, regularly arrayed immobilization matrix that should eventually allow the controlled and periodic surface display of "functional" glycosylation motifs. Nanobiotechnological applications of tailored S-layer *neo*glycoproteins may include the fields of receptor mimics, vaccine design, and drug delivery using carbohydrate recognition.

16.9 Conclusions

Nanobiotechnology as a multidisciplinary field covers a great variety of knowledge domains including biology, chemistry, physics, and material sciences.

One of the main strategies in nanobiotechnology concerns the design and creation of novel materials on the nanoscale, exploiting self-assembly systems with building blocks at the molecular level. It is now apparent that S-layer proteins are

exceptional proteins in their capability to self-assemble into monomolecular arrays, in suspension, and on surfaces and interfaces. Furthermore, S-layers stabilizing lipid membranes mimic the supramolecular assembly of archaeal cell envelopes.

Basic studies on the structure, chemistry, genetics, assembly, and function of S-layer proteins have demonstrated that they represent ideal patterning elements for nanobiotechnological and biomimetic applications. The repetitive structural and physicochemical properties and isoporosity of the (glyco)protein lattices make them ideal matrices and building blocks for generating highly reproducible layered and 3D supramolecular structures.

Moreover, S-layers have proven to be particularly suited to "bottom-up" strategies in combination of all major classes of biological molecules and nanoparticles.

During the last decade S-layer technologies have particularly advanced by the construction of S-layer fusion proteins comprising the self-assembly domain and a fused functional sequence (Egelseer et al. 2008; Sleytr et al. 2007a).

Although a broad range of applications for S-layer has been suggested, many other areas of utilization may emerge in life and nonlife sciences.

Acknowledgements Financial support from the Austrian Science Fund (FWF, projects P18013-B10 (P.M.), P18510-B12 (E.M.E.), P19047-B12 (C.S), and 20256-B11 (B.S.)), the Erwin-Schrödinger Society for Nanosciences, the FP6 EC STREP NASSAP (project 13352; to U.B.S.), the Hochschuljubiläumsstiftung der Stadt Wien, project H-1809/2006 (to C.S.), and the Air Force Office of Scientific Research, USA (AFOSR, projects F49620-03-1-0222 (U.B.S.), FA9550-06-1-0208 (D.P.), and FA9550-07-1-0313 (U.B.S.)) is gratefully acknowledged.

References

Angenendt P (2005) Progress in protein and antibody microarray technology. Drug Discov Today 10:503–511

Åvall-Jääskeläinen S, Kyla-Nikkila K, Kahala M, Miikkulainen-Lahti T, Palva A (2002) Surface display of foreign epitopes on the *Lactobacillus brevis* S-layer. Appl Environ Microbiol 68:5943–5951

Bayley H, Cremer PS (2001) Stochastic sensors inspired by biology. Nature 413:226–230

Bhatnagar PK, Awasthi A, Nomellini JF, Smit J, Suresh MR (2006) Anti-tumor effects of the bacterium *Caulobacter crescentus* in murine tumor models. Cancer Biol Ther 5:485–491

Bingle WH, Nomellini JF, Smit J (1997) Cell-surface display of a *Pseudomonas aeruginosa* strain K pilin peptide within the paracrystalline S-layer of *Caulobacter crescentus*. Mol Microbiol 26:277–288

Bohle B, Breitwieser A, Zwolfer B, Jahn-Schmid B, Sara M, Sleytr UB, Ebner C (2004) A novel approach to specific allergy treatment: the recombinant fusion protein of a bacterial cell surface (S-layer) protein and the major birch pollen allergen Bet v 1 (rSbsC-Bet v 1) combines reduced allergenicity with immunomodulating capacity. J Immunol 172:6642–6648

Bora U, Sharma P, Krishnamoorthy K, Nahar P (2006) Photoreactive cellulose membrane – a novel matrix for covalent immobilization of biomolecules. J Biotechnol 126:220–229

Breitwieser A, Egelseer EM, Moll D, Ilk N, Hotzy C, Bohle B, Ebner C, Sleytr UB, Sára M (2002) A recombinant bacterial cell surface (S-layer) – major birch pollen allergen-fusion protein (rSbsC/Bet v1) maintains the ability to self-assemble into regularly structured monomolecular lattices and the functionality of the allergen. Protein Eng 15:243–249

Brown FGD, Hoey EM, Martin S, Rima BK, Trudgett A (1993) Vaccine design. In: James K, Morris A (eds) Molecular medical science series. Wiley, Chichester, UK

Campbell CT, Sampathkumar S-G, Yarema KJ (2007) Metabolic oligosaccharide engineering: perspectives, applications, and future directions. Mol BioSyst 3:187–194

Dieluweit S, Pum D, Sleytr UB (1998) Formation of a gold superlattice on an S-layer with square lattice symmetry. Supramolec Sci 5:15–19

Egelseer EM, Sára M, Pum D, Schuster B, Sleytr UB (2008) Genetically engineered S-layer proteins, S-layer-specific polysaccharides and lipids as components of a versatile molecular construction kit for application in nanobiotechnology. In: Shoseyov O, Levy I (eds) NanoBio-Technology: bioinspired devices and materials of the future. Science of Knowledge Press, Little Falls, NJ, pp 55–86

Fletcher CM, Coyne MJ, Bentley DL, Villa OF, Comstock LE (2007) Phase-variable expression of a family of glycoproteins imparts a dynamic surface to a symbiont in its human intestinal ecosystem. Proc Natl Acad Sci USA 104:2413–2418

Ford LA, Thune RL (1992) Immunization of channel catfish with crude, acid-extracted preparation of motile aeromonad S-layer protein. Biomed Lett 47:355–362

Georgiou G, Stathopoulos C, Daugherty PS, Nayak AR, Iverson BL, Curtis R (1997) Display of heterologous proteins on the surface of micro-organisms: from the screening of combinatorial libraries to live recombinant vaccines. Nat Biotechnol 15:29–34

Gerstmayr M, Ilk N, Schabussova I, Jahn-Schmid B, Egelseer EM, Sleytr UB, Ebner C, Bohle B (2007) A novel approach to specific allergy treatment: the recombinant allergen-S-layer fusion protein rSbsC-Bet v 1 matures dendritic cells that prime Th0/Th1 and IL-10-producing regulatory T cells. J Immunol 179:7270–7275

Gufler PC, Pum D, Sleytr UB, Schuster B (2004) Highly robust lipid membranes on crystalline S-layer supports investigated by electrochemical impedance spectroscopy. Biochim Biophys Acta 166:154–165

Györvary E, Stein O, Pum D, Sleytr UB (2003) Self-assembly and recrystallization of bacterial S-layer proteins at silicon supports imaged in real time by atomic force microscopy. J Microscopy 212:300–306

Györvary E, Schroedter A, Talapin DV, Weller H, Pum D, Sleytr UB (2004) Formation of nanoparticle arrays on S-layer protein lattices. J Nanosci Nanotech 4:115–120

Hall SR, Shenton W, Engelhardt H, Mann S (2001) Site-specific organization of gold nanoparticles by biomolecular templating. Chem Phys Phys Chem 3:184–186

Hilal N, Kochkodan V, Nigmatullin R, Goncharuk V, Al-Khatib L (2006) Lipase-immobilized biocatalytic membranes for enzymatic esterification: comparison of various approaches to membrane preparation. J Membrane Sci 268:198–207

Huber C, Liu J, Egelseer EM, Moll D, Knoll W, Sleytr UB, Sára M (2006) Heterotetramers formed by an S-layer-streptavidin fusion protein and core-streptavidin as nanoarrayed template for biochip development. Small 2:142–150

Ilk N, Völlenkle C, Egelseer EM, Breitwieser A, Sleytr UB, Sára M (2002) Molecular characterization of the S-layer gene, sbpA, of *Bacillus sphaericus* CCM 2177 and production of a functional S-layer fusion protein with the ability to recrystallize in a defined orientation while presenting the fused allergen. Appl Environ Microbiol 68:3251–3260

Ilk N, Küpcü S, Moncayo G, Klimt S, Ecker RC, Hofer-Warbinek R, Egelseer EM, Sleytr UB, Sára M (2004) A functional chimaeric S-layer-enhanced green fluorescent protein to follow the uptake of S-layer-coated liposomes into eukaryotic cells. Biochem J 379:441–448

Jahn-Schmid B, Graninger M, Glozik M, Küpcü S, Ebner C, Unger FM, Sleytr UB, Messner P (1996a) Immunoreactivity of allergen (Bet v 1) conjugated to crystalline bacterial cell surface layers (S-layers). Immunotechnol 2:103–113

Jahn-Schmid B, Messner P, Unger FM, Sleytr UB, Scheiner O, Kraft D (1996b) Toward selective elicitation of TH1-controlled vaccination responses: vaccine applications of bacterial surface layer proteins. J Biotechnol 44:225–231

Jahn-Schmid B, Siemann U, Zenker A, Bohle B, Messner P, Unger FM, Sleytr UB, Scheiner O, Kraft D, Ebner C (1997) Bet v 1, the major birch pollen allergen, conjugated to crystalline

bacterial cell surface proteins, expands allergen-specific T cells of the Th1/Th0 phenotype in vitro by induction of IL-12. Int Immunol 9:1867–1874
Kasianowicz JJ, Brandin E, Branton D, Deamer D (1996) Characterization of individual polynucleotide molecules using a membrane channel. Proc Natl Acad Sci USA 93:13770–13773
König H (1988) Archaebacterial cell envelopes. Can J Microbiol 34:395–406
Koo OM, Rubinstein I, Onyuksel H (2005) Role of nanotechnology in targeted drug delivery and imaging: a concise review. Nanomedicine 1:193–212
Küpcü S, Sára M, Sleytr UB (1991) Chemical modification of crystalline ultrafiltration membranes and immobilization of macromolecules. J Membr Sci 61:167–175
Küpcü S, Sára M, Sleytr UB (1993) Influence of covalent attachment of low molecular weight substances on the rejection and adsorption properties of crystalline proteinaceous ultrafiltration membranes. Desalination 90:65–76
Küpcü S, Sára M, Sleytr UB (1995) Liposomes coated with crystalline bacterial cell surface protein (S-layer) as immobilization structures for macromolecules. Biochim Biophys Acta 1235:263–269
Küpcü S, Sleytr UB, Sára M (1996) Two-dimensional paracrystalline glycoprotein S-layers as a novel matrix for the immobilization of human IgG and their use as microparticles in immunoassays. J Immunol Methods 196:73–84
Lee S-W, Sabet M, Um H-S, Yang J, Kim HC, Zhu W (2006) Identification and characterization of the genes encoding a unique surface (S-) layer of *Tannerella forsythia*. Gene 371:102–111
Mader C, Küpcü S, Sára M, Sleytr UB (1999) Stabilizing effect of an S-layer on liposomes towards thermal or mechanical stress. Biochim Biophys Acta 1418:106–116
Mader C, Küpcü S, Sleytr UB, Sára M (2000) S-layer coated liposomes as a versatile system for entrapping and binding target molecules. Biochim Biophys Acta 1463:142–150
Mader C, Huber C, Moll D, Sleytr UB, Sára M (2004) Interaction of the crystalline bacterial cell surface layer protein SbsB and the secondary cell wall polymer of *Geobacillus stearothermophilus* PV72 assessed by real-time surface plasmon resonance biosensor technology. J Bacteriol 186:1758–1768
Malcolm AJ, Best MW, Szarka RJ, Mosleh Z, Unger FM, Messner P, Sleytr UB (1993a) Surface layers of *Bacillus alvei* as a carrier for a *Streptococcus pneunoniae* conjugate vaccine. In: Beveridge TJ, Koval SF (eds) Bacterial Paracrystalline Surface Layers. Plenum Press, New York, pp 219–233
Malcolm AJ, Messner P, Sleytr UB, Smith RH, Unger FM (1993b) Crystalline bacterial cell surface layers (S-layers) as combined carrier/adjuvants for conjugated vaccines. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Immobilized macromolecules: application potentials. Springer, London, UK, pp 195–207
Meller A, Nivon L, Brandin E, Golovchenko J, Branton D (2000) Rapid nanopore discrimination between single polynucleotide molecules. Proc Natl Acad Sci USA 97:1079–1084
Mertig M, Kirsch R, Pompe W, Engelhardt H (1999) Fabrication of highly oriented nanocluster arrays by biomolecular templating. Eur Phys J D 9:45–48
Mescher MF, Strominger JL (1976) Purification and characterization of a prokaryotic glycoprotein from the cell envelope of *Halobacterium salinarium*. J Biol Chem 251:2005–2014
Mesnage S, Weber-Levy M, Haustant M, Mock M, Fouet A (1999) Cell surface-exposed tetanus toxin fragment C produced by recombinant *Bacillus anthracis* protects against tetanus toxin. Infect Immun 67:4847–4850
Messner P (2004) Prokaryotic glycoproteins: unexplored but important. J Bacteriol 186:2517–2519
Messner P, Schäffer C (2003) Prokaryotic glycoproteins. In: Herz W, Falk H, Kirby GW (eds) Progress in the chemistry of organic natural products, vol 85. Springer, Wien, pp 51–124
Messner P, Sleytr UB (1988) Separation and purification of S-layers from Gram-positive and Gram-negative bacteria. In: Hancock IC, Poxton I (eds) Bacterial cell surface techniques. Wiley, Chichester, pp 97–104
Messner P, Sleytr UB (1992) Crystalline bacterial cell-surface layers. In: Rose AH (ed) Advances in microbial physiology, vol 33. Academic Press, London, pp 213–275

Messner P, Mazid MA, Unger FM, Sleytr UB (1992) Artificial antigens. Synthetic carbohydrate haptens immobilized on crystalline bacterial surface layer glycoproteins. Carbohydr Res 233:175–184

Messner P, Unger FM, Sleytr UB (1996) Vaccine development based on S-layer technology. In: Sleytr UB, Messner P, Pum D, Sara M (eds) Crystalline bacterial cell surface proteins. R.G. Landes, Academic Press, Austin, TX, pp 161–173

Messner P, Steiner K, Zarschler K, Schäffer C (2008) S-layer nanoglycobiology of bacteria. Carbohydr Res. doi:10.1016/j.carres.2007.12.025

Moll D, Huber C, Schlegel B, Pum D, Sleytr UB, Sára M (2002) S-layer-streptavidin fusion proteins as template for nanopatterned molecular arrays. Proc Natl Acad Sci USA 99:14646–14651

Naik RR, Stringer SJ, Agarwal G, Jones SE, Stone MO (2002) Biomimetic synthesis and patterning of silver nanoparticles. Nat Mater 1:169–172

Neubauer A, Pum D, Sleytr UB (1993) An amperometric glucose sensor based on isoporous crystalline protein membranes as immobilization matrix. Anal Lett 26:1347–1360

Neubauer A, Hödl C, Pum D, Sleytr UB (1994) A multistep enzyme sensor for sucrose based on S layer microparticles as immobilization matrix. Anal Lett 27:849–865

Nomellini JF, Küpcü S, Sleytr UB, Smit J (1997) Factors controlling *in vivo* recrystallization of the *Caulobacter crescentus* paracrystalline S-layer. J Bacteriol 179:6349–6354

Nomellini JF, Toporowski MC, Smit J (2004) Secretion or presentation of recombinant proteins and peptides mediated by the S-layer of *Caulobacter crescentus*. In: Banex F (ed) Expression technologies: current status and future trends. Horizon Scientific Press, Norfolk, UK, pp 477–524

Novotny R, Schäffer C, Strauss J, Messner P (2004) S-layer glycan-specific loci on the chromosome of *Geobacillus stearothermophilus* NRS 2004/3a and dTDP-L-rhamnose biosynthesis potential of *G. stearothermophilus* strains. Microbiology 150:953–965

Novotny R, Scheberl A, Giry-Laterriere M, Messner P, Schäffer C (2005) Gene cloning, functional expression and secretion of the S-layer protein SgsE from *Geobacillus stearothermophilus* NRS 2004/3a in *Lactococcus lactis*. FEMS Microbiol Lett 242:27–35

Pleschberger M, Neubauer A, Egelseer EM, Weigert S, Lindner B, Sleytr UB, Muyldermans S, Sara M (2003) Generation of a functional monomolecular protein lattice consisting of an s-layer fusion protein comprising the variable domain of a camel heavy chain antibody. Bioconjug Chem 14:440–448

Pleschberger M, Saerens D, Weigert S, Sleytr UB, Muyldermans S, Sára M, Egelseer EM (2004) An S-layer heavy chain camel antibody fusion protein for generation of a nanopatterned sensing layer to detect the prostate-specific antigen by surface plasmon resonance technology. Bioconjug Chem 15:664–671

Powell MF, Newman MJ (1995) Vaccine design: the subunit and adjuvant approach. Plenum Press, New York, USA

Pum D, Sleytr UB (1994) Large-scale reconstitution of crystalline bacterial surface layer proteins at the air/water interface and on lipid films. Thin Solid Films 244:882–886

Pum D, Sleytr UB (1995) Monomolecular reassembly of a crystalline bacterial cell surface layer (S-layer) on untreated and modified silicon surfaces. Supramol Science 2:193–197

Pum D, Weinhandl M, Hödl C, Sleytr UB (1993a) Large scale recrystallization of the S-layer of *Bacillus coagulans* 38–66 at the air/water interface and on lipid films. J Bacteriol 175:2762–2766

Pum D, Sára M, Sleytr UB (1993b) Two-dimensional (glyco)protein crystals as patterning elements and immobilisation matrices for the development of biosensors. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Immobilised macromolecules: application potentials. Springer, London, UK, pp 141–160

Pum D, Sàra M, Schuster B, Sleytr UB (2006) Bacterial surface layer proteins: a simple but versatile biological self-assembly system in nature. In: Chen J, Jonoska N, Rozenberg G (eds) Nanotechnology: science and computation. Springer, Berlin, pp 277–290

Riedmann EM, Kyd JM, Smith AM, Gomez-Gallego S, Jalava K, Cripps AW, Lubitz W (2003) Construction of recombinant S-layer proteins (rSbsA) and their expression in bacterial

ghosts – a delivery system for the nontypeable *Haemophilus influenzae* antigen Omp26. FEMS Immunol Med Microbiol 37:185–192
Samuelson P, Gunneriusson E, Nygren P-Å, Ståhl S (2002) Display of proteins on bacteria. J Biotechnol 96:129–154
Sára M (2001) Conserved anchoring mechanisms between crystalline cell surface S-layer proteins and secondary cell wall polymers in Gram-positive bacteria. Trends Microbiol 9:47–49
Sára M, Sleytr UB (1987a) Molecular sieving through S-layers of *Bacillus stearothermophilus* strains. J Bacteriol 169:4092–4098
Sára M, Sleytr UB (1987b) Production and characteristics of ultrafiltration membranes with uniform pores from two-dimensional arrays of proteins. J Membr Sci 33:27–49
Sára M, Sleytr UB (1988) Membrane biotechnology: two-dimensional protein crystals for ultrafiltration purposes. In: Rehm HJ (ed) Biotechnology, vol 6b. VCH, Weinheim, pp 615–636
Sára M, Sleytr UB (2000) S-layer proteins. J Bacteriol 182:859–868
Sára M, Manigley C, Wolf G, Sleytr UB (1988) Isoporous ultrafiltration membranes from bacterial cell envelope layers. J Membr Sci 36:179–186
Sára M, Küpcü S, Weiner C, Weigert S, Sleytr UB (1993) Crystalline protein layers as isoporous molecular sieves and immobilisation and affinity matrices. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Immobilised macromolecules: application potentials. Springer, London, UK, pp 71–86
Sára M, Küpcü S, Sleytr UB (1996) Biotechnological applications of S-layers. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Crystalline bacterial cell surface layer proteins (S-layers). R. G. Landes, Academic Press, Austin, TX, pp 133–159
Sára M, Pum D, Schuster B, Sleytr UB (2005) S-layers as patterning elements for application in nanobiotechnology. J Nanosci Nanotechnol 5:1939–1953
Sára M, Egelseer EM, Huber C, Ilk N, Pleschberger M, Pum D, Sleytr UB (2006) S-Layer proteins: potential applications in nano(bio)technology. In: Rehm B (ed) Microbial bionanotechnology: biological self-assembly systems and biopolymer-based nanostructures. Horizon Scientific Press, Norwich, UK, pp 307–338
Schäffer C, Messner P (2004) Surface-layer glycoproteins: an example for the diversity of bacterial glycosylation with promising impacts on nanobiotechnology. Glycobiology 14:31R–42R
Schäffer C, Wugeditsch T, Kählig H, Scheberl A, Zayni S, Messner P (2002) The surface layer (S-layer) glycoprotein of *Geobacillus stearothermophilus* NRS 2004/3a. Analysis of its glycosylation. J Biol Chem 277:6230–6239
Schäffer C, Novotny R, Küpcü S, Zayni S, Scheberl A, Friedmann J, Sleytr UB, Messner P (2007) Novel biocatalysts based on S-layer self-assembly of *Geobacillus stearothermophilus* NRS 2004/3a: a nanobiotechnological approach. Small 3:1549–1559
Schuster B (2005) Biomimetic design of nano-patterned membranes. NanoBiotechnology 1:153–164
Schuster B, Sleytr UB (2002) Single channel recordings of α-hemolysin reconstituted in S-layer-supported lipid bilayers. Bioelectrochemistry 55:5–7
Schuster B, Sleytr UB (2005) 2D-Protein crystals (S-Layers) as support for lipid membranes. In: Tien TH, Ottova A (eds) Advances in planar lipid bilayers and liposomes, vol 1. Elsevier Science, Amsterdam, The Netherlands, pp 247–293
Schuster B, Sleytr UB (2006) Biomimetic S-layer supported lipid membranes. Curr Nanosci 2:143–152
Schuster B, Pum D, Braha O, Bayley H, Sleytr UB (1998) Self-assembled α-hemolysin pores in an S-layer-supported lipid bilayer. Biochim Biophys Acta 1370:280–288
Schuster B, Pum D, Sára M, Braha O, Bayley H, Sleytr UB (2001) S-layer ultrafiltration membranes: a new support for stabilizing functionalized lipid membranes. Langmuir 17:499–503
Schuster B, Weigert S, Pum D, Sára M, Sleytr UB (2003) New method for generating tetraether lipid membranes on porous supports. Langmuir 19:2392–2397
Schuster B, Györvary E, Pum D, Sleytr UB (2005) Nanotechnology with S-layer proteins. In: Vo-Dinh T (ed) Protein nanotechnology: protocols, instrumentation and application, vol 00, Book series: methods mol biol. Humana Press, Totowa, NJ, pp 101–124

Shenton W, Pum D, Sleytr UB, Mann S (1997) Synthesis of CdS superlattices using self-assembled bacterial S-layers. Nature 389:585–587

Simon B, Nomellini JF, Chiou P, Binale W, Thornton J, Smit J, Leong JA (2001) Recombinant vaccines against infectious hematopoietic necrosis virus: production line by the *Caulobacter crescentus* S-layer protein secretion system and evaluation in laboratory trials. Dis Aqua Org 44:400–402

Sleytr UB (1975) Heterologous reattachment of regular arrays of glycoproteins on bacterial surfaces. Nature 257:400–402

Sleytr UB (1978) Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly and function. Int Rev Cytol 53:1–64

Sleytr UB (1981) Morphopoietic and functional aspects of regular protein membranes present on prokaryotic cell walls. In: Kiermayer O (ed.) Cytomorphogenesis in plants. Cell Biology Monographs, vol 8. Springer Wien, NY, pp. 1–26

Sleytr UB, Messner P (1989) Self-assembly of crystalline bacterial cell surface layers (S-layers). In: Plattner H (ed) Electron microscopy of subcellular dynamics. CRC Press, Boca Raton, FL, pp 13–31

Sleytr UB, Plohberger R (1980) The dynamic process of assembly of two-dimensional arrays of macromolecules on bacterial cell walls. In: Baumeister W, Vogell W (eds) Electron microscopy at molecular dimensions. Springer, Berlin, pp 36–47

Sleytr UB, Thorne KJI (1976) Chemical characterization of the regularly arrayed surface layers of *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. J Bacteriol 126:377–383

Sleytr UB, Mundt W, Messner P (1991) Immunogenic composition containing ordered carriers. US Patent Nr 5,043,158

Sleytr UB, Messner P, Pum D, Sara M (1993) Crystalline bacterial cell surface layers. Mol Microbiol 10:911–916

Sleytr UB, Messner P, Pum D, Sára M (1996) Occurence, location, ultrastructure and morphogenesis of S-layers. In: Sleytr UB, Messner P, Pum D, Sára M (eds) Crystalline bacterial cell surface layer proteins (S-layers). R.G. Landes, Academic Press, Austin, TX, pp 5–33

Sleytr UB, Sára M, Pum D, Schuster B (2001) Molecular nanotechnology and nanobiotechnology with two-dimensional protein crystals (S-layers). In: Rosoff M (ed) Nano-surface chemistry. Marcel Dekker, New York, pp 333–389

Sleytr UB, Sára M, Pum D, Schuster B, Messner P, Schäffer C (2003) Self assembly protein systems: microbial S-layers. In: Steinbüchel A, Fahnestock S (eds) Biopolymers, vol 7. Wiley-VCH, Weinheim, pp 285–338

Sleytr UB, Pum D, Sára M, Schuster B (2004a) Molecular nanotechnology with 2-D protein crystals. In: Nalwa HS (ed) Encyclopedia of nanoscience and nanotechnology, vol 5. Academic Press, San Diego, pp 693–702

Sleytr UB, Mader C, Schuster B, Unger FM (2004b) Use of a secondary cell wall polymer of prokaryotic microorganisms. European Patent 1 285 003 B1

Sleytr UB, Egelseer EM, Pum D, Schuster B (2004c) S-Layers. In: Niemeyer CM, Mirkin CA (eds) NanoBiotechnology: concepts, methods and perspectives. Wiley-VCH, Weinheim, pp 77–92

Sleytr UB, Sára M, Pum D, Schuster B (2005) Crystalline bacterial cell surface layers (S-layers): a versatile self-assembly system. In: Ciferri A (ed) Supra-molecular polymers, 2nd edn. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp 583–616

Sleytr UB, Egelseer EM, Ilk N, Pum D, Schuster B (2007a) S-layers as a basic building block in a molecular construction kit. FEBS J 274:323–334

Sleytr UB, Huber C, Ilk N, Pum D, Schuster B, Egelseer EM (2007b) S-layers as a tool kit for nanobiotechnological applications. FEMS Microbiol Lett 267:131–144

Smith RH, Messner P, Lamontagne LR, Sleytr UB, Unger FM (1993) Induction of T-cell immunity to oligosaccharide antigens immobilized on crystalline bacterial surface layers (S-layers). Vaccine 11:919–924

Spiro RG (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology 12:43R–56R

Steiner K, Novotny R, Patel K, Vinogradov E, Whitfield C, Valvano MA, Messner P, Schäffer C (2007) Functional characterization of the initiation enzyme of S-layer glycoprotein glycan biosynthesis in *Geobacillus stearothermophilus* NRS 2004/3a. J Bacteriol 189:2590–2598

Thibodeaux CJ, Melançon CE, Liu H-W (2007) Unusual sugar biosynthesis and natural product glycodiversification. Nature 446:1008–1016

Tschiggerl H, Breitwieser A, de Roo G, Verwoerd T, Schäffer C, Sleytr UB (2008a) Exploitation of the S-layer self-assembly system for site directed immobilization of enzymes demonstrated for an extremophilic laminarinase from *Pyrococcus furiosus*. J Biotechnol 133:403–411

Tschiggerl H, Casey JL, Parisi K, Foley M, Sleytr UB (2008b) Display of a peptide mimotope on a crystalline bacterial cell surface layer (S-layer) lattice for diagnosis of Epstein-Barr virus infection. J Bioconj Chem 19(4):860–865

Umelo-Njaka E, Nomellini JF, Bingle WH, Glasier LG, Irvin RT, Smit J (2001) Expression and testing of *Pseudomonas aeruginosa* vaccine candidate proteins prepared with the *Caulobacter crescentus* S-layer protein expression system. Vaccine 19:1406–1415

Völlenkle C, Weigert S, Ilk N, Egelseer EM, Weber V, Loth F, Falkenhagen D, Sleytr UB, Sára M (2004) Construction of a functional S-layer fusion protein comprising an IgG-binding domain for the development of specific adsorbents for extracorporal blood purification. Appl Environ Microbiol 70:1514–1521

Weigert S, Sára M (1995) Surface modification of an ultrafiltration membrane with crystalline structure and studies on interaction with selected protein molecules. J Membr Sci 106:147–159

Wetzer B, Pfandler A, Györvary E, Pum D, Lösche M, Sleytr UB (1998) S-layer reconstitution at phospholipid monolayers. Langmuir 14:6899–6906

Weygand M, Schalke M, Howes PB, Kjaer K, Friedmann J, Wetzer B, Pum D, Sleytr UB, Lösche M (2000) Coupling of protein sheet crystals (S-layers) to phospholipid monolayers. J Mater Chem 10:141–148

Wieland F, Lechner J, Sumper M (1982) The cell wall glycoprotein of halobacteria: structural, functional and biosynthetic aspects. Zbl Bakt Hyg I Abt Orig C 3:161–170

Zhu H, Snyder M (2003) Protein chip technology. Curr Opin Chem Biol 7:55–63

Chapter 17
Accumulation of Heavy Metals by Micro-organisms: Biomineralization and Nanocluster Formation

Sonja Selenska-Pobell and Mohamed Merroun

17.1 Introduction

The unicellular Prokaryotes (Bacteria and Archaea) are the most widely distributed organisms on earth. They inhabit in quite high density and diversity not only the so-called life-friendly habitats of humans, animals, plants, insects and other Eukarya, but they also represent the only form of life in niches with very harsh conditions, such as extremely hot thermal springs, permafrost, highly acidic mine drainages, etc. This is possible for them because a large variety of prokaryotic organisms exhibit fascinating resistant mechanisms to extreme temperatures, acidity, alkalinity, high metal, radionuclide, and salt concentrations. In addition, many of them possess the capability to metabolize an enormous number of different organic and inorganic compounds, some of them highly poisonous to other living organisms. The universal distribution and the robustness of bacteria and archaea are due not only to their diverse and unusual metabolic activities but also to the very varied, phylum-specific structural organization and functionality of their outermost component – the cell wall. The cell wall of prokaryotes plays an important role in the following vital functions: protection of the cells from toxicity and other kinds of environmental stress, uptake of nutrients and other life-essential elements, including metals, as well as secretion and anchoring of important molecules, such as enzymes which often support the metabolism and the life of particular species. On the other hand, the cell wall is involved in cell detoxification processes such as efflux of poisonous or other metals accumulated in toxic concentrations (Nies 2003), and secretion of specific ligands (Bonthrone et al. 2000; Macaskie et al. 1992) or waste products of cellular metabolism (Rosen et al. 2004). Here, we describe the accumulation of heavy metals by micro-organisms.

S. Selenska-Pobell (✉)
Institute of Radiochemistry, Research Center Dresden-Rossendorf, 01314 Dresden, Germany
e-mail: s.selenska-pobell@fzd.de

H. König et al. (eds.), *Prokaryotic Cell Wall Compounds*,
DOI 10.1007/978-3-642-05062-6_17,

17.2 Role of Prokaryotic Cell Wall in Biologically Induced Mineralization

Analyses of microfossils demonstrate that prokaryotic organisms were the first forms of life established on our planet about 3.6 billion years ago and they also show that those ancient micro-organisms were involved in precipitation and biomineralization of inorganic compounds (Bäuerlein 2003; Beveridge 1989, 2005; Ehrlich 1998). Because the shape of the ancient microfossils looks very similar to those of prokaryotes currently living, it is believed that their cell wall structure and function were close or equivalent to that of modern bacteria and archaea (Beveridge 1989; Ehrlich 1998). Hence, interactions with metals and the biomineralization accompanied prokaryotic life from its beginning.

Due to their ubiquitous distribution in surface and subsurface terrestrial and aquatic environments, bacteria and archaea play a major role in deposition and weathering in the earth's crust of a large variety of minerals enriched with or consisting mainly of different metals, such as iron, manganese (Beveridge 1989; Douglas and Beveridge 1998; Frankel and Bazylinski 2003; Lovley 1993), copper (Sillitoe et al. 1996), or gold (Kashefi et al. 2001), and even radionuclides (e.g., uranium) (Lovley et al. 1991). The structure of biologically synthesized minerals is strongly influenced by the metabolic properties of the bacterial or archaeal strains involved in their production and also by the differences in their cell wall organization (for the latter see details in Parts I and II of this volume).

The main metal-binding groups of the cell walls of Gram-positive and Gram-negative bacteria, where the primary accumulation of metals occurs, are the carboxylic and phosphate groups, which are provided from different biopolymers characteristic of the bacterial groups mentioned. The concentration of metals at the sites enriched with metal-binding ligands increases above the saturation point, and this results in precipitation and nucleation of mineral building.

In the case of Gram-negative bacteria, the highest concentration of metal=binding groups is in the highly anionic lipopolysacharide wrap which represents the outer face of their outer membrane, which is a complex mosaic bilayered structure containing in addition phospholipids and proteins (see Chap. 5, this volume). The carboxylic and phosphate groups of the rather thin (about 3 nm) peptidoglycan layer of Gram-negative bacteria lying in the periplasm, between the outer and the plasma membranes, also contribute to metal binding but to a significantly lesser extent than in Gram-positive bacteria (Beveridge 1989).

The highly cross-linked and multiple peptidoglycan layer, consisting of sugar derivatives and a particular small group of amino acids (Chap. 2, this volume) is between 15 and 25 nm thick in Gram-positive bacteria, and plays the major role in metal binding due to the lack of an outer membrane in the latter. Additional phosphate-containing components, called secondary polymers, such as teichoic acids and/or teichuronic acids (Chap. 6, this volume), are often bound to the peptidoglycan of Gram-positive but not of Gram-negative bacteria. They contribute

further to the net negative charge of the cell wall and enhance its metal-binding capacity (Beveridge 1989; Urrutia Mera et al. 1992).

The accumulation of metals in both Gram-positive and Gram-negative bacteria described above is a passive process and it is not controlled by the living cells. Moreover, it was demonstrated that spores (Beveridge 1989) and dead cells of some Gram-positive bacteria can be also implicated in metal binding and that in some cases this binding is even more effective than in the case of the active, metabolizing cells (Urrutia Mera et al. 1992). The reason for this is that the metabolizing cells continuously pump protons into the cell wall which compete with the metal ions for the negatively charged carboxylic and phosphate groups. In many Gram-negative bacteria it is suggested that the metal-saturated parts of their outer cell membranes can bleb off from the cells, forming vesicles which are responsible for the further precipitation of metals and development of minerals (Beveridge 1989).

The periplasm, as a space component in the cell wall mainly in Gram-negative bacteria, plays an important role in metal precipitation and biomineralization due to the enzymes harbored there, such as oxido-reductases and hydrogenases involved in bio-transformation, immobilization, and consequent biomineralization of a large variety of metals (De Luca et al. 2001; Elias et al. 2004; Glasauer et al. 2007; Lloyd 2003). Because of the limited space, the biominerals formed in the periplasm are often nanoscale (Creamer et al. 2007). When metal reductases are situated on the outer membranes of the Gram-negative bacteria, they are involved in extracellular metal accumulation and nucleation with consequent formation of larger biominerals (DiChristina et al. 2002; Marshall et al. 2006). This kind of biomineralization, which involves direct redox transformations of the accumulated metals, is referred as active bacteria-induced mineralization (Frankel and Bazylinski 2003).

The cell walls of Archaea are very different chemically from those of Bacteria, and they vary significantly between the phylogenetic groups of this prokaryotic kingdom, possessing various electrochemical charges and metal-binding properties. The interactions of these micro-organisms with metals are poorly studied (Kashefi et al. 2001, 2008).

Most archaea and a large number of bacteria possess proteinaceous layers external to their cell walls (Sleytr et al. 1996; see also Chap. 3 of this volume). These layers are in some cases glycosylated (Sleytr et al. 1996), possess a large number of carboxylated amino acids (Sleytr et al. 1996; Pollmann et al. 2005), and can be also phosphorylated (Merroun et al. 2005). As a result, they have an affinity to metal cations (Fahmy et al. 2006; Pollmann et al. 2006; Sleytr et al. 1996). S-layers of some photosynthetic bacteria can be implicated in processes of active biomineralization (Schulze-Lam et al. 1992). The authors demonstrated that at moderate photosynthetic activity the S-layer of a *Synechococcus* sp. strain serves as a template for formation of gypsum ($CaSO_4.2H_2O$). In strong light, however, an alkalization of the surrounding cells takes place due to photosynthesis and as a result calcite ($CaCO_3$) is formed.

Other surface layers such as capsules, slimes, and sheaths as outermost cell structures also play an important role in the biomineralization processes of many

prokaryotic organisms due to their richness in carboxylic and phosphate groups (Beveridge 1989; Bäuerlein 2003; Hanert 2006).

A combination of passive metal sorption by the negatively charged cell wall extracellular polymers and active secretion of phosphate groups due to phosphatase activity was described as an interesting kind of biomineralization by Macaskie et al. (2000); it was confirmed for many bacterial isolates recovered from extreme (Martinez et al. 2007; Merroun et al. 2006; Nedelkova et al. 2007) and non-extreme habitats (Jroundi et al. 2007).

There are many interesting cases of microbe-controlled intracellular biomineralization which are not discussed in this chapter but can be found in other reviews and publications (Bazylinski and Frankel 2003; Glasauer et al. 2002, 2007).

The next two sections of this chapter will focus, firstly, on bacterial and archaeal cell wall-dependent accumulation and biomineralization of metals (mainly iron and uranium) and, secondly, on the cell wall-supported formation of metallic palladium bio-nanocatalysts by particular Gram-positive and Gram-negative bacteria.

17.3 Prokaryotic Cell Wall-Dependent Accumulation and Biomineralization of Iron and Uranium

The biologically induced mineralization processes by prokaryotes include passive (Beveridge 1989, 2005; Brown et al. 1998) or active (Fortin et al. 1996; Hanert 2006; Lloyd 2003; Lovley 2002; Macaskie et al. 1992, 2000) interactions with and accumulation of a large variety of metals on the cell wall. The metal accumulates can serve as nucleation sites for growth of cell minerals under suitable biogeochemical conditions (Bäuerlein 2003; Beveridge 1989, 2005; Frankel and Bazylinski 2003; Macaskie et al. 2000; Martinez et al. 2007; Renninger et al. 2004).

The largest and best studied group of biominerals is based on iron. This is due to the wide distribution of this element, which is the fourth most abundant element in the earth's crust (after oxygen, silicon, and aluminum), and also because of its importance for life. It is supposed that some of the earliest inhabitants of the anoxic prebiotic Earth were able to conserve energy via Fe(III) reduction with H_2 as an electron donor and were implicated in this way in the earliest biological deposition of magnetite (Lovley 2002; Vargas et al. 1998).

Fe(III) reduction is an ubiquitous process in modern Earth habitats and is performed by a large variety of psychrophylic, mesophilic, thermophilic, and even hyperthermophilic bacteria and archaea (Kashefi et al. 2008; Lloyd 2003; Roh et al. 2006).

In a large variety of Gram-negative bacteria, the dissimilatory Fe(III) reduction is supported by periplasmic, outer-membrane, or extracellularly located c-type of cytochromes which are responsible for direct transfer of electrons to Fe(III) oxides which are highly insoluble at circum-neutral pH (DiChristina et al. 2002; Elias et al. 2004; Lloyd 2003). Part of the resultant ferrous iron is usually up taken by the cells

and used for the metabolic needs of the organisms. A significant amount of the solubilized metal is adsorbed by the negatively charged ligands (mainly carboxylic and phosphate groups) of the cell wall and usually stimulates an extracellular formation of iron minerals. Beveridge and colleagues were able to demonstrate that in one representative of these organisms, the gammaproteobacterial strain *Shewanella putrefaciens* CN32, not only does deposition of de novo formed magnetite precipitates on the cell surface occur, but also precipitation and even penetration and deposition of mineral nanocrystals from the environment into the periplasm and within the peptidoglycan layers (Glasauer et al. 2001). In the cell cytoplasm of this strain, intracellular magnetite-like granules were found which were more reduced than the extracellularly and cell-wall-deposited mineral particles (Glasauer et al. 2002, 2007). The authors relate the formation of these granules to an unique mechanism closely linked to the dissimilatory Fe(III) reduction which is driven in the periplasm by enzymes located at the cytoplasm membrane. The latter also explains the release of mineral-containing blebs into the cytoplasm and not into the environment. This process, however, differs significantly from the biologically controlled formation of magnetite in the magnetosomes of magnetotactic bacteria (Glasauer et al. 2002; Bazylinski and Frankel 2003).

In natural environments the formation of mixed Fe(II)/Fe(III) minerals such as magnetite is not only a result of iron (hydr)oxides reduction but also o Fe(II) oxidation under anaerobic conditions (Chaudhuri et al. 2001) or in aerobic/microanaerobic environments with acidic pH, such as mine drainages, where Fe(III) is highly soluble (Fortin et al. 1996). Moreover, in distinct micro-aerophilic sites of the latter environments, synergetic consortia consisting of iron-oxidizing and sulfate/iron-reducing micro-organisms are established which provide iron in both oxidation forms and facilitate its mineralization. The initial precipitation of the iron minerals occurs onto the cell walls of these prokaryotic organisms (Fortin et al. 1996) and also in the acidic extracellular phosphate-containing lipopolysaccharide layers of the iron-oxidizing organisms, such as *Acidithiobacillus ferrooxidans* (Gehrke et al. 1998). A process of direct passive precipitation of regularly distributed iron arrays onto the highly ordered lattices of the S-layers enveloping some bacteria in natural biofilms was demonstrated by Brown et al. (1998).

In addition to iron, prokaryotic cells are able to accumulate, in either active or passive ways, a large variety of other metals, such as Mn, V, Cr, Co, Pt, Pd, Au, and Ag, and even radionuclides such as Cm, Np, Pu, Tc, and U (Lloyd 2003). In natural conditions a co-precipitation of several metals and formation of mixed minerals can occur as well (Beveridge 1989; Lloyd 2003).

The bioaccumulation and biologically induced mineralization of uranium is a subject of particular interest due to the increased pollution of the environment with this rare element as a result of its mining and processing for nuclear fuel and military applications during recent decades. Bacteria, because of their fascinating resistance to heavy metals and radionuclides and also to their capability to bind metals from both concentrated and also highly diluted solutions, offer effective and cheap solutions for reclamation of polluted sites. The interaction mechanisms of prokaryotes with uranium, although dependent on the chemistry of this rare earth

element, are similar to the interactions with iron and most other metals. For instance, cell wall-located cytochromes are involved in U(VI) reduction as in Fe (III) reduction (Elias et al. 2004; Marshall et al. 2006; Lloyd 2003; Lovley et al. 1993).

The reduction of the soluble U(VI) to insoluble U(IV) was induced in many uranium-contaminated liquid wastes and sediments via addition of electron donors such as lactate, pyruvate, acetate, or ethanol (Istok et al. 2004; Nevin et al. 2003; Suzuki et al. 2003). However, it was demonstrated that after depletion of the added electron donors, the immobilized uranium was reoxidized and returned back to the treated habitat (Anderson et al. 2003; Nyman et al. 2006; Wan et al. 2005).

It seems that the reduction of U(VI), which is attributed to the formation of uranium deposits in the remote past (Lovley et al. 1991), has only a temporary and rather limited success in modern industrially produced uranium-containing environments (Anderson et al. 2003; Nyman et al. 2006; Wan et al. 2005), at least at the early stages of their remediation. This is not surprising bearing in mind that the biogeochemical conditions in the wastes are obviously not comparable with the ancient Earth conditions in which most of the uranium deposits were formed (Lovley et al. 1991).

For instance, in uranium mill-tailings, nitrate is the most common co-contaminant of uranium from its processing (Finneran et al. 2002; Istok et al. 2004; Nevin et al. 2003). In the solid waste material of uranium mining, large amounts of iron and other minerals are present as well, deposited during the long geologic history of the uranium ores (Finneran et al. 2002; Geissler and Selenska-Pobell 2005; Petrie et al. 2003; Roh et al. 2006). After mining, this material is exposed to aeration, temperature, and moisture conditions which are rather different from those in its natural surroundings.

In addition, it was demonstrated that uranium waste water, sediment, and soil material is occupied by a large variety of active and/or resting micro-organisms which have been subjected to a long evolution and have developed different and very effective protection mechanisms against toxic metals and radionuclides (Francis et al. 2004; Macaskie et al 2000; Merroun et al. 2005, 2006; Nies 2003; Renninger et al. 2004; Selenska-Pobell 2002). It was demonstrated that microorganisms in the subsurface of the uranium wastes preferentially use nitrate and Fe (III) for anaerobic respiration because their reduction is thermodynamically more favorable than the reduction of U(VI) (Finneran et al. 2002; Istok et al. 2004; Suzuki et al. 2003). In model microcosm experiments performed under oligotrophic and anaerobic conditions, close to the natural, with samples collected from depleted and lightly contaminated uranium mining wastes near the city of Johanngeorgenstadt (Germany), it was found that the addition of uranyl nitrate results in immobilization of the added U(VI) in mixed inorganic and organic phosphate compounds (Geissler et al. 2009). The latter was attributed to the activity of a surprisingly large number of various bacterial species which were induced by the treatments. Most of the newly induced bacterial populations were denitrifiers which were not identified in the untreated samples, most probably due to their low density which was below the limit of detection of the identification methods used (Geissler et al. 2009).

No detectable reduction of U(VI) was found in the samples treated with uranyl nitrate during the experiments, which were monitored for about 100 days. Instead, an increased reduction of Fe(III) to Fe(II) with increasing incubation time was demonstrated, which signified that after the reduction of the added nitrate, Fe(III) was the main electron acceptor used by the bacterial community established at the later stages of treatment with uranyl nitrate. At these late stages of the treatments, establishment of uranium-sensitive bacterial populations and recovery to the initial bacterial composition occurred, which indicated that the added uranium was no longer bioavailable. Time-resolved laser-induced fluorescence spectroscopic analysis demonstrated that most of the added U(VI) was bound by phosphate phases of biotic origin (Geissler et al. 2009).

In order to study the mechanisms of the uranium immobilization and to characterize the uranyl phosphate mineral phases formed, bacterial strains were cultured from different uranium mining waste samples. Their interactions with uranium were studied by transmission electron microscopy combined with energy dispersive X-ray (TEM-EDX) and extended X-ray absorption fine structure (EXAFS) spectroscopic analysis (Francis et al. 2004; Merroun and Selenska-Pobell 2001; Merroun et al. 2002, 2003, 2005, 2006, 2008; Nedelkova et al. 2007).

These studies demonstrated that U(VI) was predominantly precipitated in uranyl phosphate phases and that the form and localization of the precipitates were dependent on the phylogenetic affiliation of the strains and especially on their cell wall structural characteristics.

Several examples of U(VI) bioaccumulation and mineralization by Gram-positive and Gram-negative bacterial isolates are presented in Figs. 17.1 and 17.2. It is important to note that the experiments with all strains were performed under the same conditions: 48 h of intensive shaking in 0.5 mmol/L solution of uranyl nitrate at pH 4.5, which corresponds to the conditions in the above-mentioned uranium waste near the city of Johanngeorgenstadt in Germany.

In the case of the *Bacillus sphaericus* isolates JG-7B and JG-A12 (Fig. 17.1a–c), uranium was bound exclusively by the components of the cell wall. Most of it was accumulated onto the internal side of the S-layer and also into the cross-linked peptidoglycan layers (see the arrows in Fig. 17.1b). No intracellular precipitates of uranium were found in these two strains (Merroun et al. 2005, 2008). Due to its ability to accumulate selectively and reversibly high amounts of U(VI) from mill-tailing waters (Selenska-Pobell et al. 1999), the strain JG-A12 was successfully used for construction of biological ceramics for bioremediation (Raff et al. 2003). The S-layer of *B. sphaericus* JG-A12 is involved in interactions not only with uranium but also with other radionuclides. Fig. 17.1d shows, for instance, an example of accumulation of the chemical analog of Cm(III), namely Eu(III), onto the surface of the strain. In this case the S-layer heavily loaded with Eu(III) is removed from the cell and is further mineralized. The cell is, however, protected from the remaining Eu(III) in the solution by the newly synthesized S-layer which continues to accumulate the metal (arrows).

The presence of empty uranium-encrusted cell walls was very typical for the studied actinobacterial isolates of the species *Microbacterium oxydans* (Fig. 17.1e, f).

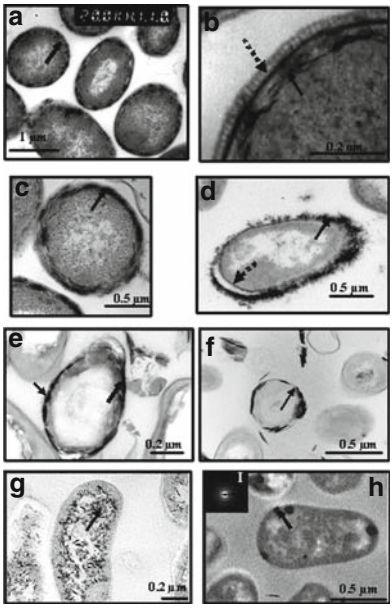

Fig. 17.1 Transmission electron micrographs of thin sections of the cells of *B. sphaericus* JG-7B treated with U(VI) (**a**, **b**), *B. sphaericus* JG-A12 treated with U(VI) (**c**) and with Eu(III) (**d**), *M. oxydans* SW-3 (**e**) and S15-M2 treated with U(VI) (**f**), *Arthrobacter* sp. JG37-iso3 treated with U(VI) (**g**) and with Pb(II) (**h**). I in Fig. 17.1h corresponds to the electron diffractogram of the pyromorphite phase (shown by an *arrow*) deposited at the cells of the strain JG37-iso3. The uranium-phosphate nature of the crystals and granules was confirmed by EDX (not shown). The strains JG-A12, JG-7B and JG37-iso 3 were recovered from the uranium waste pile near Johanngeorgenstadt, SW3 from water of the flooded uranium mill tailings near the Seelingstadt, Germany, and S15-M2 from a deep-well monitoring site S15 of a Siberian radioactive waste repository, Russia (Nedelkova et al. 2007)

As already reported, these strains accumulated uranium exclusively in their extremely thick peptidoglycan layer (Nedelkova et al. 2007). In contrast, the cells of another group of actinobacterial isolates, which were affiliated with the genus *Arthrobacter*, accumulated uranium only intracellularly (Fig. 17.1g). The latter is concurrent with the results of Suzuki and Banfield (2004) who demonstrated using TEM-EDX that the intracellularly accumulated uranium is associated with polyphosphate granules. In our case, we demonstrated not only association with the polyphosphate granules but also growth of intracellular nanocrystals. It is not clear why the cell wall is not protecting this kind of bacteria from the extremely toxic U(VI) at the studied acidic pH. Moreover, it was demonstrated that about 80% of the *Arthrobacter* cells are able to up take the dissolved uranium very quickly and that they remain viable after that (Suzuki and Banfield 2004; Geissler 2007). Interestingly, it seems that the cell wall of *Arthrobacter* plays an important role in detoxification of lead (Fig. 17.1h). Most of the metal added in the form of Pb $(N0_3)_2$ was accumulated at the cell walls in the form of a phosphate-containing

mineral pyromorphite ($Pb_5(PO_4)_3Cl$) (Geissler 2007). The interaction of the *Arthrobacter* isolates with uranium is very unusual and seems to be an exception within the Gram-positive bacteria. In general, the cell wall in these bacteria plays the main role in protection against uranium toxicity (Francis et al. 2004; Merroun et al. 2005, 2006; Nedelkova et al. 2007).

It was demonstrated that the accumulation of U(VI) by Gram-negative bacteria is also species-specific and occurs mainly on the cell wall as phosphate complexes (Francis et al. 2004, Merroun et al. 2002, 2003, 2005, 2006, 2008; Renninger et al. 2004; Suzuki and Banfield 2004). Examples shown in Fig. 17.2 demonstrate various

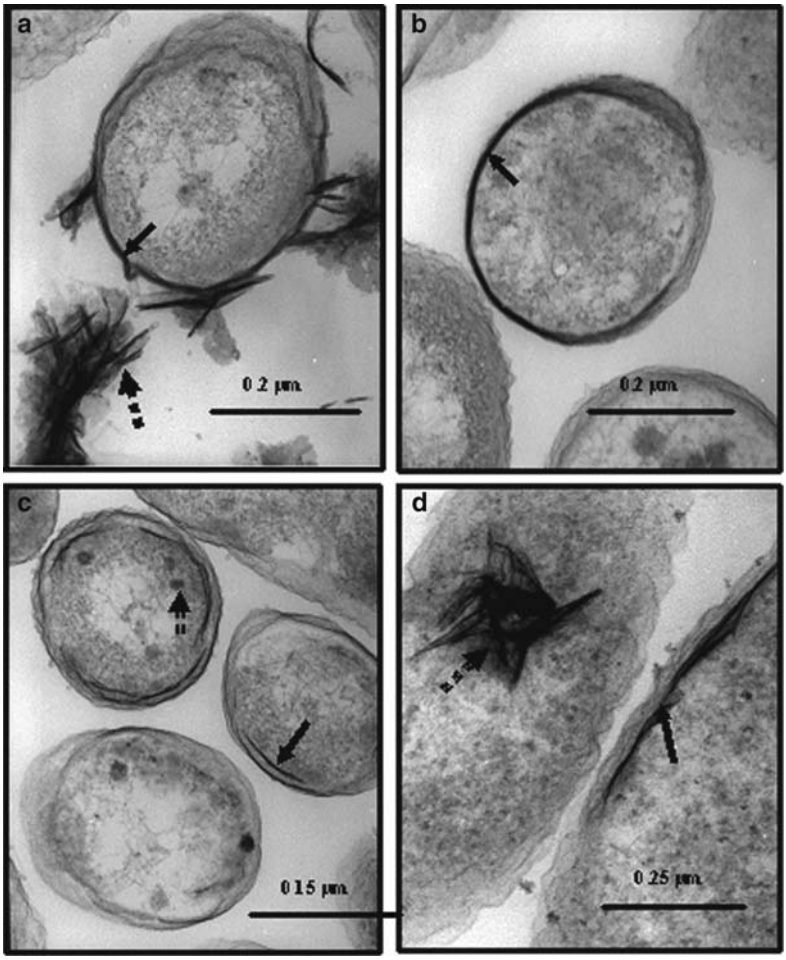

Fig. 17.2 Transmission electron micrographs of thin sections of the cells of *P. stutzeri* DSMZ 5190 (**a**), *P. rhodesiae* R5 (**b**), *P. migulae* CIP 105470 (**c**), and *S. maltophilia* JG-2 (**d**) treated with uranium. *S. maltophilia* JG-2 was recovered from a sediment of the uranium mining waste pile near the town of Johanngeorgenstadt, Germany. *P. rhodesiae* R5 from heavy metal contaminated soil in Bulgaria

U(VI) accumulation mechanisms in some *Pseudomonas* species of the Gamma subdivision of Proteobacteria which are widely distributed in heavily contaminated uranium wastes (Selenska-Pobell 2002; Geissler et al. 2009). It is noticeable that not all bacterial cells are equally loaded with uranium and that some of them were even free of uranium. This phenomenon might be connected with the different physiological state of the cells in the treated bacterial cultures which were not synchronized. *Pseudomonas stutzeri* DSMZ 5190, which possesses a thick layer of mucous extracellular polymers, accumulates U(VI) initially onto its outer membrane, where at particular sites growth of bigger crystals is initiated which migrate from the cell surface into the surrounding mucous layer and continue to grow further, forming big needle-like crystals (Fig. 17.2a). According to Macaskie et al. (2000) similar extracellular lipopolysaccharide layers to those in *P. stutzeri* DSMZ 5190 play the role of a supporting matrix for further growth of uranyl phosphate crystals, due to the secretion of inorganic phosphate groups by the stressed bacteria.

Representatives of the species *Pseudomonas rhodesie* form fine uranium precipitates on their cell wall (Fig. 17.2b), whereas in the case of *Pseudomonas migulae* a multilayer of fine uranium accumulates was formed which is associated with the outer membrane, with the thin peptidoglycan layer, and also with the cytoplasmic membrane (Fig. 17.2c). Occasionally, uranium associated with polyphosphate bodies was also found in the cells of the latter strain. Renninger et al. (2004) have demonstrated that in *Pseudomonas aeruginosa* uranium is initially sorbed onto the surface in the form of uranyl hydroxides and then precipitated as uranyl phosphate due to the release of orthophosphate from the cells as a result of stress-activated degradation of the polyphosphate bodies. This mechanism seems also to be the case with the above-mentioned *P. rhodesiae* strain, because no intracellular accumulates of uranium have ever been found in its cells. The observed intracellular accumulation of uranium in the case of *P. migulae* is possibly due to the properties of its outer membrane which permits metal diffusion inside the cells where the metal is complexed into the polyphosphate bodies and detoxified. Similar observations were made with other Gram-negative bacteria such as *Halomonas* sp. (Francis et al. 2004) and *Acidithiobacillus ferrooxidans* (Merroun et al. 2002). Another interesting case of uranium mineralization was found in the uranium mining waste isolate *Stenotrophomonas maltophilia* JG-2 (Fig. 17.2d). In most of the cells of this strain, uranium was associated with the cytoplasmic membrane and in some of the cells intracellular needle-like uranium crystals were formed. Such formations were reported earlier for a *Pseudomonas* sp. strain (Marqués et al. 1991). The growth of such crystals may be associated with interactions with the polyphosphate bodies but they can be also attributed to the uranium precipitates formed onto the plasma membrane which were released into the cell cytoplasm and rearranged there. This case is similar to the case described above of formation of intracellular iron nanoparticles by *S. putrefaciens* CN32 (Glasauer et al. 2007).

The atomic structure of the uranyl precipitates formed by different bacterial strains recovered from both extreme and non-extreme habitats was characterized by using extended X-ray absorption fine structure (EXAFS) (Francis et al. 2004;

Jroundi et al. 2007; Beazley et al. 2007; Merroun et al. 2002, 2003, 2005, 2006, 2008; Nedelkova et al. 2007). It was demonstrated that the fine structure of the complexes formed (organic and/or inorganic phases) is affected by the pH of the U (VI) solution (Kelly et al. 2002; Merroun et al. 2006; Nedelkova et al. 2007). At pHs ranging from 4 to 7, formation of autunite/meta-autunite (Beazley et al. 2007; Jroundi et al. 2007; Merroun et al. 2006; Nedelkova et al. 2007) or H-autunite (Macaskie et al. 1992, 2000) mineral phases was reported.

The EXAFS spectra of the uranium complexes formed by the cells of two of the above-mentioned strains, namely *P. stutzeri* DSMZ 5190 and *B. sphaericus* JG-7B, are similar to those of m-autunite (Fig. 17.1) (U is co-ordinated to phosphate groups in a monodentate binding mode with a U–P bond distance of about 3.60 ± 0.02 Å) indicating that the cells of these two strains are able to precipitate U in a m-autunite-like mineral phase. However, as shown in Fig. 17.3, the local co-ordination of U associated with the purified S-layer of *B. sphaericus* JG-7B is different from that in the case of the whole cells and its EXAFS spectrum possesses different structural parameters. In the S-layer protein sample U(VI) is co-ordinated to carboxyl groups in a bidentate fashion with an average distance between the U atom and the C atom of 2.89 ± 0.02 Å, and to phosphate groups in a monodentate fashion with an average distance between the U atom and the P atom of 3.61 ± 0.02 Å. The implication of both functional groups (phosphate and carboxyl) in the co-ordination of U has also been observed in the case of the S-layer of the strain *B. sphaericus* JG-A12 (Merroun et al. 2005), and also in the purified cell wall of *Bacillus subtilis* (Kelly et al. 2002).

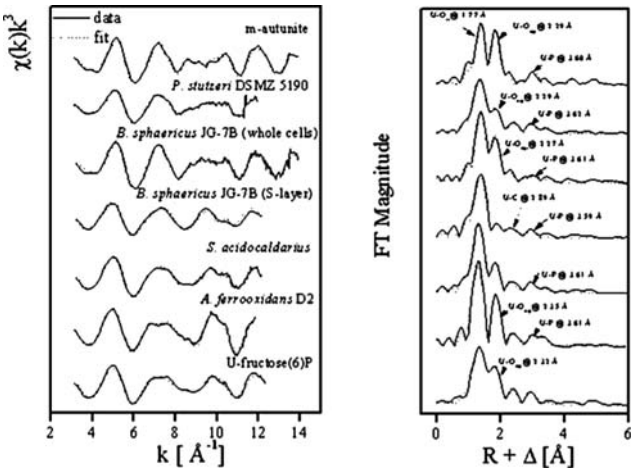

Fig. 17.3 Uranium L_{III}-edge k^3–weighted EXAFS spectra (*left*) and corresponding Fourier transform (FT) (*right*) of the uranium complexes formed on microbial cells and on an isolated S-layer protein at pH 4.5. Meta-autunite and fructose-1,6-phosphate are presented as reference compounds

In the case of the acidophilic uranium mining isolate *A. ferrooxidans* D2, U(VI) was co-ordinated to organic phosphate groups in a monodentate binding mode (U–P bond distance 3.61 ± 0.02 Å), and the EXAFS spectrum of the corresponding U complexes is similar to that of U/fructose phosphate complexes (Koban et al. 2004).

A different local co-ordination of U(VI) was found recently for the cells of the acidophilic and thermophilic archaeal strain *Sulfolobus acidocaldarius* (Fig. 17.3) which inhabits different uranium mill tailings. The high Debye–Waller factor of the U–O_{eq1} (second co-ordination shell in the FT) shell suggests that this equatorial shell is split into more than one oxygen bond. This splitting may involve two distances, a shorter U–O_{eq1} from the backscattering contribution of the phosphate oxygen(s) (monodentate binding mode) and a longer U–O_{eq1} bond distance probably from co-ordinated water or from carboxyl groups. These results indicate that two types of ligands are implicated in the co-ordination of U(VI): phosphate groups (most probably from the plasma membrane) and possibly hydroxyl/carboxyl groups. The latter groups can originate from the S-layer which envelops the cells of the archaeon and which, in contrast to that of *B. sphaericus* 7B, is glucosylated. No information about the localization of the uranium accumulates is available at the moment and we cannot exclude intracellular accumulation of U(VI) in this case. Interestingly, the halophilic archaeon *Halobacterium halobium* complexes U predominantly by inorganic phosphate groups (Francis et al. 2004). Bearing in mind that archaea possess very diverse cell surfaces, it is possible to expect a large variety of biominerals to be formed on them.

It is necessary to stress once again that in uranium-contaminated environments many additional biotic and abiotic factors influence the biomineralization of uranium. In addition to the bacterial populations mentioned, actively interacting with the radionuclide, there is a large amount of dead biomass due to the uranium toxicity. For this reason the organic uranyl phosphate complexes found along with the inorganic phases in the natural samples (Geissler et al. 2009) can be connected to interactions with phosphorylated biopolymers supplied by both dead and live bacteria which, besides the orthophosphate released by particular populations, contribute to the observed U(VI) immobilization and biomineralization. The reduction of U(VI) from the mixed phosphate minerals formed could take place when the other, thermodynamically more favorable electron acceptors, such as nitrate or Fe(III)), are reduced and when enough electron donors, possibly from the dead microbial populations, are released and supplied to support the reduction.

17.4 Cell Wall-Supported Formation of Metallic Palladium Nanoparticles

Bioreductive formation of Pd(0) nanoparticles from Pd(II)-containing solutions was demonstrated on the cell surfaces of different bacteria including *Desulfovibrio desulfuricans* (Lloyd et al. 1998), *Desulfovibrio fructosovorans* (Macaskie et al. 2005),

Rhodobacter sphaeroides (Redwood et al. 2007), and *Shewanella oneidensis* (De Windt et al. 2006). The so-called palladized cells have a high catalytic activity in test reactions involving production of hydrogen from hypophosphite (Yong et al. 2002), hydrogenolytic dehalogenation of polychlorinated biphenyls (De Windt et al. 2006), reduction of toxic Cr(VI) to less toxic Cr(III) (Macaskie et al. 2005), or chloride release from chlorinated aromatic compounds (Redwood et al. 2007). The formation of the Pd(0) starts with Pd(II) complexation to carboxylic or amino groups of the proteins in the cell's periplasm, where the reduction of the deposited metal is mediated by one of the three major hydrogenases via hydrogen oxidation (Macaskie et al. 2005). The size of the particles can be modulated by loading with different Pd amounts and the resulting catalytically active nanoparticles with a size of about 5 nm are stabilized by the architecture of the cell wall. These nanoparticles are ferromagnetic, in contrast to the bulk Pd which is paramagnetic (Creamer et al. 2007).

Nanoparticles with the same size, similar hydrogenation catalytic activity, and ferromagnetic properties were produced on the surface of the S-layer-enveloped Gram-positive bacterium *B. sphaericus* JG-A12 (Creamer et al. 2007). The formation mechanism of the latter nanoclusters is, however, completely different. It was demonstrated that in this case Pd(II) is deposited in the pores of the inner side of the S-layer (Fig. 17.4). This localization is similar to the accumulation of U(VI) described in the preceding section (compare with Fig. 17.1b). However, in contrast to U(VI) which is co-ordinated by phosphate and carboxylic groups, Pd(II) is co-ordinated only by the carboxylic groups of the S-layer (Fahmy et al. 2006).

As confirmed by EXAFS spectroscopic analysis, the formation of the Pd(0) nanoclusters occurred after addition of H_2 as a reducing agent (Creamer et al. 2007). The reduction of Pd(II) was also achieved by using purified and recrystallized S-layers of *B. sphaericus* and applying hydrogen (Pollmann et al. 2006) or electron irradiation (Wahl et al. 2001). The advantage of the S-layer-supported Pd nanoparticles is that they not only possess a regular size of about 2 nm, but that they are also regularly distributed following the lattice symmetry of the protein matrix. The latter is of great importance for nano-electronics and other nanotechnological applications.

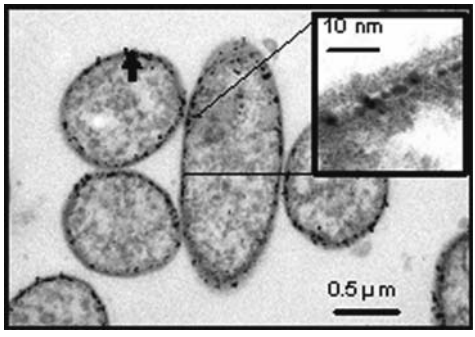

Fig. 17.4 TEM micrograph of Pd(0) nanoclusters on the cells of *B. sphaericus* JG-A12

17.5 Conclusions

The biologically induced biomineralization of iron and uranium strongly depends on the cell wall structure of bacteria and archaea. The main metal binding ligands of the cell wall components of these organisms are carboxylic and phosphate groups. Due to the diverse organization of prokaryotic cell walls, these two chemical groups are surrounded by different biopolymers in Gram-negative and Gram-positive bacteria, as well as in Archaea, which strongly influences the form and the size of the biominerals formed.

In natural environments, mixed Fe(II)/Fe(III) minerals are formed due to the activities of different groups of prokaryotic organisms able to oxidize or reduce this element on the cell surface or in the periplasm and to facilitate its biomineralization in this way.

In the oligotrophic and acidic conditions of uranium mining wastes, an increase of the U(VI) concentration in the absence of electron donors leads to formation of uranyl phosphate minerals.

In laboratory experiments under conditions similar to those of the natural uranium mining waste environments, bacteria bind soluble U(VI) mainly via phosphate groups on their cell walls forming meta-autunite mineral phases. In addition, both archaea and bacteria are able to precipitate uranyl phosphate mineral phases due to the secretion of orthophosphate groups into their extracellular surface polymers.

The cell walls of some Gram-positive and Gram-negative bacteria can serve as a template for fabrication of Pd nanoparticles which are requisites for development of bionanocatalysts and for other technological applications.

References

Anderson RT, Vrionis HA, Ortiz-Bernad I, Resch CT, Lonh PE, Dayvault R, Karp K, Marutzky S, Metzler DR, Peacock A, White DC, Lowe M, Lovley DR (2003) Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium contaminated aquifer. Appl Environ Microbiol 69:5884–5891

Bäuerlein E (2003) Biomineralization of unicellular organisms: unusual membrane biochemistry for the production of inorganic nano- and microstructures. Angew Chem Int Ed 42(6):615–641

Bazylinski DA, Frankel RB (2003) Biologically controlled mineralization in prokaryotes. Rev Mineral Geochem 54(1):217–247

Beazley MJ, Martinez RJ, Sobecky PA, Webb SM, Taillefert M (2007) Uranium biomineralization as a result of bacterial phosphatase activity: insight from bacterial isolates from a contaminated subsurface. Environ Sci Technol 41:5701–5707

Beveridge TJ (1989) Role of cellular design in bacterial metal accumulation and mineralisation. Annu Rev Microbiol 43:147–71

Beveridge TJ (2005) Bacterial cell wall structures and implications for interactions with metal ions and minerals. J Nucl Radiochem Sci 6(1):7–10

Bonthrone KM, Quarmby J, Hewitt CJ, Allan VJM, Paterson-Beedle M, Kennedy JF, Macaskie LE (2000) The effect of the growth medium on the composition and metal binding behaviour of

the extracellular polymeric material of a metal-accumulating *Citrobacter* sp. Environ Technol 2:123–134

Brown DA, Beveridge TJ, Keevil CW, Sherriff BL (1998) Evaluation of microscopic techniques to observe iron precipitation in a natural microbial biofilm. FEMS Microbiol Ecol 26:297–310

Chaudhuri SK, Lack JG, Goates JI (2001) Biogenic magnetite formation through anaerobic bio-oxidation of Fe (II). Appl Environ Microbiol 67:2844–2848

Creamer NJ, Mikheenko IP, Yong P, Deplanche K, Sanyahumbi WDJ, Pollmann K, Merroun M, Selenska-Pobell S, Macaskie LE (2007) Novel supported Pd hydrogenation bionanocatalyst for hybrid homogeneous/heterogeneous catalysis. Catal Today 128:80–87

De Luca G, De Philip P, Dermoun Z, Rousset M, Vermeglio A (2001) Reduction of technetium (VII) by *Desulfovibrio fructosovorans* is mediated by the nickel-iron hydrogenase. Appl Environ Microbiol 67:4583–4587

De Windt W, Boon N, Van den Bulcke J, Rubberecht L, Prata F, Mast J, Hennebel T, Verstraete W (2006) Biological control of the size and the reactivity of catalytic Pd(0) produced *Shewanella oneidensis*. Antonie Van Leeuwenhoek 90:377–389

DiChristina T, Moore CM, Haller CA (2002) Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires ferE, a homolog of the pulE (gspE) type II protein secretion gene. J Bacteriol 184:142–151

Douglas S, Beveridge TJ (1998) Mineral formation by bacteria in natural microbial communities. FEMS Microbiol Ecol 26:79–88

Ehrlich HL (1998) Geomicrobiology: its significance for geology. Earth-Sci Rev 45:45–60

Elias DA, Suflita JM, McInerney MJ, Krumholz LR (2004) Periplasmic cytochrome C_3 of *Desulfovibrio vulgaris* is directly involved in H_2-mediated metal but not sulphate reduction. Appl Environ Microbiol 70:413–420

Fahmy K, Merroun M, Raff J, Pollmann K, Hennig Ch, Savchuk O, Selenska-Pobell S (2006) Secondary structure and Pd(II) co-ordination in S-layer proteins from *Bacillus sphaericus* studied by infrared and X-ray absorption spectroscopy. Biophys J 91:996–1007

Finneran KT, Housewright ME, Lovley DR (2002) Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. Environ Microbiol 4:510–516

Fortin D, Davis B, Beveridge TJ (1996) Role of *Thiobacillus* and sulfate-reducing bacteria in iron biocycling in oxic and acidic mine tailings. FEMS Microbiol Ecol 21:11–24

Francis AL, Gillow JB, Dodge CJ, Harris R, Beveredge TJ, Papenguth HW (2004) Uranium association with halophilic and non-halophilic bacteria and archaea. Radiochim Acta 92:481–488

Frankel RB, Bazylinski DA (2003) Biologically induced mineralization by bacteria. Rev Mineral Geochem 54(1):95–114

Gehrke T, Telegi J, Thierry D, Sand W (1998) Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. Appl Environ Microbiol 64:2743–2747

Geissler A (2007) Prokaryotic micro-organisms in uranium mining waste piles and their interactions with uranium and other heavy metals. PhD Thesis. TU Bergakademie Freiberg, Freiberg, Germany

Geissler A, Selenska-Pobell S (2005) Behavior of U(VI) added to a uranium mining waste pile sample and the resulting changes in the indigenous bacterial community. Geobiology 3:275–285

Geissler A, Merroun M, Geipel G, Reuther H, Selenska-Pobell S (2009) Biogeochemical changes induced in uranium mining waste pile samples by uranyl nitrate treatments under anaerobic conditions. Geobiology 7:282–294

Glasauer S, Landley S, Beveridge TJ (2001) Sorption of Fe(hydr)oxides to the surface of *Shewanella putrefaciens*: cell-bound fine grained minerals are not always formed de novo. Appl Environ Microbiol 67:5544–5550

Glasauer S, Landley S, Beveridge TJ (2002) Intracellular iron minerals in a dissimilatory iron reducing bacterium. Science 295(4):117–119

Glasauer S, Landley S, Boyanov M, Lai B, Kemner K, Beveridge TJ (2007) Mixed valence cytoplasmic iron granules are linked to anaerobic respiration. Appl Environ Microbiol 73:993–996

Hanert HH (2006) The genus *Siderocapsa* (and other iron- and manganese-oxidizing Eubacteria). In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes, vol 7. Springer, New York, pp 1005–1015

Istok JD, Senko JM, Krumholz LR, Watson D, Bogle MA, Peacock A, Chang Y-J, White DC (2004) In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ Sci Technol 38:468–475

Jroundi F, Merroun M, Arias JM, Rossberg A, Selenska-Pobell S, Gonzalez-Munoz MT (2007) Spectroscopic and microscopic characterization of uranium biomineralization by *Myxococcus xanthus*. Geomicrobiol J 24:441–449

Kashefi K, Tor JM, Nevin KP, Lovley D (2001) Reductive precipitation of gold by dissimilatory Fe(III)-reducing bacteria and archaea. Appl Environ Microbiol 67:3275–3279

Kashefi K, Shelobolina ES, Elliot WC, Lovley DR (2008) Growth of thermophilic and hyperthermophilic Fe(III)-reducing micro-organisms on a ferruginous smectite as a sole electron acceptor. Appl Environ Microbiol 74:251–258

Kelly SD, Kemner KM, Fein JB, Fowle DA, Boyanov MI, Bunker BA, Yee N (2002) X-ray absorption fine structure determination of pH-dependent U-bacterial cell wall interactions. Geochim Cosmochim Acta 65:3855–3871

Koban A, Geipel G, Roßberg A, Bernhard G (2004) Uranyl (VI) complexes with sugar phosphates in aqueous solution. Radiochim Acta 92:903–908

Lloyd JR (2003) Microbial reduction of metals and radionuclides. FEMS Microb Rev 27: 411–425

Lloyd JR, Yong P, Macaskie L (1998) Enzymatic recovery of elemental palladium by using sulphate-reducing bacteria. Appl Environ Microbiol 64:4607–4609

Lovley DR (1993) Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol Rev 55:259–287

Lovley DR (2002) Dissimilatory metal reduction: from early life to bioremediation. ASM News 68(5):231–237

Lovley DR, Phillips GYA, Landa ER (1991) Microbial reduction of uranium. Nature 350:413–416

Lovley DR, Widman PK, Woodward JC, Phillips EJP (1993) Reduction of uranium by cytochrome c_3 of *Desulfovibrio vulgaris*. Appl Environ Microbiol 59:3572–3576

Macaskie LE, Empson RM, Cheetham AK, Grey CP, Scarnulis AJ (1992) Uranium bioaccumulation by *Citrobacter* sp. as a result of enzymatically mediated growth of polycrystalline HUO_2PO_4. Science 257:782–784

Macaskie LE, Bonthrone KM, Yong P, Goddart DT (2000) Enzymatically mediated bioprecipitation of uranium by a *Citrobacter* sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation. Microbiology 146:1855–1867

Macaskie LE, Baxter-Plant VS, Creamer NJ, Humphries AC, Mikheenko IP, Mikheenko PM, Penfold DW, Yong P (2005) Applications of bacterial hydrogenases in waste decontamination, manufacture of novel bionanocatalysts and in sustainable energy. Biochem Soc Trans 33:76–79

Marqués AM, Roca X, Simon-Pujol MD, Fuste MC, Congregado F (1991) Uranium accumulation by *Pseudomonas* sp. ESP-5028. Appl Microbiol Biotechnol 35:406–410

Marshall MJ, Beliaev AS, Dohnalkova AC, Kennedy DW, Shi L, Wang Z, Boyanov MI, Lai B, Kemner K, McLean JS, Reed SB, Culley DE, Bailey VL, Simonson CJ, Saffarini DA, Romine MF, Zachara JM, Fredrickson JK (2006) C-type cytochrome-dependent formation of U(VI) nanoparticles by *Shewanella onediensis*. PLos 4(8):1324–1333

Martinez RJM, Beazley J, Teillefert M, Arakaki AK, Skolnick J, Sobecky PA (2007) Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated from radionuclide- and metal-contaminated subsurface soils. Environ Microbiol 9:3122–3133

Merroun M, Selenska-Pobell S (2001) Interactions of three eco-types of *Acidithiobacillus ferrooxidans* strains with U(VI). BioMetals 14:171–179

Merroun M, Hennig C, Rosberg A, Geipel G, Reich T, Selenska-Pobell S (2002) Molecular and atomic analysis of uranium complexes formed by three eco-types of *Acidithiobacillus ferrooxidans*. Biochem Soc Trans 30:669–672

Merroun M, Hennig C, Rossberg A, Reich T, Selenska-Pobell S (2003) Characterization of U(VI)-*Acidithiobacillus ferrooxidans* complexes using EXAFS, transmission electron microscopy, and energy-dispersive X-ray analysis. Radiochim Acta 91:583–592

Merroun M, Raff J, Rossberg A, Hennig C, Reich T, Selenska-Pobell S (2005) Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. Appl Environ Microbiol 71:5532–5543

Merroun M, Nedelkova M, Rossberg A, Hennig C, Selenska-Pobell S (2006) Interaction mechanisms of bacteria from extreme habitats with uranium as a function of pH. Radiochim Acta 94:723–729

Merroun ML, Nedelkova M, Ojeda, J Hennig C, Rossberg A, Romero-Gonzalez M, Selenska-Pobell S (2008) Bio-precipitation of uranium by natural bacterial isolates: a combined potentiometric titration, TEM and X-ray absorption spectroscopy study. FEMS Microbiol Ecol (In press)

Nedelkova M, Merroun ML, Rossberg A, Hennig C, Selenska-Pobell S (2007) Microbacterial isolates from the vicinity of a radioactive waste depository and their interactions with uranium. FEMS Microbiol Ecol 59:694–705

Nevin KP, Finneran KT, Lovley DR (2003) Micro-organisms associated with uranium bioremediation in a high-salinity subsurface sediment. Appl Environ Microbiol 69:3672–3675

Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313–339

Nyman JL, Marsh TL, Ginder-Vogel MA, Gentile M, Fendorf S, Criddle C (2006) Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms. Biodegradation 17:303–316

Petrie L, North NN, Dollhopf SL, Balkwill DL, Kostka JE (2003) Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl Environ Microbiol 69:7467–7479

Pollmann K, Raff J, Schnorpfeil M, Radeva G, Selenska-Pobell S (2005) Novel surface layer protein genes in *Bacillus sphaericus* associated with unusual insertion elements. Microbiology 151:2961–2973

Pollmann K, Raff J, Merroun M, Fahmy K, Mikheenko I, Selenska-Pobell S (2006) Bacteria from uranium mining waste piles and their technological applications. Biotechnol Adv 24:58–68

Raff J, Soltmann U, Matys S, Selenska-Pobell S, Böttcher H, Pompe W (2003) Biosorption of uranium and copper by biocers. Chem Mater 15:240–244

Redwood MD, Deplanche K, Baxter-Plant VS, Macaskie LE (2007) Biomass-supported palladium catalysts on *Desulfovibrio desulfuricans* and *Rhodobacter sphaeroides*. Biotechnol Bioengin. DOI:On line publication: 10.1002/bit.21689

Renninger N, Knopp R, Nitsche H, Clark DS, Keasling JD (2004) Uranyl precipitation by *Pseudomonas aeruginosa* via controlled polyphosphate metabolism. Appl Environ Microbiol 70:7404–7412

Roh Y, Gao H, Vali H, Kennedy DW, Yang ZK, Gao W, Dohnalkova AC, Stapelton RD, Moon J-W, Phels TJ, Fredricson JK, Zhou J (2006) Metal reduction and iron iomineralization by a psychrotolerant Fe(III)-reducing bacterium, *Shewanella* sp. strain PV-4. Appl Environ Microbiol 72:3236–3244

Rosen R, Becher D, Buttner K, Biran D, Hecker M, Ron EZ (2004) Highly phosphorylated bacterial proteins. Proteomics 4:3068–3077

Schulze-Lam S, Harauz G, Beveridge TJ (1992) Participation of a cyanobacterial S-layer in fine-grain mineral formation. J Bacteriol 174:7971–7981

Selenska-Pobell S (2002) Diversity and activity of bacteria in uranium waste piles. In: Keith-Roach MJ, Livens FR (eds) Interactions of micro-organisms with radionuclides. Elsevier Sciences Ltd, Oxford, UK, pp 225–254

Selenska-Pobell S, Miteva V, Boudakov I, Panak P, Bernhard G, Nitsche H (1999) Selective accumulation of heavy metals by three indigenous *Bacillus* isolates, *B. cereus, B. megaterium and B. sphaericus* in drain waters from a uranium waste pile. FEMS Microbiol Ecol 29:59–67

Sillitoe RH, Folk RL, Saric N (1996) Bacteria as mediators of copper sulfide enrichment during weathering. Science 272:1153–1155

Sleytr UB, Messner P, Pum D, Sára MRG (1996) Crystalline bacterial cell surface proteins. Academic Press, Landes Company

Suzuki Y, Banfield JF (2004) Resistance to and accumulation of uranium by bacteria from a uranium-contaminated site. Geomicrobiol J 21:113–121

Suzuki Y, Kelly SD, Kemner KM, Banfield JF (2003) Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. Appl Environ Microbiol 69:1337–1346

Urrutia Mera M, Kemper M, Doyle R, Beveridge TJ (1992) The membrane-induced proton motive force influences the metal binding ability of *Bacillus subtilis* cell walls. Appl Environ Microbiol 58:3834–3844

Vargas M, Kashefi K, Blunt-Harris EL, Lovley DR (1998) Microbiological evidence for Fe(III) reduction on early Earth. Nature 395:65–67

Wahl R, Mertig M, Raff J, Selenska-Pobell S, Pompe W (2001) Electron-beam induced formation of highly ordered palladium and platinum nanoparticle arrays on the S-layer of *Bacillus sphaericus* NCTC 9602. Adv Mater 13:736–740

Wan J, Tokunaga TK, Brodie E, Wang Z, Zheng Z, Herman D, Hazen TC, Firestone MK, Sutton SR (2005) Reoxidation of bioreduced uranium under reducing conditions. Environ Sci Technol 39:6162–6169

Yong P, Rowson NA, Farr JPG, Harris IR, Macaskie LM (2002) Bioreduction and biocrystallization of palladium by *Desulfovibrio desulfuricans* NCIMB 8307. Biotechnol Bioeng 80:369–379

Index

C

D

I

M

P

R

S

T