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Polyextremophiles

Life Under Multiple Forms of Stress

POLYEXTREMOPHILES

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 27

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Joseph Seckbach

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INTRODUCTION

The current volume presents a collection of essays, review papers, and results of original research dealing with different aspects of life at more than one environmental extreme. We know many types of “extremophilic” microorganisms (and some macroorganisms as well) that are adapted to life at high or low temperature, high or low pH, high salt concentrations, high pressure, extreme levels of radiation, etc. Each of these modes of life requires special mechanisms that enable the organisms to withstand the environmental conditions that are too harsh for most other organisms. The existence of “polyextremophilic” organisms is even more intriguing, as here the organisms must be able to tolerate a combination of stressful factors. The microorganisms that lived on early Earth were most probably polyextremophiles, and their offspring are still found in specialized niches. These polyextremophiles may serve as analogues or models for extraterrestrial life-forms that may exist or may have existed on Mars, Europa, and other celestial bodies.

This book contains 32 chapters, contributed by 61 authors from 17 countries. The present volume complements the other volumes in the **COLE** series (www.springer.com/series/5775) that deal with selected aspects of extremophiles, notably volumes 5, 9, 11, 17, and 21. We hope that this volume will prove valuable as an up-to-date overview of the current state of the research on extremophiles in general and polyextremophiles in particular.

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and
Helga Stan-Lotter
November 2012

FOREWORD

Almost all of the chapters in this volume involve microorganisms exclusively. This foreword will restrict comments to such organisms. There are two perspectives to consider. Extreme environments for most scientists are those in which the number or type of species is severely limited by chemical, physical, or biological features. Many microbial environments that are considered extreme on the basis of two or more factors are represented in this volume, although in many of these cases, the organisms in these environments are well adapted to those extremes. In contrast, there are extreme conditions that cause stresses, one or more, severe or mild, for organisms that are not in their optimal milieu. In the latter situation the organisms are not well adapted to these extremes, but are stressed, and may be close to their tolerance limits. In natural habitats, it is thought that microorganisms are nearly always experiencing stress at various times during their life or growth cycles, and usually there are several factors imparting this stress. For example, seldom are one or more nutrients or substrates available in sufficient quantities to allow maximum growth rates. However, in oligotrophic environments, the optimal strategy is normally not to exercise maximum growth rates, but rather partially adapt by growing slowly, by not depleting nutrients, and therefore showing greater fitness than copiotrophs that tend to show a burst of growth and then starve. Nevertheless, no matter what strategy is employed, a low nutrient environment may be considered an extreme, even for oligotrophs. It is difficult and often impossible to assess the degree of stress imparted by nutrient insufficiency. For this reason, other stresses and extremes that can be measured and evaluated more readily are described in this book. A condensed review of many of the chapters follows.

Some chapters are concerned with haloalkaliphily or haloalkalitolerance in environments such as soda lakes or the famous saline wadis of Egypt. In most of these cases the microorganisms represent adaptations to these environments, but stresses are surely involved if these environments do not represent the optima for the relatively few species. High pH tolerant as well as thermophilic or thermotolerant microorganisms have been isolated from normal soils and sediments, indicating inactivity (stress) in the habitat from which they were isolated. Halobacteria (Archaea), normally aerobic heterotrophs, regularly encounter low O_2 as a stress but have adapted in various ways by utilizing other oxidants, such as nitrate, or by synthesizing and utilizing a photo-ATP-producing rhodopsin. "Brine lakes," found at depths in the Mediterranean and Red Seas, harbor microorganisms under high pressure and high salt. These microbes exist in darkness and anoxia, neither of which are extremes, but are they under stress or optimally adapted to the conditions in the brine, conditions that may have isolated these organisms from the mixolimnion for thousands of years? High salinity of a different sort

exists in only a few rare environments, e.g., Dead Sea, where chlorides (e.g., Mg, Ca) comprise the salt stress, in which these ions also greatly reduce the availability of inorganic phosphate. It is doubtful whether any of these microorganisms are well adapted. However, other microbes are not tolerant enough to compete in this environment.

There are chapters that focus on cold environments such as snow and ice where it is apparent that many microorganisms are psychrotolerant rather than psychrophilic. Low temperature or its fluctuation may be a stress but particularly with other environmental factors that enhance this negative effect, such as high solar radiation or nutrient limitation. In one chapter it is described how low and fluctuating temperature and UV radiation had an effect on several freshwater green algae from Antarctica by altering gene expression, morphology, ultrastructure, and physiology. An aspect of low temperature not often identified is freezing and assessing its effect, which may be lethal or tolerated by some organisms but not necessarily those that grow in cold environments. High temperature along with low pH, high pressure, or high salt is also addressed. Ultraviolet radiation and its protection by melanin in black fungi are considered. In one chapter, ionizing radiation, the potentially lethal reactive oxygen that is produced, and the protective Mn antioxidant complex induced in a halophilic archaeon are described. Although many of the chapters present results of current or novel research, others are speculative. Besides this summary of many of the subjects presented in this volume, other examples of microbial polyextremophiles and of environmental stresses that are not specifically included in this volume will be mentioned.

Low temperature (i.e., below 8 °C) in Antarctic freshwater and saline melt ponds on the ablation moraine of the McMurdo Ice Shelf is itself a stress, since some of the cyanobacteria from these ponds showed maximal growth and photosynthetic rates above 18 °C. Continuous sunlight was inhibitory to photosynthesis in midday for some cyanobacteria (e.g., *Oscillatoria* cf. *priestleyi*) if they were to move to the surface of a microbial mat. The same inhibition by UV radiation in particular was demonstrated in cyanobacterial mats of hypersaline ponds in Mexico if the motile cyanobacteria moved to the surface of the mat during periods of high solar radiation. Neither these nor the motile cyanobacteria in Antarctica moved to the surface, however, but remained 1–2 mm below the soft mat surface except during periods of very low light intensity or darkness, natural or artificial. Low temperature and darkness are two further stressors for polar phototrophs and are exemplified by the numerous cyanobacteria and diatoms and other algae that survive the many months of darkness at 78° south or 78° north latitudes (e.g., McMurdo Ice Shelf, Antarctica; Svalbard, Norway). Low temperature, darkness, and brine accumulate in the benthos of somewhat saline ponds in Antarctica (~77–78° S) while the water column is frozen; thus, some cyanobacteria and other phototrophs there are stressed by cold, long-term darkness, and extremely high salinity. On a daily basis, mats of cyanobacteria in meltwater streams (largely *Nostoc*) freeze during low sun angles in the Antarctic dry valleys

and thaw during higher sun angles (e.g., stream from the Canada Glacier, Taylor Valley). In addition, the high temperature of hot springs in Iceland (~66° N) and eastern Greenland (~70° N) is maintained through the winter darkness without loss of the cyanobacterial mats. For the unicellular algae of the class Cyanidiophyceae that grow optimally at pH 2–3 and temperatures of 40–50 °C, the conditions are to which these algae are adapted and are therefore not considered stresses. However, within conditions realized in acidic hot springs and in acidic streams such as the Rio Tinto of Spain, there are numerous potentially toxic compounds, namely metals or metalloids such as Al, Hg, As(III), As(V), Fe(II), Fe(III), and Mn. Thus, single or multiple factors may inhibit these algae. Higher pH, i.e., >pH 4 is another stressor that may be encountered by the species of the acidophilic Cyanidiophyceae but may be overcome by slow growth in confined environments and eventually by their lowering of the surrounding pH to optimal levels. Suboptimal temperature constitutes another stress for this algal group. Sulfide (H₂S, HS⁻) almost always interacts with temperature and light intensity as a stressor in the case of some photosynthetic cyanobacteria. Some thermophilic forms are adapted to moderately high temperatures (45–60 °C) but show a great sensitivity to environmental sulfide, while a few others adapt to sulfide with partial tolerance or by shifting to anoxygenic photosynthesis. The use of sulfide as an electron donor in a species of *Oscillatoria* may occur only under low light, while O₂ production under high light resulted in a shift to oxygenic photosynthesis when the sulfide was exhausted. Sulfide or thiosulfate, as electron donors in the case of phototrophic purple and green sulfur bacteria, may be limited and therefore cause stress, as in the case of low nutrients, and further stress may be caused by high light intensity or aerobic conditions for these anaerobic phototrophs.

In almost all cases, UVB and UVA radiation may be considered stresses that act in concordance with other factors. When one or more toxic substances limited photosynthesis in a warm stream where a cyanobacterium (i.e., *Calothrix*) thrived, UV radiation stimulated the production of the UV-shielding sheath compound, scytonemin, whereas in a non-toxic spring where the same cyanobacterium thrived, UV damage was circumvented by more active metabolism and scytonemin was not synthesized. In another example (the cyanobacterium *Nostoc*), the synthesis of scytonemin occurred under UV stimulation only when the organism was under the stress of having to fix N₂ rather than depending on combined nitrogen. In still another example, high UV radiation at an alkaline hot spring caused measurable inhibition of photosynthesis although overall photosynthesis was highest in mid-morning to midday. However, photosynthesis declined in the afternoon even at solar irradiances comparable to the morning, indicating damage sustained earlier in the day by both high light and UV radiation. Full photosynthetic potential was restored by the next morning.

Desiccation is another extreme not covered extensively in this volume, but it is important to note that microbial (cyanobacterial) crusts in deserts and elsewhere ent availability, compaction, and surely other negative factors. One of the most ubiquitous extreme habitats is that of endolithotrophs in which cyanobacteria,

algae, and sometimes fungi form a narrow (1–3 mm) band a few millimeters below the surface of semi-porous rocks such as limestone, dolomite, and sandstone. This extreme habitat, often in cold or hot deserts, is also advantageous in some ways, protecting the microbiota from wind abrasion, grazers, and high solar radiation (including UV). However, the negative stressors are periodic or long-term desiccation and a probable shortage of nutrients even when moisture is available because of the crowding of the microbes and the limitation by low light intensity that determines the lower border of the band.

Many other examples exist. However, this volume encompasses a large range of multiple stresses in many extreme environments. The reading of this book should be of great value to all microbiologists.

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Biodata of **Richard W. Castenholz**, author of the “*Foreword*” to this volume.

Dr. Richard W. Castenholz is Professor Emeritus in the Institute of Ecology and Evolution, University of Oregon, Eugene, Oregon, USA. He received his B.S. in Botany at the University of Michigan (1952) and his Ph.D. in Botany at Washington State University (1957). He has been a faculty member at Oregon since 1957 and was promoted to Professor in 1969. His early research was on the ecology of freshwater and marine epilithic diatoms. In the 1960s he began his research on thermophilic cyanobacteria. In the 1970s he and Beverly K. Pierson isolated and characterized thermophilic anoxygenic *Chloroflexus*, the first described member of the current phylum Chloroflexi. Later studies of hot spring mats developed into research of phototrophs in other environments, including hypersaline ponds, Antarctic meltwater ponds, and endolithic habitats. As an outcome of these studies, he and Ferran Garcia-Pichel characterized the UV-shielding pigment scytonemin, present in the sheaths of cyanobacteria in sun-exposed habitats, and the motile escape strategy in several motile cyanobacteria in thermal, temperate, and polar microbial mats. Currently his main focus is on the phylogenetic and geographic diversity of the thermoacidophilic unicellular algae of the Rhodophytan class Cyanidiophyceae (order Cyanidiales). He has maintained a large collection of cultures of microbial phototrophs from extreme environments for over 40 years.

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PREFACE

*Imagination is everything. It is the preview of life's coming attractions.
Imagination is more important than knowledge.*

Albert Einstein

Recently, I had the chance to see one of the largest exhibitions of Claude Monet's most famous series of paintings in London. As Kenneth Clark explains in his famous book *Civilization*, Monet attempted a kind of color symbolism to express the changing effects of light. For example, he painted a series of cathedral facades in different lights—pink, blue, and yellow—which seem to me too far from my own experience. The colors of these objects depend on the physical environment, such as sunlight, snow, the time of the day, the season etc.

Under different conditions, one object may show quite different properties. Who can be sure what is the absolute property? The biological world may have the same uncertainty.

Pioneering Studies of Extremophiles

THERMOPHILES

In June 1965, Thomas Brock, a microbiologist at Indiana University, discovered a new form of bacteria in the thermal vents of Yellowstone National Park. They can survive at near-boiling temperatures. At that time the upper temperature for life was thought to be 73 °C. He found that one particular spring, Octopus Spring, had large amounts of pink, filamentous bacteria at temperatures of 82–88 °C. Here were organisms living at temperatures above the “upper temperature for life.” He isolated and collected many microbes from this geothermal area (Brock and Freeze, 1969). It is worth mentioning that the strain YT-1 was the first to be used as source of *Taq* polymerase (Brock, 1997). His findings paved the way for a new microbiology: taxonomy, physiology, enzymology, molecular biology, genetics, etc.

ALKALIPHILES

In 1968, I was looking at the Renaissance buildings in Florence in Italy which were so very different from Japanese architectures. About 500 years ago no Japanese could have imagined this Renaissance culture. Then suddenly a voice whispered in my ear, “There might be a whole new world of microorganisms in

different unexplored cultures.” “Could there be an entirely unknown domain of microorganisms at alkaline pH?” The acidic environment was being studied, probably because most food is acidic. Science, just as much as the arts, relies upon a sense of romance and intuition. Upon my return to Japan, I prepared an alkaline medium and inoculated it with small amounts of soil collected from various sites on the campus of the Institute of Physical and Chemical Research (RIKEN). To my surprise, after overnight incubation at 37 °C, various microorganisms flourished in all 30 test tubes. Here was a new alkaline world that was utterly different from the neutral world discovered by Pasteur. I named these microorganisms that thrive in alkaline environments “alkaliphiles.” This was my first encounter with extremophiles (Horikoshi, 1971).

One of the enzymes isolated from alkaliphiles is cyclodextrin glycosyltransferase which produces very high yield of cyclodextrin (CD). CD can encapsulate many volatile compounds. For instance, Japanese horseradish (wasabi) is encapsulated in CD and its flavor and taste are stabilized for long times (Horikoshi, 2006).

Wasabi is one of the most important spices for Japanese food. Nowadays you may have typical Japanese food sushi all over the world. This means CD popularizes Japanese food culture throughout the world.

Time is the best appreciator of scientific work, and we know that an industrial discovery rarely bears all its fruits in the hands of its first inventor.

Louis Pasteur

Extreme Environments

Not too many years ago, almost all biologists believed that life could survive only within a very narrow range of temperature, pressure, acidity, alkalinity, salinity, and so on. Nature, however, contains many extreme environments, such as hot springs, saline lakes, deserts, alkaline or acidic lakes, and the deep sea. All of these environments would seem to be too harsh for life to survive.

However, in recent times many organisms have been found in such extreme environments. Moreover, some of them cannot survive in a so-called “moderate” environment. Thermophilic bacteria grow in environments with extremely high temperatures, but cannot grow at 20–40 °C. Some alkali-loving bacteria cannot grow in a nutrient broth at pH 7.0 but flourish at pH 13. These properties depend on growth conditions (pH values of media for thermophiles, culture temperatures for alkaliphiles and piezophiles, etc.). Thus, the idea of extreme environments is relative, not absolute. Clearly we have been too anthropocentric in our way of thinking. We should therefore extend our consideration to other environments having multi/poly extreme conditions for life. They metabolize inorganic sulfur or iron as their energy source. Some of them isolated from deep sea or sub-deep seafloor sediment have an entirely different metabolic pathway from conventional life. It is distinctly possible that very ancient life-forms may be in hibernation in the world’s largest refrigerator.

Definition of Extremophiles

Extremophiles are organisms that are adapted/evolved to grow optimally at or near the extreme ranges of environmental variables. R.D. MacElroy first coined the term “extremophiles” in a 1974 paper entitled “Some comments on the evolution of extremophiles,” but definitions of extreme and extremophiles are of course anthropocentric. A much larger diversity of organisms are known that can tolerate extreme conditions and grow but not necessarily optimally in extreme habitats; these organisms are defined as extremophiles.

Distribution of Polyextremophiles

At the wider scale, extreme environments on Earth have arisen and continue to arise as a consequence of plate tectonic activity, the dynamic nature of the cryosphere, and the formation of endorheic basins. Plate boundaries occur wherever two tectonic plates collide and result in the formation of mid-ocean ridges, mountains, deep-ocean trenches, volcanoes, and other geothermal phenomena such as marine hydrothermal vent systems.

Hydrothermal vent systems are found in abundance worldwide and are presumed to have existed as soon as liquid water accumulated on Earth. Black smoker and carbonate chimney vents are different environments: black smokers arise at diverging plate boundaries above magma chambers, are highly acidic (pH 1–3), are very hot (up to 405 °C), with vent fluids rich in Fe and Mn, and CO₂, H₂S, H₂, and CH₄; carbonate chimneys in contrast are found off-axis (away from diverging boundaries), are highly alkaline (pH 9–12), moderately hot (up to 90 °C), and rich in H₂, CH₄, and low molecular weight hydrocarbons.

A high proportion of the Earth’s surface contains water in solid form (sea ice, ice caps and sheets, glaciers, snowfields, permafrost), the longevity of which may be thousands or even a few million years. Cryosphere-climate dynamics are complex and influence precipitation, hydrology, and ocean circulation. Deserts develop in regions where precipitation is very low (or zero) and also unpredictable. Highly saline lakes and pans often develop under these circumstances.

The average depth of the world’s oceans is about 3,800 m; high pressure generates yet another extreme environment. Oligotrophic environments are defined as those presenting very low nutrient concentrations; they include oceans depleted in iron, nitrate, phosphate, tropical laterite soils, and white sands. Finally, a range of environments are deemed to be extreme by virtue of chemically and/or physically caused toxicity (e.g., soils high in arsenic, lakes exposed to high incident radiation).

New extreme ecosystems continue to be discovered and investigated including the deep biosphere that exists at great depths in sub-seafloor sediments and in subterranean rock formations, and the carbonate chimney vent system. Extreme environments almost invariably are affected by two or more extreme conditions. Is our current knowledge of extremophile diversity comprehensive? It is highly

possible that acidopsychrophiles, acidohalophiles, and thermohalophiles exist, and these should not be neglected by microbiologists!

It may appear overhasty to introduce the evolution and discussions of the origin of life in the preface. Assuming a thermophilic beginning, acidophily probably arose at an early stage, while alkaliphily evolved only after certain mineral precipitation and sufficient buffer concentration of CO₂ was established in the atmosphere. Moreover, halophily could have developed only after an arid climate was imposed on land and psychrophily only after a major temperature fall.

The question “what is life?” is precisely the question “what is evolution?”

Carl R. Woese

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**PART I:
GENERAL ASPECTS**

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Biodata of **Mark C. Capece**, **Evan Clark**, **Jamal K. Saleh**, **Daniel Halford**, **Nicole Heintz**, **Samuel Hoskins**, and **Lynn J. Rothschild**, authors of “*Polyextremophiles and the Constraints for Terrestrial Habitability*.”

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POLYEXTREMOPHILES AND THE CONSTRAINTS FOR TERRESTRIAL HABITABILITY

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1. Introduction

Space has been touted as “The Final Frontier,” but the harsh environments found in the cosmos are largely inhospitable to humans. Extremophiles, however, already flourish in extreme terrestrial habitats and may represent the only known organisms capable of surviving in these hostile extraterrestrial environments. Our knowledge of life in extreme environments has progressed considerably in the past several decades. Thermophilic life was unknown until the discovery of *Thermus aquaticus* in 1969, which dramatically expanded the list of known habitats (Brock and Freeze, 1969). Since then, hundreds of new extremophiles have been identified. While thermophiles, organisms able to tolerate extremely high temperatures, were the first focus of extremophile research, attention soon diverted to the discovery of other extreme inhabited niches, such as acidic, cold, high pressure, and even radioactive environments, revealing a diversity of biochemical mechanisms that organisms have adapted to live in seemingly inhospitable conditions.

The environments inhabited by polyextremophiles, which thrive at multiple extremes, provide excellent models for potential extraterrestrial habitats. We now know that sulfur-oxidizing bacteria reside in the high-pressure, acidic hydrothermal vents in the Guaymas Basin and psychrophilic, halotolerant bacteria live in the Siberian permafrost (Lonsdale, 1977; Shcherbakova et al., 2009). Polyextremophiles living in the cold, highly pressurized environments of subglacial lakes on Earth lend

credence to the possibility of extraterrestrial life residing within the subsurface lakes found on the Jovian moon Europa and the Kronian moon Enceladus (Marion et al., 2003; Parrilli et al., 2011).

While extremophiles as a group can survive in any individual extreme, but there are some combinations of polyextremophily that have yet to be characterized on Earth. Synthetic biology may be able to fill these gaps. Novel polyextremophiles may be engineered through the combination of multiple biochemical survival mechanisms. For example, the introduction of extreme genome repair pathways into a psychrophile could instill radioresistance into an organism already capable of surviving at low temperatures (Galhardo and Rosenberg, 2009). The product of this transformation would be an organism that could thrive in extremely cold, radiation-exposed habitats. It may very well be that the first organism to inhabit extraterrestrial environments will be designed, rather than discovered.

In this chapter, we make a first effort to compile what is known about organisms living at major environmental extremes and their corresponding mechanisms of survival in order to estimate the niche space for life on Earth. After presenting the extremes individually, we discuss the combination of extremes to delineate synergistic and antagonistic effects of multiple biochemical adaptations. We then analyze parameters that affect life at the interface of extremes in temperature and pH. The combination of these two extremes produces a two-dimensional niche space model whose population by terrestrial organisms may indicate biological constraints to terrestrial and extraterrestrial habitability. We conclude with a brief discussion of the potential for synthetic biology to engineer artificial polyextremophiles that expand the niche space for terrestrial life and could potentially allow survival in extraterrestrial environments.

2. Adaptations for Life at Individual Extremes

Extremophiles have been studied extensively to answer questions in biogeography, food preservation, biotechnology, and astrobiology. As we learn more about the extremes of terrestrial life, we will develop constraints on the search for extraterrestrial life (Rampelotto, 2010; Dartnell, 2011). The individual extremes that have been studied most extensively include temperature, pH, salinity, pressure, radiation, desiccation, and oxygen stress, as shown in Table 1. The following sections explore the limits of terrestrial life and biochemical adaptations that allow organisms to thrive at each of these individual extremes.

2.1. TEMPERATURE: THERMOPHILES AND PSYCHROPHILES

About 75 % of the Earth's biosphere, including polar, deep ocean, and atmospheric habitats, is permanently cold, and 70 % of the surface of the planet has a temperature between 1 and 5 °C (Feller and Gerday, 2003; Cavicchioli, 2006).

Table 1. Summary of extremophile classifications and their corresponding biochemical adaptations.

Adaptations		
Extremophile	Environmental parameter	Membrane and cell wall Cytoplasm, metabolome, and bioenergetics Proteome and genome
Psychrophile	$T < 15\text{ }^{\circ}\text{C}$	Unsaturated fatty acids (B) Glycerol ether lipids (A) EPS Teichoic acid
Thermophile	$T > 60\text{ }^{\circ}\text{C}$	Saturated fatty acids (B) ω -Alicyclic fatty acids (B) Glycerol ether lipids (A)
Acidophile	$\text{pH} < 3$	ω -Alicyclic fatty acids (B) Glycerol ether lipids (A) Channels with small pores Channels with positive charge K^+/H^+ antiporters
Alkaliphile	$\text{pH} > 9$	Negative surface charge $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporters $\text{Na}^+/\text{solute}$ symporters
Halophile	$[\text{NaCl}] > 5\% (\text{w/v})$	Ion accumulation Polar organic osmolyte accumulation
Piezophile	$P > 60\text{ MPa}$	Unsaturated fatty acids (B) Glycerol ether lipids (A) EPS

Cold-shock pathway
Active-site flexibility
T-dependent transcription factors

Heat-shock pathway
Chaperonins
Uncharged surface
DNA repair pathways

Acid-shock pathway
Chaperonins
Positive surface charge
“Iron rivets” (A)
pH-dependent transcription factors
DNA repair pathways

Negative surface charge

Negative surface charge
Ion-subunit bridges

Cold-shock pathway
Heat-shock pathway
Uncharged surface
16S rRNA helix elongation

(continued)

Table 1. (continued)

Extremophile	Environmental parameter	Adaptations		
		Membrane and cell wall	Cytoplasm, metabolome, and bioenergetics	Proteome and genome
Radioreistant	Radiation survival		Mn ²⁺ accumulation Polar organic solute accumulation	Polyploidy DNA repair pathways
Xerophile	Anhydrobiosis		Mn ²⁺ accumulation Polar organic lyoprotectant accumulation	DNA repair pathways
Aerobe	[O ₂] ≈ 21 % (v/v)		Aerobic respiration	Superoxide dismutase
Microaerophile	[O ₂] < 5 % (v/v)		Aerobic respiration	Superoxide reductase
Anaerobe	[O ₂] ≈ 0 % (v/v)		Anaerobic respiration	

A archaea only, *B* bacteria only, *P* pressure, *T* temperature.

Thus, the existence of psychrophiles, or organisms that live in these cold environments, is intuitive. Psychrophiles are commonly divided into two categories: stenopsychrophiles and eurypsychrophiles. Stenopsychrophiles, or obligate psychrophiles, can only survive at temperatures below 15 °C, while eurypsychrophilic, or mesotolerant, organisms grow optimally below 15 °C but can also survive at higher temperatures (Feller and Gerday, 2003; Cavicchioli, 2006).

It is well known that many organisms remain metabolically active at temperatures well below freezing; trees, penguins, polar bears, and the Himalayan midge are common examples (Koshima, 1984). Psychrophilic microorganisms, including the bacterium *Psychromonas ingrahamii*, the archaeon *Methanosarcina baltica*, and the fungus *Humicola marvinii*, metabolize and reproduce optimally at temperatures below 15 °C and, in some cases, have been reported to survive with greatly reduced metabolism down to -20 °C (Weinstein et al., 1997; Von Klein et al., 2002; Auman et al., 2006; Kumar et al., 2007).

Life at low temperatures has many challenges, including membrane rigidity, protein misfolding, and slower reaction rates. Many psychrophilic microorganisms, such as the alga *Chlamydomonas* sp. ICE-L, have been found to increase membrane fluidity by incorporating more unsaturated fatty acids into the phospholipid bilayer (Zhang et al., 2011). Methanogens also express a variety of unique protein adaptations designed to assist both anabolic and catabolic metabolism (Von Klein et al., 2002; Kumar et al., 2007). For example, a psychrophilic α -amylase was found to have a higher turnover rate (k_{cat}), lower activation energy (ΔG^\ddagger), and lower change in enthalpy (ΔH^\ddagger) than mesophilic and thermophilic amylases (Feller and Gerday, 2003). This reduction in activation energy is partially achieved through increased flexibility in the psychrophilic enzyme's active site, which reduces the substrate-enzyme complex binding energy and activation energy (Roulling et al., 2011). Eurypsychrophilic methanogens have also been found to possess temperature-dependent transcription factors that respond to variations in temperature by changing proteome composition (Goodchild et al., 2004). Finally, extracellular polysaccharide substances (EPS) and teichoic acid are cryoprotectants, and intracellular fumarate and glycerol are chaotropes commonly produced upon cold shock (Rice et al., 2008; Marx et al., 2009; Chin et al., 2010).

Thermophilic and hyperthermophilic microorganisms are defined by optimal growth above 60 and 80 °C, respectively, and in the case of *Pyrolobus fumarii*, temperatures up to 113 °C have been reported to sustain proliferation (Blöchl et al., 1997; Rothschild and Mancinelli, 2001; Kashefi and Lovley, 2003). Thermophiles are, for the most part, prokaryotic, and the most hyperthermophilic are archaea, which possess a large number of high-temperature adaptations. Many thermophiles that appear closely related to the last universal common ancestor (LUCA) have been discovered in hot springs, leading to the hypothesis that the origin of life on Earth may be integrally related to hot spring biogeochemistry, although this proposition is controversial (Miller and Bada, 1988; Koonin and Martin, 2005). What is not typically stressed is that hot springs, such



Figure 1. Octopus Springs, Yellowstone National Park, WY, USA, has been found to contain a wide variety of thermophiles.

as Octopus Springs in Yellowstone National Park (Fig. 1), exist with a wide variety of pH, elemental composition, and radiation parameters. These special environments often create ideal model systems for investigating the interaction of temperature with other environmental parameters in polyextremophiles. Deep-sea vents also provide opportunities to study thermophiles at high pressures and in the absence of light and oxygen.

Common challenges endured by thermophilic organisms include high membrane fluidity, increased protein misfolding, and nucleic acid degradation (Kumar et al., 2007; Toueille and Sommer, 2011). Adaptations to high membrane fluidity typically involve increases in the production of saturated fatty acids and ω -alicyclic fatty acids, both of which preserve a more crystalline, ordered structure (Chang and Kang, 2004). Archaea have, more than any other domain, mastered the control of membrane fluidity through the use of glycerol ethers, which can maintain a liquid crystalline state at temperatures over 100 °C (Koga and Morii, 2005). Since the early Earth was much warmer than contemporary temperatures, this archaeal adaptation is consistent with the idea that archaea arose early in evolution. Thermophiles have been found to improve protein thermostability by expressing heat-shock chaperonins to assist protein folding, prevent protein denaturation, and, in the case of small heat-shock protein “holdases,” disaggregate amyloids (Trent, 1996; Luo and Robb, 2011). Protein folding is also aided by moving charged residues to the interior of the protein, effectively neutralizing the surface charge (Fukuchi et al., 2003). At high temperatures, neutral proteins have lower dielectric properties and desolvation penalties (Thomas and Elcock, 2004). In addition, more densely packed protein folding and exposed hydrophobic shells

minimize regions where high-temperature water can destabilize the protein (Vetriani et al., 1998). Interior salt and disulfide bridges further improve thermostability (Robb and Maeder, 1998).

Proteins and other macromolecular complexes are also stabilized by organic solute thermolytes (Santos et al., 2011). These compatible solutes are usually either polyol-phosphodiester, like di-*myo*-inositol phosphate, or α -hexose derivatives, like mannosylglycerate, although aspartate accumulation has also been linked to hyperthermophily. While these thermolytes have been shown to play a physiological role in high-temperature adaptation, the accumulation of compatible solutes has not been observed in all thermophiles and, thus, must not be essential for thermophily (Santos et al., 2011). Finally, high temperatures promote the degradation of nucleic acids, so many thermophiles upregulate DNA repair pathways in order to preserve genetic stability (Touaille and Sommer, 2011).

2.2. pH: ACIDOPHILES AND ALKALIPHILES

Organisms that thrive at the extremes of pH are classified as either acidophiles, which exhibit optimal growth below pH 3, or alkaliphiles, which grow optimally at pH greater than 9 (Rothschild and Mancinelli, 2001; Wiegel, 2011). Dozens of acidophilic and alkaliphilic species have been discovered and represent every domain of life. Furthermore, many neutrophiles, organisms that grow optimally at neutral pH, have demonstrated coping strategies similar to their extremophilic counterparts that allow suboptimal growth at low and high pH. These species are typically classified as acidotolerant or alkalitolerant, respectively.

Acidophiles and alkaliphiles have been discovered in habitats all over the world. Acidophiles thrive in sites of acid mine drainage, solfataric fields, acidothermal hot springs (Fig. 2) and fumaroles, coal spoils, and bioreactors. These environments feature low pH values, temperatures ranging from 25 to over 90 °C, pressures up to 5 MPa, low salinity, some heavy metals, and either anaerobic or aerobic conditions (Seckbach and Libby, 1970; Hallberg and Lindström, 1994; Golyshina et al., 2000; He et al., 2004; Ferris et al., 2005; Yoshida et al., 2006; Hallberg et al., 2010; Reeb and Bhattacharya, 2010). Alkaliphiles have been found to proliferate in alkalithermal hot springs, shallow hydrothermal systems, sewage, and hypersaline soda lakes, such as Mono Lake, CA, USA, and Lake Elementaita in the Kenyan-Tanzanian Rift Valley. The conditions at these locations include a wide range of temperatures but usually feature high pH and moderate to high concentrations of dissolved salts (Xu et al., 1999; Hoover et al., 2003; Ma et al., 2004; Kanekar et al., 2012).

Over the past three decades, many novel adaptive mechanisms have been discovered that allow organisms to live in extremes of pH. These mechanisms are designed to maintain pH homeostasis in order to prevent genomic and proteomic



Figure 2. Acidophilic algae, such as *Cyanidium caldarium*, form green mats in Nymph Creek, Yellowstone National Park, WY, USA.

damage and energetic dysfunction (Zychlinsky and Matin, 1983; Matin, 1990; Krulwich, 1995; Padan et al., 2005; Baker-Austin and Dopson, 2007).

Acidophiles face the unique challenge of living in an environment with a high $[H^+]$. Thus, these organisms must moderate the use of H^+ influx to drive ATP synthesis in order to prevent cytoplasmic acidification. This balance is best achieved by simply restricting the influx of H^+ through nonspecific avenues. One method for restricting H^+ influx is by reducing the permeability of the cell membrane. Acidophilic archaea, such as *Ferroplasma acidiphilum*, have the advantage of possessing membranes composed of glycerol ethers, as ethers are less susceptible to acid hydrolysis than the glycerol esters of bacterial phospholipids (Baker-Austin and Dopson, 2007). Some acidophilic bacteria incorporate ω -alicyclic fatty acids into the membrane in order to increase acid resistance (Chang and Kang, 2004), although these acid-resistant lipids can lose structural integrity at neutral pH (van de Vossenberg et al., 1998). In addition, the genome of some bacterial species contains significant cell membrane biosynthetic enzyme diversity, which has been postulated to allow membrane adaptation to fluctuating acidic pH (Baker-Austin and Dopson, 2007). The second strategy is to reduce H^+ influx through transmembrane channels. At low extracellular pH, *Acidithiobacillus ferrooxidans* upregulates the expression of Omp40, a channel with a smaller pore size. Similarly, acidotolerant *Escherichia coli* strains replace negatively-charged channels with positively-charged channels when the extracellular pH falls below 5 (Baker-Austin and Dopson, 2007). A third adaptive mechanism is the generation of a Donnan potential through the accumulation of monovalent cations in the cytoplasm. The high intracellular cation concentration generates a positive charge

gradient $\Delta\psi$, thereby inhibiting H^+ influx despite the favorable concentration gradient. K^+/H^+ antiporters with stoichiometries of $>1:1$ are often employed to promote the formation of this Donnan potential. These antiporters, as well as ATP-dependent H^+ pumps, which are found in acidophiles of all three domains, also serve to promote the efflux of H^+ and resist cytoplasmic acidification (Matin, 1990; Baker-Austin and Dopson, 2007; Das et al., 2009; Enami et al., 2010). Furthermore, since organic acids behave as H^+ transporters, many acidophiles are chemoorganoheterotrophs that oxidize the compound to produce H_2 rather than H^+ . The catabolism of formic acid ($HCOOH$), for example, would generate one molecule of carbon dioxide (CO_2) and one molecule of hydrogen (H_2) without affecting the cytoplasmic pH (Baker-Austin and Dopson, 2007).

When these mechanisms fail to prevent excessive H^+ influx, many acidophilic and acidotolerant organisms can survive minor acidification by buffering the cytoplasm with a high-pI proteome, decarboxylated glutamate and arginine, and phosphoric acid (Matin, 1990). Any minor proteomic or genomic damage incurred due to this acidification may then be repaired with an acid-shock response of molecular chaperones and DNA lesion repair mechanisms (Baker-Austin and Dopson, 2007). Interestingly, some of the most acidophilic archaea employ Fe cofactors, which have been found to stabilize proteins at low pH. Removal of these “iron rivets” results in immediate loss of protein tertiary structure (Baker-Austin and Dopson, 2007).

Alkaliphiles live in an environment with a low $[H^+]$, so the challenge for these organisms is to continuously neutralize the cytoplasm and encourage H^+ influx to drive ATP synthesis. The first and most prominent adaptation is the use of Na^+/H^+ and K^+/H^+ antiporters to move H^+ into and monovalent cations out of the cell. The anaerobic alkaliphiles *Natronaerobius thermophilus* and *Desulfovibrio vulgaris* typically utilize NhaC (Na^+/H^+) antiporters, while the aerobic alkaliphiles *Bacillus pseudofirmus* and *Synechococcus elongatus* primarily express $Na^+(K^+)/H^+$ antiporters of the CPA-1 and CPA-2 families (Krulwich et al., 2009; Mesbah et al., 2009; Mesbah and Wiegel, 2011). The most alkaliphilic species have genomes that encode more than a dozen different transport proteins, each with a different pH range for activity (Mesbah et al., 2009, 2011). Intracellular salt concentrations are restored via Na^+ - and K^+ -coupled solute uptake and Na^+ -coupled flagellar rotation (Krulwich, 1995; Horikoshi, 2011). The use of sodium motive force (SMF) to drive flagellar motors is a result of unique flagellar stators found primarily in extreme alkaliphiles (Ito et al., 2011). Acidic cell walls and cytoplasmic buffering by low-pI proteins promote desirable cation and H^+ influx (Horikoshi, 2011; Mesbah and Wiegel, 2011). These strategies allow alkaliphiles to maintain an intracellular pH up to 2.5 units lower than the extracellular environment (Horikoshi, 2011; Krulwich et al., 2011a). For oxidative phosphorylation to occur in an alkaline environment, the organism must somehow recouple ionic influx with ATP synthesis. This goal may be achieved in a variety of ways, including the sequestration of H^+ within the membrane and modifications to the

α -subunit of the F_1F_0 -ATPase that increase the rate of H^+ capture and prevent premature H^+ loss (Krulwich et al., 1996, 1998, 2011a). Although Na^+ -coupled ATPases have been discovered in anaerobic alkaliphiles, these complexes operate as Na^+ pumps driven by ATP hydrolysis and are used to generate SMF under physiological conditions (Krulwich et al., 2011a).

2.3. SALINITY: HALOPHILES

Halophiles are extremophiles that grow optimally in hypersaline environments. Halotolerant species, while not bona fide extremophiles, typically grow optimally in environments with trace amounts of salt, but can survive up to 5 % (w/v) NaCl. Slight halophiles have a higher minimum salt requirement than halotolerant microorganisms, but still cannot grow above 5 % NaCl. Moderate halophiles grow in waters with up to 20 % salt, and some species, categorized as extreme halophiles, require near-saturated solutions of 20–30 % NaCl to grow (Kanekar et al., 2012). These concentrations are much higher than the average ocean salinity of only 3.5 % NaCl. Halophilic prokaryotes have been isolated from a diverse range of environments, including saline marshes and lakes; the Dead Sea; Great Salt Lake, Utah, USA (Fig. 3); the Wadi An Natrun, Egypt; Lake Magadi, Kenya; ancient rock salts; and various seawater brine solutions (Wainø et al., 2000; Kanekar et al., 2012). While marine sources feature NaCl as the dominant solute, athalassohaline waters, which are saline lakes not of marine origin, contain other salts and may require distinct adaptive mechanisms (Kanekar et al., 2012). Bacterial and archaeal halophiles have been characterized and are concentrated in the families *Halomonadaceae* and *Halobacteriaceae*, respectively (Kanekar et al., 2012). Eukaryotic halophiles include the alga *Dunaliella salina*, the fungus *Hortaea werneckii*, the brine fly *Ephydra salina*, and brine shrimp *Artemia salina* (Oren, 2005; Eggermont et al., 2008; Ma et al., 2010; Gajardo and Beardmore, 2012).

Extreme hypersaline environments are not conducive to life, because high concentrations of extracellular salts challenge the ability to maintain turgor pressure and typically lead to intracellular desiccation. To adapt to these conditions, most halophiles follow either a “high-salt-in” or “compatible-solutes-in” strategy (Mesbah and Wiegel, 2011; Zaccai, 2011; Kanekar et al., 2012).

The “high-salt-in” strategy involves the intracellular accumulation of high concentrations of inorganic ions, such as Na^+ , K^+ , and Cl^- , preventing substantial water loss to the environment. However, a highly ionic cytoplasm could affect protein stability through interactions with exposed hydrophobic domains. Thus, many halophilic proteomes are composed of proteins that shield core hydrophobic domains behind an acidic surface (Fukuchi et al., 2003; Paul et al., 2008; Siglioccolo et al., 2011). However, this adaptation means that ions are then required to stabilize these negatively charged proteins (Lanyi, 1974). These ions take positions in the hydration shell around halophilic proteins and interact with the local surface charge (Zaccai, 2011). In addition, the quaternary struc-



Figure 3. Great Salt Lake, UT, USA, is home to the extreme halophile *Halorhabdus utahensis*, which exhibits optimal growth at 27 % NaCl.

tures of multimeric protein complexes in *Haloarcula marismortui* are maintained by the binding of ions between subunits (Zaccai, 2011). The “high-salt-in” adaptation of increasing the influx of salts is common in anaerobic bacterial and archaeal halophiles of the orders *Halanaerobiales* and *Halobacteriales*, respectively (Mesbah and Wiegel, 2011).

The “compatible-solutes-in” method features the accumulation of polar organic osmolytes, such as glycine betaine, glutamate, glycerol, proline, and trehalose, in the cytoplasm (Zaccai, 2011; Kanekar et al., 2012). The algae *D. salina* and *Galdieria sulphuraria*, for example, maintain high intracellular concentrations of glycerol and floridoside, respectively (Reeb and Bhattacharya, 2010; Zaccai, 2011). In contrast to the ions of the “high-salt-in” strategy, osmolytes of the “compatible-solutes-in” strategy are excluded from the hydration shell of proteins and primarily act as modulators of osmotic equilibrium between free and bound water molecules (Oren, 2002). In addition, some bacteria adjust the compatible solute to different extracellular salt concentrations and growth stages. *Halobacillus halophilus* accumulates intracellular glutamine and glutamate at 9 % NaCl but shifts to proline at 17 % NaCl in the exponential phase and ectoine at 17 % NaCl in the stationary phase (Müller and Köcher, 2011). These solutes do not have a significant negative effect on protein stability, so hydrophobic shielding is not a defining characteristic. This strategy is common in eukaryotes and many bacteria. Microorganisms utilizing this approach are typically aerobic and do not exhibit extreme halophily (Mesbah and Wiegel, 2011; Zaccai, 2011).

Finally, regulation of saline response pathways is commonly achieved through the “chloride regulon” (Saum and Müller, 2008). This network was elucidated through the discovery that both *H. halophilus* and *Natranaerobius*

thermophilus require Cl^- anions for growth (Mesbah and Wiegel, 2011; Müller and Köcher, 2011). In *H. halophilus*, Cl^- uptake activates expression of glutamine synthetase, and glutamate accumulation upregulates proline production (Müller and Köcher, 2011). These osmolytes confer resistance to salinity-induced desiccation.

2.4. PRESSURE: PIEZOPHILES

It has been estimated that less than 1 % of the total volume of the biosphere exists in terrestrial habitats at pressures of one standard atmosphere (0.1 MPa) or less (Somero, 1992). Thus, the vast majority of organisms live under high pressures that can reach up to 1,000 times greater than one atmosphere. These environments give rise to extremophiles known as piezophiles, or “pressure lovers.” Piezophiles have been found in the γ -proteobacteria class of Bacteria, Crenarchaeota of Archaea, and assorted genera of Eukarya (Bartlett et al., 2007; Abe, 2011). Piezophilic organisms are considered either facultative or obligate piezophiles, depending on their ability to grow at standard pressure, and are distinguished from piezotolerant species, whose growth rates are slow but do not halt above 0.1 MPa. Most piezophiles have been discovered in deep-sea environments, such as the Mariana Trench, Challenger Deep, where hydrostatic pressures are 38 MPa on average and can reach values up to 110 MPa (Abe and Horikoshi, 2001). These environments feature temperatures that range from 1 to 300 °C, pH values as low as 3, low-oxygen availability, sparse nutrients, and relatively high doses of natural radiation (Cherry and Heyraud, 1982; Abe and Horikoshi, 2001; Takai et al., 2001; Margesin and Miteva, 2011).

Extreme pressures place stress on an organism through the inverse pressure-volume thermodynamic relationship. In other words, structures that must maintain a relatively large volume and reactions that result in an increase in volume are disfavored at high pressure. This relationship affects membrane fluidity, protein stability, and reaction kinetics. To combat the loss of membrane fluidity at high pressure, many piezophiles incorporate large amounts of monounsaturated and polyunsaturated fatty acids in their cell membranes (Casadei et al., 2002). *cis*-Unsaturated phospholipids resist pressure-favored ordering within the membrane. Eicosapentaenoic acid and docosahexaenoic acid are two common polyunsaturated fatty acids found in piezophilic Gammaproteobacteria (Abe et al., 1999; Bartlett et al., 2008; Kato, 2011). Organic compounds called piezolytes, like β -hydroxybutyrate, and EPS are also produced in response to high-pressure stress (Martin et al., 2002; Marx et al., 2009). In addition, high pressure can cause the dissociation of voluminous multimeric complexes, such as the ribosome, into their monomeric constituents (Gross et al., 1993; Abe et al., 1999; Abe and Horikoshi, 2001). Recent work has shown that extended helices in the 16S rRNA of many deep-sea microorganisms greatly improve piezostability of prokaryotic ribosomes (Lauro et al., 2007; Bartlett and Kerman, 2011). Furthermore, proteins with highly hydrophobic

domains, which are typically found in piezophiles and thermophiles, are more stable than polar and charged proteins at high pressure due to the disfavored increase in volume induced by the solvation of hydrophobic residues. High pressure also influences metabolism through variations in the electron transport chain, upregulation of heat-shock and cold-shock proteins, and shifting the rate constants and equilibria of all metabolic processes (Abe et al., 1999; Abe and Horikoshi, 2001).

The low-pressure extreme has been studied to a far lesser extent. Although organisms have been discovered at 0.03 MPa near the top of Mt. Everest, no documented cases of growth have been observed at pressures below the Kármán line of about 10^{-6} MPa (West et al., 1983; Liu et al., 2007). The absence of growth, however, does not indicate lethality, as cyanobacteria, firmicutes, tardigrades, and ticks have been observed to survive exposure to low Earth orbit (LEO) and high vacuum (Saffary et al., 2002; Jönsson et al., 2008; Olsson-Francis et al., 2010; Mozetic and Vratnica, 2011; Ishigaki et al., 2012).

2.5. RADIATION: RADIORESISTANCE

Terrestrial life is shielded from large doses of cosmic radiation by the ozone layer and planetary magnetic field. As a result, life on the surface is subjected to an average of only 2 mGy of ionizing radiation each year (United Nations, 2000). However, organisms in deep-sea hydrothermal vents such as those in the Guaymas Basin, are exposed to over 200 mGy of terrestrial-based ionizing radiation each year (Cherry and Heyraud, 1982; Jolivet et al., 2004). Radiation-induced stress in these environments has led to the rise of a variety of radioresistant microorganisms.

Ultraviolet (UV) and ionizing radiations pose major threats to cellular life. When a cell is exposed to high-energy radiation, damage is inflicted to protein, lipids, and nucleic acids. UV radiation, particularly short-wavelength UVB and UVC, causes pyrimidine dimerization in DNA, and terrestrial ionizing radiation, which is mostly X-, β -, and γ -rays from nuclear decay, generates reactive radicals (Cox and Battista, 2005; Cadet and Douki, 2011). Proteins containing cysteine residues, iron-sulfur or heme groups, and cation-binding sites are particularly susceptible to oxidation from superoxides and other reactive oxygen species. Recent research even suggests that radiation-induced proteomic damage is far more lethal than genetic damage to a cell (Daly et al., 2007; Daly, 2009, 2011). Thus, the ability to combat this free radical formation distinguishes the extremely radioresistant species from organisms that can only tolerate small doses of radiation. *Deinococcus radiodurans* has been shown to survive ionizing radiation levels greater than 12 kGy, making this bacterium an excellent model for studying radioresistance (Cox and Battista, 2005). Studies on *D. radiodurans* have revealed a pool of small molecules that can preserve the activity of enzymes exposed to 50 kGy of radiation. The active component of this pool is Mn^{2+} , which complexes with phosphate to reduce reactive superoxides to peroxides (Daly et al., 2010). When grown in the absence of Mn^{2+} , *D. radiodurans* becomes much more

susceptible to radiation (Daly et al., 2004). Large concentrations of Mn^{2+} have been observed to accumulate in several radioresistant microorganisms, including *Lactobacillus plantarum*, *Synechocystis* sp. PCC 68034, and *D. radiodurans* (Daly et al., 2007, 2010, Daly, 2009). Although to a lesser extent than Mn^{2+} , *D. radiodurans* also exhibits trehalose accumulation to help stabilize proteins (Santos et al., 2011).

Mn^{2+} ions, however, have not been observed to prevent radiation-induced genetic damage (Daly et al., 2007). To survive single-stranded and double-stranded DNA cleavage caused by radiation, many radioresistant species employ a variety of preventive and repair mechanisms. One of the most common strategies is polyploidy, in which the cell maintains multiple identical copies of the chromosome. Damage to one chromosome would then have minimal impact on survivability (Daly et al., 2004). In addition, many breaks can be repaired with conventional DNA repair mechanisms. Some DNA repair processes even require additional chromosomes to operate. *D. radiodurans*, for example, employs a novel RecA-independent double-strand break repair mechanism known as synthesis-dependent single-strand annealing (Cox and Battista, 2005).

2.6. DESICCATION: XEROPHILES

Water is the solvent of life; thus, dehydration is one of the most serious stresses to all organisms. In the absence of water, biochemical reactions cannot take place, metabolism ceases, and the structures of cell membranes and proteins collapse. Water comprises 70 % of the average invertebrate's body mass, and water molecules account for more than 95 % of the total molecules in an invertebrate's body (Edney, 1977; Hadley, 1994). Desiccating environments occur in many contexts, from large deserts to small dried ponds. Xerotolerant organisms are able to survive desiccation by inhibiting water loss, yet die once water content declines past a critical level. On the other hand, xerophiles can survive near complete dehydration for extended periods of time by entering a state of ametabolism known as anhydrobiosis, then resuming metabolism when water becomes present. Xerophilic species cover a wide spectrum of taxa, from unicellular organisms to plants, tardigrades, nematode worms, insect larvae, and the cysts of primitive crustaceans. The longest records of recovery in nematode worms, tardigrades, insects, and crustaceans are 39, 20, 17, and 16 years, respectively (Baumann, 1922; Steiner and Albin, 1946; Clegg, 1967; Adams, 1985; Guidetti and Jönsson, 2002; Schill, 2010).

Strategies for desiccation survival fall into two main categories: prevention of water loss and repair of damage upon rehydration. The ability to survive is dependent not only on the absolute degree of desiccation but also on the rate of water loss (Wharton and Marshall, 2002). Many organisms employ morphological changes to help limit these losses. For example, xerophilic tardigrades decrease their rate of water loss by entering a cryptobiotic "tun" state

to decrease their surface area (Sømme, 1995; Wright, 2001). Tun formation involves longitudinal contraction and invagination of the limbs. Similarly, nematode worms coil to decrease external surface area, and some species form tightly packed aggregates meant to protect those worms in the center of the mass from the environment (Ellenby, 1969). Furthermore, xerophiles have been found to accumulate large concentrations of lyoprotectant polyols, particularly trehalose, immediately before entering anhydrobiosis (Dose et al., 1992; Santos et al., 2011). Despite these protective mechanisms, DNA damage is a common consequence of desiccation (Dose et al., 1992). Thus, extensive single- and double-strand repair mechanisms are typical of xerophilic species (Torsvik and Øvreås, 2008).

2.7. OXYGEN: AEROBES, ANAEROBES, AND MICROAEROPHILES

Although many details of the composition of the atmosphere on early Earth are still debated, it is widely accepted that carbon dioxide and nitrogen gas were the dominant constituents (Kasting and Howard, 2006). About 3.4 Ga, the combination of carbon dioxide in the atmosphere and reduced mineral surfaces promoted the development of methanogenesis, the metabolic process in which sulfide is oxidized to elemental sulfur and carbon dioxide or formate is reduced to methane (Liu et al., 2012; Trevors et al., 2012). Proliferation of methanogens enriched the atmospheric methane content (Zerkle et al., 2012). However, the rise of oxygenic photosynthesis approximately 2.7 Ga led to bursts of oxygen production between 2.6 and 2.5 Ga (Dismukes et al., 2001; Anbar et al., 2007; Zerkle et al., 2012). During this time, localized oxygen production was neutralized by the reducing atmosphere (Liu et al., 2012). Finally, about 2.4 Ga, cyanobacterial oxygen production surpassed the reducing potential of the atmosphere, leading to the Great Oxygenation Event (GOE) (Anbar et al., 2007; Zerkle et al., 2012). In addition to atmospheric accumulation, this massive release of oxygen led to the oxidation of sulfur to sulfate (Canfield et al., 2000). Since many species, especially methanogens, required reduced sulfur for metabolism and possessed oxygen-sensitive enzymes, global oxidation led to the obliteration of many early archaea (Liu et al., 2012). Only those environments sufficiently isolated from the atmosphere could remain anoxic, severely restricting the habitable regions for obligate anaerobes. Since the GOE, there have been several more changes in atmospheric oxygen concentrations, and periodic fluctuations between hypoxic and hyperoxic atmospheres have precipitated significant evolutionary changes. The Cambrian explosion about 550 Ma has been associated with the rise in oxygen to 1 % of current levels (Dole, 1965). Furthermore, hyperoxic periods, in which the oxygen content of the atmosphere was greater than contemporary levels, have promoted the development of the earliest animal body plans, the ability of vertebrates to adapt to life on land, flapping flight, and gigantism (McAlester, 1970; Graham et al., 1995; Dudley, 1998; Berner et al., 2007; Than, 2011; Verberk and Bilton, 2011).

Adaptations to the rise in atmospheric oxygen have led to the evolution of aerobic respiration, a process by which the terminal reduction of oxygen aids the production of large amounts of energy in the form of ATP. The production of energy does not come without a cost, however, as the formation of reactive oxygen species can lead to rampant oxidation. These superoxides are often reduced by antioxidant proteins, such as superoxide dismutase, glutathione, and thioredoxin. The resultant peroxides are then decomposed to water and oxygen by the enzyme catalase. Thus, these antioxidants are necessary adaptive mechanisms for the obligately aerobic and facultatively anaerobic archaea, bacteria, and eukaryotes.

Some organisms require oxygen for respiration but do not possess these classes of antioxidants. Known as microaerophiles, these species exist at a very narrow range of oxygen concentrations. Research on the microaerophilic bacteria *Treponema pallidum* and *Desulfoarculus baarsii* has revealed a novel antioxidative enzyme called superoxide reductase (Lombard et al., 2000; Bonnot et al., 2010). While superoxide dismutase uses an active site composed of [Cu(His)₄] and [Zn(His)₃Asp] complexes to oxidize one superoxide to oxygen and reduce another superoxide to peroxide in a four-step mechanism, superoxide reductase employs nonheme [Fe(His)₄Cys] active sites and a distinct mechanism (Tainer et al., 1983; Bonnot et al., 2010). Therefore, superoxide reductase is a novel adaptation to life in low-oxygen environments.

Anaerobic respiration is the anoxic response to respiration in aerobes. This process utilizes inorganic molecules, such as sulfate and nitrate, or elemental sulfur as terminal electron acceptors rather than oxygen. Although anaerobic respiration yields more energy than fermentative substrate-level phosphorylation, the reductive potentials of these inorganic substrates are significantly lower than oxygen. As a result, what is gained from not requiring antioxidative potential is lost in energetic yield and growth rate of obligate anaerobes.

3. Polyextremophiles: Life at the Interface of Extremes

Many terrestrial and extraterrestrial environments feature conditions that fall within more than one extreme. Studying polyextremophiles has the potential to delineate the envelope of habitability by providing both empirical and theoretical constraints on biological processes, as all organisms fall somewhere in a multidimensional niche space (Pikuta et al., 2007a; Dartnell, 2011). In an effort to provide a comprehensive look at the overlap of the extremes of temperature and pH, we have tabulated available literature data (Table 2) and then plotted the environmental tolerances of over 200 species two-dimensionally with pH as a function of temperature (Fig. 5). The interaction of the physicochemical properties of temperature and pH and the constraints imposed by the interactions of other extremes are discussed in the following sections.

Table 2. Compilation of temperature and pH growth ranges and optima for known extremophiles.

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Acaryochloris marina</i>	20–35	29	6.5–10.0	8.5	Miyashita et al. (2003)
<i>Acidianus ambivalens</i>	ND–87	80	1.0–3.5	ND	Zillig et al. (1985, 1986) and Fuchs et al. (1996)
<i>Acidianus infernus</i>	65–96	85	1.0–5.5	2.0	Segerer et al. (1986)
<i>Acidianus manzaensis</i>	60–90	80	1.0–5.0	1.2	Yoshida et al. (2006)
<i>Acidianus tengchongensis</i>	55–80	70	1.0–5.5	2.5	He et al. (2004)
<i>Acidilobus aceticus</i>	60–92	85	2.0–6.0	3.8	Prokofeva et al. (2000)
<i>Acidithiobacillus caldus</i>	32–52	45	1.0–3.5	2.3	Hallberg and Lindström (1994) and Kelly and Wood (2000)
<i>Acidithiobacillus ferrivorans</i>	4–37	31	1.9–3.4	2.5	Hallberg et al. (2010)
<i>Acidithiobacillus ferrooxidans</i>	10–37	33	1.3–4.5	2.5	Temple and Colmer (1951) and Kelly and Wood (2000)
<i>Acidithiobacillus thiooxidans</i>	10–37	29	0.5–5.5	2.5	Waksman and Joffe (1922) and Kelly and Wood (2000)
<i>Aeribacillus pallidus</i>	30–70	63	ND	8.5	Scholz et al. (1987), Wiegel (1998), Banat et al. (2004), and Miñana-Galbis et al. (2010)
<i>Aeropyrum camini</i>	10–97	85	6.5–8.8	8.0	Nakagawa et al. (2004)
<i>Aeropyrum pernix</i>	70–100	90	5.0–9.0	7.0	Sako et al. (1996a) and Kawarabayasi et al. (1999)
<i>Alicyclobacillus acidocaldarius</i>	45–70	65	2.0–6.0	4.0	Darland and Brock (1971) and Wisotzkey et al. (1992)
<i>Alicyclobacillus acidoterrestris</i>	35–55	48	2.2–5.8	ND	Wisotzkey et al. (1992)
<i>Alicyclobacillus cycloheptanicus</i>	40–53	48	3.0–5.5	4.0	Wisotzkey et al. (1992)
<i>Alkalibacter saccharofermentans</i>	6–50	35	7.2–10.2	9.0	Garnova et al. (2004)
<i>Alkalibacterium olivoapovlriticus</i>	4–35	30	8.0–11.0	9.2	Ntougias and Russell (2001)
<i>Alkalibacterium psychrotolerans</i>	5–45	34	9.0–12.0	10.0	Yumoto et al. (2004)
<i>Alkalilimnicola halodurans</i>	20–55	35	ND	8.5	Yakimov et al. (2001) and Kanekar et al. (2012)
<i>Alkalimonas amylytica</i>	10–42	37	7.5–11.0	10.0	Ma et al. (2004)
<i>Alkalimonas collagenimarina</i>	5–37	33	7.0–10.5	9.3	Kurata et al. (2007)
<i>Alkalimonas delamerensis</i>	10–42	37	8.0–11.0	10.3	Ma et al. (2004)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Alkalispirillum mobile</i>	30–45	37	5.0–12.0	9.5	Rijkenberg et al. (2001) and Kanekar et al. (2012)
<i>Anaerobranca horikoshii</i>	34–66	57	6.9–10.3	8.5	Engle et al. (1995) and Wiegel (1998)
<i>Aquifex aeolicus</i>	58–95	85	5.5–8.0	6.5	Deckert et al. (1998) and Nübel et al. (2000)
<i>Aquifex pyrophilus</i>	67–95	85	5.4–7.5	6.8	Burggraf et al. (1992) and Huber et al. (1992b)
<i>Archaeoglobus fulgidus</i>	60–95	83	6.4–7.4	6.9	Stetter (1988), Beeder et al. (1994), Klenk et al. (1997), and Lovley et al. (2000)
<i>Archaeoglobus lithotrophicus</i>	55–87	80	6.0–8.5	6.0	Stetter et al. (1993) and Vorholt et al. (1995)
<i>Archaeoglobus profundus</i>	65–90	82	4.5–7.5	6.0	Burggraf et al. (1990)
<i>Archaeoglobus veneficus</i>	65–80	80	6.5–8.0	7.0	Huber et al. (1997)
<i>Bacillus thermoaerophilus</i>	40–60	ND	7.0–8.0	ND	Meier-Stauffler et al. (1996) and Wiegel (1998)
<i>Caldariella acidophila</i>	63–89	87	3.0–4.5	ND	De Rosa et al. (1974) and De rosa et al. (1975)
<i>Caldivirga maquilingensis</i>	60–92	85	2.3–5.9	3.7	Itoh et al. (1999)
<i>Caldococcus noboribetus</i>	70–96	92	1.5–4.0	3.0	Aoshima et al. (1996) and Aoshima and Oshima (1997)
<i>Caloramator indicus</i>	37–75	63	6.2–9.2	8.1	Chrisostomos et al. (1996) and Wiegel (1998)
<i>Chlamydomonas acidophila</i>	10–27	18	2.0–7.0	3.8	Moser and Weisse (2011)
<i>Chloroflexus aurantiacus</i>	30–70	56	7.0–ND	8.0	Pierson and Castenholz (1974) and Wiegel (1998)
<i>Clostridium paradoxum</i>	30–63	56	6.9–11.1	9.3	Li et al. (1993) and Wiegel (1998)
<i>Clostridium thermoalcaliphilum</i>	27–58	51	7.0–11.0	9.2	Li et al. (1993) and Wiegel (1998)
<i>Cyanidium caldarium</i>	20–60	55	0–5.0	2.5	Doemel and Brock (1970), Seckbach and Libby (1970), Seckbach and Kaplan (1973), and Kao et al. (1975)
<i>Deinococcus geothermalis</i>	30–55	47	4.5–8.5	6.5	Ferreira et al. (1997)
<i>Deinococcus murrayi</i>	30–55	47	5.5–10.0	8.0	Ferreira et al. (1997)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Desulfurococcus amylolyticus</i>	68–97	90	5.7–7.5	6.4	Bonch-Osmolovskaya et al. (1988)
<i>Desulfurococcus mobilis</i>	55–97	85	4.5–7.0	6.0	Zillig et al. (1982)
<i>Desulfurococcus mucosus</i>	55–97	85	2.2–6.5	6.0	Zillig et al. (1982)
<i>Escherichia coli</i>	4–45	37	3.6–9.2	7.0	Parhad and Rao (1974)
<i>Falsibacillus pallidus</i>	15–42	34	6.0–8.5	7.5	Zhou et al. (2009)
<i>Ferroglobus placidus</i>	65–95	85	6.0–8.5	7.0	Hafenbradl et al. (1996) and Tor et al. (2001)
<i>Ferroplasma acidarmanus</i>	23–46	42	0–1.5	1.2	Edwards et al. (2000), Okibe et al. (2003), and Dopson et al. (2004)
<i>Ferroplasma acidiphilum</i>	15–45	35	1.3–2.2	1.7	Golyshina et al. (2000)
<i>Fervidobacterium islandicum</i>	50–80	65	6.0–8.0	7.0	Huber et al. (1990)
<i>Fervidobacterium nodosum</i>	41–79	70	6.0–8.0	7.0	Patel et al. (1985)
<i>Filobacillus milosensis</i>	ND–42	36	6.5–8.9	7.6	Schlesner et al. (2001) and Kanekar et al. (2012)
<i>Geoglobus ahangari</i>	65–95	88	5.0–7.6	7.0	Kashefi et al. (2002)
<i>Geothermobacterium ferrireducens</i>	65–100	85	ND	6.8	Kashefi et al. (2002)
<i>Geotoga subterranea</i>	30–60	45	5.5–9.0	6.5	Ravot et al. (1995b)
<i>Halobacillus litoralis</i>	10–43	35	6.0–9.5	7.5	Spring et al. (1996)
<i>Halobacillus trueperi</i>	10–44	35	6.0–9.5	7.5	Spring et al. (1996)
<i>Halobiforma haloterrestris</i>	ND–58	42	6.0–9.2	7.5	Hezayen et al. (2002) and Kanekar et al. (2012)
<i>Halococcus dombrowskii</i>	28–50	39	5.8–8.0	7.5	Stan-Lotter et al. (2002) and Kanekar et al. (2012)
<i>Haloferax lucentense</i>	10–45	37	5.0–9.0	7.5	Gutierrez et al. (2002) and Kanekar et al. (2012)
<i>Haloferax prahovense</i>	23–51	43	6.0–8.5	7.3	Enache et al. (2007) and Kanekar et al. (2012)
<i>Halomarina oriensis</i>	ND–60	37	ND	7.5	Inoue et al. (2011)
<i>Halomicrobium mukohataei</i>	ND	43	6.2–8.0	ND	Oren et al. (2002)
<i>Halomonas alimentaria</i>	4–45	30	5.0–ND	7.0	Yoon et al. (2002) and Kanekar et al. (2012)
<i>Halomonas campisalis</i>	4–50	30	6.0–11.0	9.5	Joshi et al. (2007) and Kanekar et al. (2012)
<i>Halomonas halocynthiae</i>	7–35	27	5.0–11.0	8.0	Romanenko et al. (2002b) and Kanekar et al. (2012)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Halomonas muralis</i>	10–35	30	5.0–10.0	7.3	Heyrman et al. (2002) and Kanekar et al. (2012)
<i>Halonatronum saccharophilum</i>	ND–60	46	7.7–10.3	8.3	Zhilina et al. (2001) and Kanekar et al. (2012)
<i>Haloplasma contractile</i>	10–44	34	6.0–8.0	7.0	Antunes et al. (2008a)
<i>Haloquadratum walsbyi</i>	25–45	ND	5.5–8.5	6.5	Burns et al. (2007)
<i>Halorhabdus tiamatea</i>	15–55	45	5.5–8.5	6.8	Antunes et al. (2008b)
<i>Halorhabdus utahensis</i>	17–55	50	5.5–8.5	6.9	Wainø et al. (2000)
<i>Halorhodospira halochloris</i>	33–50	47	7.5–10.0	8.5	Imhoff and Truper (1977)
<i>Halorhodospira halophila</i>	ND–56	47	ND	7.7	Raymond and Sistrof (1969) and Imhoff and Süling (1996)
<i>Halorubrum tebenquichense</i>	35–50	ND	7.0–10.0	ND	Lizama et al. (2002) and Kanekar et al. (2012)
<i>Halosimplex carlsbadense</i>	22–50	39	7.0–8.0	7.5	Vreeland et al. (2002) and Kanekar et al. (2012)
<i>Hyperthermus butylicus</i>	77–108	95	ND	7.0	Zillig et al. (1990)
<i>Ignicoccus hospitalis</i>	73–98	90	4.5–7.0	5.5	Paper et al. (2007)
<i>Ignicoccus islandicus</i>	70–98	90	3.8–6.5	5.8	Huber et al. (2000a)
<i>Ignicoccus pacificus</i>	75–98	90	4.5–7.0	6.0	Huber et al. (2000a)
<i>Ignisphaera aggregans</i>	85–98	92	5.4–7.0	6.4	Niederberger et al. (2006)
<i>Isochrysis galbana</i>	19–32	27	5.0–9.0	7.0	Kaplan et al. (1986)
<i>Isosphaera pallida</i>	40–55	ND	ND	8.3	Giovannoni et al. (1987) and Wiegel (1998)
<i>Kineococcus radiotolerans</i>	11–41	ND	5.0–9.0	ND	Phillips et al. (2002)
<i>Marinilactibacillus psychrotolerans</i>	–1.8 to 45	39	6.0–10.0	8.8	Ishikawa et al. (2003)
<i>Meiothermus chliarophilus</i>	40–60	50	5.0–10.0	8.0	Nobre et al. (1996a, b) and Wiegel (1998)
<i>Meiothermus ruber</i>	38–70	60	ND	8.0	Nobre et al. (1996a, b) and Wiegel (1998)
<i>Meiothermus silvanus</i>	40–65	55	5.0–10.0	8.3	Nobre et al. (1996a, b) and Wiegel (1998)
<i>Methanocaldococcus fervens</i>	48–92	85	5.5–7.6	6.5	Zhao et al. (1988) and Jeanthon et al. (1999)
<i>Methanocaldococcus indicus</i>	50–86	85	5.5–6.7	6.5	L'Haridon et al. (2003)
<i>Methanocaldococcus infernus</i>	55–91	85	5.3–7.0	6.5	Jeanthon et al. (1998)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Methanocaldococcus jannaschii</i>	50–86	85	5.2–7.0	6.0	Jones et al. (1983), Abe and Horikoshi (2001), and Holden (2009)
<i>Methanocaldococcus vulcanius</i>	49–89	80	5.3–7.0	6.5	Jeanthon et al. (1999)
<i>Methanococcoides alaskense</i>	–2 to 31	24	ND	6.9	Singh et al. (2005)
<i>Methanococcoides burtonii</i>	–3 to 30	23	6.8–8.2	ND	Franzmann et al. (1992)
<i>Methanogenium frigidum</i>	0–18	15	6.5–7.9	ND	Franzmann et al. (1997)
<i>Methanogenium marinum</i>	5–25	25	5.5–7.5	6.0	Chong et al. (2002)
<i>Methanohalophilus zhilinae</i>	ND	45	ND	9.2	Mathrani et al. (1988), Wiegel (1998), and Kanekar et al. (2012)
<i>Methanopyrus kandleri</i>	84–110	98	5.5–7.0	6.5	Kurr et al. (1991) and Takai et al. (2008)
<i>Methanosarcina baltica</i>	–22 to 27	25	4.9–8.5	7.0	Von Klein et al. (2002)
<i>Methanosarcina barkeri</i>	30–45	41	4.0–8.5	7.0	Maestrojuán and Boone (1991) and Kendrick and Kral (2006)
<i>Methanothermobacter thermautotrophicus</i>	40–75	67	6.0–8.8	7.4	Zeikus and Wolfe (1972), Blotvogel et al. (1985), and Wiegel (1998)
<i>Methanothermus fervidus</i>	65–97	83	4.0–7.0	6.5	Stetter et al. (1981)
<i>Methanothermus sociabilis</i>	55–97	88	5.5–7.5	6.5	Lauerer et al. (1986) and Garcia (1990)
<i>Methanotorris igneus</i>	45–91	88	5.0–7.5	5.7	Burggraf et al. (1990)
<i>Methylarcula marina</i>	10–42	32	5.0–10.5	8.0	Doronina et al. (2000) and Kanekar et al. (2012)
<i>Methylarcula terricola</i>	10–40	31	5.5–10.0	8.0	Doronina et al. (2000) and Kanekar et al. (2012)
<i>Natranaerobius thermophilus</i>	35–56	53	8.3–11.2	9.5	Mesbah et al. (2007)
<i>Natrialba magadii</i>	ND	39	8.0–11.5	9.5	Xu et al. (1999)
<i>Natronobacterium gregoryi</i>	ND	39	8.5–11.0	9.5	Xu et al. (1999)
<i>Natronomonas pharaonis</i>	ND	45	8.0–11.0	8.5	Xu et al. (1999), Falb et al. (2005), and Gonzalez et al. (2010)
<i>Natronorubrum bangense</i>	25–55	45	8.0–11.0	9.5	Xu et al. (1999)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Natronorubrum tibetense</i>	25–55	45	8.5–11.0	9.0	Xu et al. (1999)
<i>Nocardiopsis kunsanensis</i>	ND	37	5.0–ND	9.0	Chun et al. (2000) and Kanekar et al. (2012)
<i>Ochromonas</i> sp. strain LG	10–35	18	2.5–7.0	5.0	Moser and Weisse (2011)
<i>Palaeococcus ferrophilus</i>	60–88	83	4.0–8.0	6.0	Takai et al. (2000)
<i>Palaeococcus helgesonii</i>	45–85	80	5.0–8.0	6.5	Amend et al. (2003)
<i>Paraliobacillus ryukyuensis</i>	10–48	39	5.5–9.5	7.8	Ishikawa et al. (2002) and Kanekar et al. (2012)
<i>Petrotoga miotherma</i>	35–65	55	5.5–9.0	6.5	Ravot et al. (1995b)
<i>Picrophilus oshimae</i>	47–65	60	0–3.5	0.7	Schleper et al. (1996)
<i>Picrophilus torridus</i>	47–65	60	0–3.5	0.7	Schleper et al. (1996)
<i>Psychrobacter marincola</i>	7–35	27	5.5–9.5	7.5	Romanenko et al. (2002a) and Kanekar et al. (2012)
<i>Psychrobacter muriicola</i>	–2 to 37	17	5.8–8.5	7.0	Shcherbakova et al. (2009)
<i>Psychrobacter submarinus</i>	4–35	27	5.5–9.5	7.5	Romanenko et al. (2002a) and Kanekar et al. (2012)
<i>Psychromonas ingrahamii</i>	–12 to 10	ND	6.5–7.4	7.0	Auman et al. (2006)
<i>Pyrobaculum arsenaticum</i>	68–100	95	ND	6.5	Huber et al. (2000b)
<i>Pyrobaculum calidifontis</i>	75–100	90	5.5–7.5	6.0	Amo et al. (2002)
<i>Pyrobaculum islandicum</i>	74–102	100	5.0–7.0	6.0	Huber et al. (1987), Kashefi and Lovley (2000), Lovley et al. (2000), and Kashefi et al. (2001)
<i>Pyrobaculum oguniense</i>	70–97	90	5.4–7.4	6.3	Sako et al. (2001)
<i>Pyrobaculum organotrophum</i>	78–102	102	5.0–7.0	6.0	Huber et al. (1987)
<i>Pyrococcus abyssi</i>	67–109	96	4.0–8.5	6.8	Erauso et al. (1993) and Abe and Horikoshi (2001)
<i>Pyrococcus endeavori</i>	55–110	90	4.0–8.0	5.0	Pledger and Baross (1991)
<i>Pyrococcus furiosus</i>	70–103	100	5.0–9.0	7.0	Fiala and Stetter (1986), Lovley et al. (2000), and Kashefi et al. (2001)
<i>Pyrococcus glycovorans</i>	75–104	95	2.5–9.5	7.5	Barbier et al. (1999)
<i>Pyrococcus horikoshii</i>	80–102	98	5.0–8.0	7.0	González et al. (1998) and Kawarabayasi et al. (1998)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Pyrococcus woesei</i>	95–105	100	6.0–6.5	ND	Zillig et al. (1987)
<i>Pyrodictium abyssi</i>	80–110	97	4.7–7.1	5.5	Pley et al. (1991), Erauso et al. (1993), and Lovley et al. (2000)
<i>Pyrodictium brockii</i>	80–110	105	4.5–7.2	5.5	Stetter et al. (1983) and Pley et al. (1991)
<i>Pyrodictium occultum</i>	80–110	105	4.5–7.2	5.5	Stetter et al. (1983) and Pley et al. (1991)
<i>Pyrolobus fumarii</i>	90–113	106	4.0–6.5	5.5	Blöchl et al. (1997)
<i>Rhodothermus obamensis</i>	50–85	80	5.5–9.0	7.0	Sako et al. (1996b)
<i>Rubrobacter xylanophilus</i>	40–70	60	6.0–10.0	7.5	Carreto et al. (1996) and Wiegel (1998)
<i>Salinibacter ruber</i>	27–52	40	6.0–8.5	7.3	Kanekar et al. (2012)
<i>Salinicoccus alkaliphilus</i>	10–49	32	6.5–11.5	9.5	Zhang et al. (2002) and Kanekar et al. (2012)
<i>Salinisphaera shabaensis</i>	5–42	34	4.0–8.0	6.5	Antunes et al. (2003) and Kanekar et al. (2012)
<i>Selenihalanaerobacter shriftii</i>	16–42	38	5.3–8.9	7.2	Blum et al. (2001) and Kanekar et al. (2012)
<i>Spirochaeta americana</i>	10–44	37	8.0–10.5	9.5	Hoover et al. (2003)
<i>Staphylothermus hellenicus</i>	70–90	85	4.5–7.0	6.0	Arab et al. (2000)
<i>Staphylothermus marinus</i>	65–98	92	4.5–8.5	6.5	Fiala et al. (1986)
<i>Stetteria hydrogenophila</i>	68–102	95	4.5–7.0	6.0	Jochimsen et al. (1997)
<i>Stygiolobus azoricus</i>	57–89	80	1.0–5.5	2.5	Segerer et al. (1991)
<i>Sulfolobus acidocaldarius</i>	55–85	70	2.0–4.0	2.5	Brock et al. (1972) and Takayanagi et al. (1996)
<i>Sulfolobus hakonensis</i>	50–80	70	1.0–4.0	3.0	Takayanagi et al. (1996)
<i>Sulfolobus islandicus</i>	ND	80	3.0–3.5	ND	Zillig et al. (1998) and Stedmen et al. (2000)
<i>Sulfolobus shibatae</i>	65–86	81	2.5–4.5	3.0	Grogan et al. (1990) and Huber et al. (1992a)
<i>Sulfolobus solfataricus</i>	50–85	85	2.0–5.5	4.5	Zillig et al. (1980)
<i>Sulfolobus tokodaii</i>	70–85	80	2.0–5.0	2.5	Kawarabayasi et al. (2001) and Suzuki et al. (2002)
<i>Sulfolobus yangmingensis</i>	65–95	80	2.0–6.0	4.0	Jan et al. (1999)
<i>Sulfophobococcus zilligii</i>	70–95	85	6.5–8.5	7.5	Hensel et al. (1997)
<i>Sulfurisphaera ohwakuensis</i>	63–92	84	1.0–5.0	2.0	Kurosawa et al. (1998)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Thermoanaerobacter ethanolicus</i>	35–78	69	4.5–9.8	7.0	Wiegel and Ljungdahl (1982) and Wiegel (1998)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	35–67	55	6.0–8.5	7.8	Collins et al. (1994) and Wiegel (1998)
<i>Thermobrachium celere</i>	43–75	66	5.4–9.5	8.2	Engle et al. (1996) and Wiegel (1998)
<i>Thermococcus acidaminovorans</i>	56–93	85	5.0–9.5	9.0	Dirmeier et al. (1998) and Arab et al. (2000)
<i>Thermococcus aegaeicus</i>	50–95	90	4.5–7.5	6.0	Arab et al. (2000)
<i>Thermococcus aggregans</i>	60–94	88	5.6–7.9	7.0	Canganella et al. (1998)
<i>Thermococcus alcaliphilus</i>	56–90	85	6.5–10.5	9.0	Keller et al. (1995)
<i>Thermococcus barophilus</i>	48–100	85	4.5–9.5	7.0	Marteinsson et al. (1999)
<i>Thermococcus barossii</i>	60–92	82	4.0–9.0	6.5	Duffaud et al. (1998)
<i>Thermococcus celer</i>	ND–93	88	5.7–7.2	5.8	Zillig et al. (1983b) and Lovley et al. (2000)
<i>Thermococcus chitonophagus</i>	60–93	85	3.5–9.0	6.7	Huber et al. (1995)
<i>Thermococcus fumicolans</i>	73–103	85	4.0–9.5	8.0	Godfroy et al. (1996) and Wiegel (1998)
<i>Thermococcus gammatolerans</i>	55–96	88	4.0–8.5	6.0	Jolivet et al. (2003a)
<i>Thermococcus gorgonarius</i>	68–95	80	5.8–8.5	6.5	Miroshnichenko et al. (1998)
<i>Thermococcus guaymasensis</i>	56–90	88	5.6–8.1	7.2	Canganella et al. (1998)
<i>Thermococcus hydrothermalis</i>	55–100	80	3.5–9.5	5.5	Godfroy et al. (1997)
<i>Thermococcus kodakaraensis</i>	60–100	85	5.0–9.0	6.5	Morikawa et al. (1994) and Nakatani et al. (2000)
<i>Thermococcus litoralis</i>	65–95	88	6.2–8.5	7.2	Belkin and Jannasch (1986), Neuner et al. (1990), and Kostyukova et al. (1999)
<i>Thermococcus pacificus</i>	70–95	80	6.0–8.0	6.5	Miroshnichenko et al. (1998)
<i>Thermococcus peptonophilus</i>	60–100	85	3.0–8.0	6.0	Abe and Horikoshi (2001)
<i>Thermococcus profundus</i>	50–90	80	4.5–8.5	7.5	Kobayashi et al. (1994)
<i>Thermococcus siculi</i>	50–93	85	5.0–9.0	7.0	Grote et al. (1999)
<i>Thermococcus thioreducens</i>	55–94	83	5.0–8.5	7.0	Pikuta et al. (2007b)

(continued)

Table 2. (continued)

Name of the organism	Temp range (°C)	Temp opt (°C)	pH range	pH Opt	References
<i>Thermococcus waiotapuensis</i>	60–90	85	5.0–8.0	7.0	González et al. (1999)
<i>Thermocrinis albus</i>	55–89	ND	ND	7.5	Eder and Huber (2002)
<i>Thermocrinis ruber</i>	44–89	80	7.0–8.5	ND	Huber et al. (1998b)
<i>Thermodiscus maritimus</i>	75–98	90	5.0–7.0	5.5	Fischer et al. (1983) and Stetter et al. (1990)
<i>Thermofilum pendens</i>	70–100	85	2.8–6.7	5.0	Zillig et al. (1983a)
<i>Thermomicrobium roseum</i>	ND–85	73	6.0–9.4	8.4	Jackson et al. (1973) and Wiegel (1998)
<i>Thermoproteus neutrophilus</i>	70–97	85	5.0–7.5	6.5	Fischer et al. (1983)
<i>Thermoproteus tenax</i>	70–96	88	2.5–6.0	5.5	Fischer et al. (1983) and Zillig et al. (1983b)
<i>Thermoproteus uzoniensis</i>	74–102	90	4.6–6.8	5.6	Bonch-Osmolovskaya et al. (1990)
<i>Thermosipho africanus</i>	35–77	75	6.0–8.0	7.2	Ravot et al. (1996)
<i>Thermosipho japonicus</i>	45–80	72	5.3–9.3	7.3	Takai and Horikoshi (2000) and Abe and Horikoshi (2001)
<i>Thermosphaera aggregans</i>	67–90	85	5.0–7.0	6.5	Huber et al. (1998a)
<i>Thermosyntropha lipolytica</i>	52–70	63	7.1–9.5	7.9	Svetlitshnyi et al. (1996) and Wiegel (1998)
<i>Thermotoga elfii</i>	50–72	66	5.5–8.7	7.5	Ravot et al. (1995a)
<i>Thermotoga maritima</i>	55–90	80	5.5–9.0	6.5	Ravot et al. (1995a, b) and Abe and Horikoshi (2001)
<i>Thermotoga naphthophila</i>	48–86	80	5.4–9.0	7.0	Takahata et al. (2001)
<i>Thermotoga neapolitana</i>	55–90	80	5.5–9.0	7.0	Ravot et al. (1995b)
<i>Thermotoga petrophila</i>	47–88	80	5.2–9.0	7.0	Takahata et al. (2001)
<i>Thermotoga thermarum</i>	55–84	70	5.5–9.0	7.0	Ravot et al. (1995b)
<i>Thermus aquaticus</i>	40–79	71	6.0–9.5	7.7	Brock and Freeze (1969) and Abe and Horikoshi (2001)
<i>Truepera radiovictrix</i>	25–55	50	6.5–11.2	8.5	Albuquerque et al. (2005)
<i>Vulcanisaeta distributa</i>	70–92	90	3.5–5.6	4.5	Itoh et al. (2002)
<i>Vulcanisaeta soumiana</i>	65–89	85	3.5–5.0	4.5	Itoh et al. (2002)

ND no data available.

3.1. TEMPERATURE AND pH

Extremes of temperature and pH combine to create psychroacidophiles, psychroalkaliphiles, thermoacidophiles, and thermoalkaliphiles (Fig. 4). Since membrane fluidity decreases at low temperatures, the permeability of the membrane to H^+ is lowered in psychrophiles. This impermeability is advantageous to acidophiles and alkaliphiles, because the maintenance of cytoplasmic pH homeostasis is hindered by uncontrolled H^+ movement across the membrane. Thus, the development of psychroacidophily and psychroalkaliphily is theoretically allowed. Interestingly, only psychrotolerant alkaliphiles, such as *Alkalibacterium psychrotolerans*, have been described in this group (Yumoto et al., 2004). It is uncertain whether the absence of known psychroacidophiles is a real biological limitation or simply the rarity of that particular environmental niche on Earth. It has even been postulated that, from a geological perspective, the extremes of low pH and low temperature rarely overlapped, so there would be little chance of experiencing concomitant acidic and freezing conditions (Pikuta et al., 2007a). Although modern cold, acidic environments are known, acidophiles identified within them are not psychrophilic. Langdahl and Ingvorsen (1997) reported that acidophilic microorganisms sampled from gossan in Citronen Fjord, Greenland, which has temperatures as low as $-20^\circ C$ and pH as low as 1.9, were merely psychrotolerant.

Thermoacidophiles, like *Sulfurisphaera ohwakuensis*, thrive in extremes of both high temperature and low pH (Kurosawa et al., 1998). High temperatures and low pH values likely have antagonistic effects on the cell. Following the same

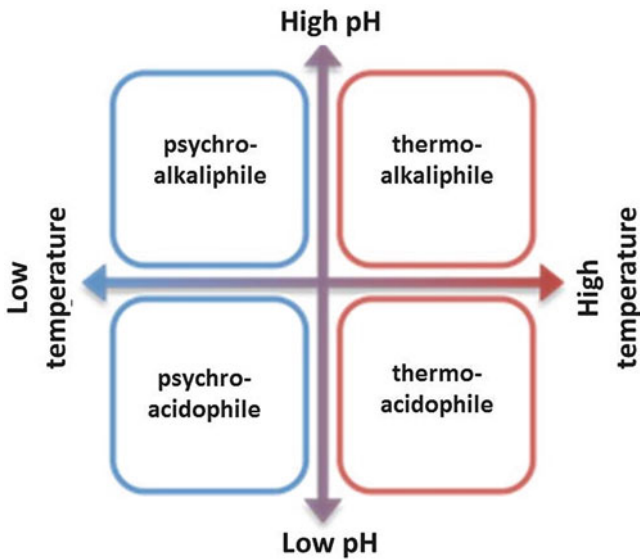


Figure 4. Classification of polyextremophiles at the interface of temperature and pH.

logic of the effect of temperature on membrane fluidity, higher temperatures would increase H^+ permeability and result in lethal cytoplasmic acidification (Padan et al., 2005). This interplay is largely avoided by archaeal glycerol ether lipids and bacterial ω -alicyclic fatty acids, however, as these membranes demonstrate incredible thermostability and resistance to acid hydrolysis (Chang and Kang, 2004; Koga and Morii, 2005; Baker-Austin and Dopson, 2007). Furthermore, RNA codon thermostability is achieved by high concentrations of purines, although the glycosidic bond in these nucleosides is particularly susceptible to acid hydrolysis (Ishikawa, 1935; Baker-Austin and Dopson, 2007). A compromise between these effects is achieved by *Picrophilus torridus* through the incorporation of fewer purines in short, unprotected open reading frames and more purines in the longer, less acid hydrolysis-susceptible open reading frames (Baker-Austin and Dopson, 2007). Protein adaptations to high temperature and low pH are also antagonistic. The thermophilic strategy of maintaining a neutral surface charge would reduce the acid-resistant buffering capacity of the proteome, and although heat-shock induction by acid stress has been observed in acidotolerant *E. coli*, the acidophilic alga *Chlamydomonas acidophila* did not modulate heat-shock protein expression in response to changes in environmental pH (Fukuchi et al., 2003; Spijkerman et al., 2007). These data indicate an absence of a strong positive high temperature-low pH correlation in biochemical adaptive mechanisms. Given this absence, it follows that acidophily above 100 °C is not seen in Fig. 5.

Thermoalkaliphiles, such as *Thermococcus alcaliphilus*, have been found at temperatures above 60 °C and pH values greater than 9 (Keller et al., 1995). As in thermoacidophily, adaptations to high temperatures and high pH values are also expected to be antagonistic. This generalization is demonstrated through the observation that higher growth temperatures for the thermoalkaliphile *Natranaerobius thermophilus* corresponds to lower optimal and maximal pH values, a trend also observed in Fig. 5 (Mesbah et al., 2007; Wiegel, 2011). As mentioned previously, thermal effects on membrane fluidity would lead to destabilization of the pH gradient and cytoplasmic alkylation, and uncharged thermophilic proteins would be destabilized by the highly charged cytoplasm (Padan et al., 2005). Interestingly, thermoalkaliphiles, particularly anaerobic taxa, have been observed to maintain a smaller pH gradient of 1.0–1.5 units, leading to consistently higher cytoplasmic pH values (Cook et al., 1996; Mesbah and Wiegel, 2008). High intracellular pH suggests that the buffering capacity of the cytoplasm may contribute more to thermoalkaliphile survival than the bioenergetic maintenance of the gradient (Wiegel, 2011).

3.2. TEMPERATURE AND SALINITY

We define psychrohalophiles as polyextremophiles that thrive at low temperatures and high concentrations of salt. At such low temperatures, the depression of the

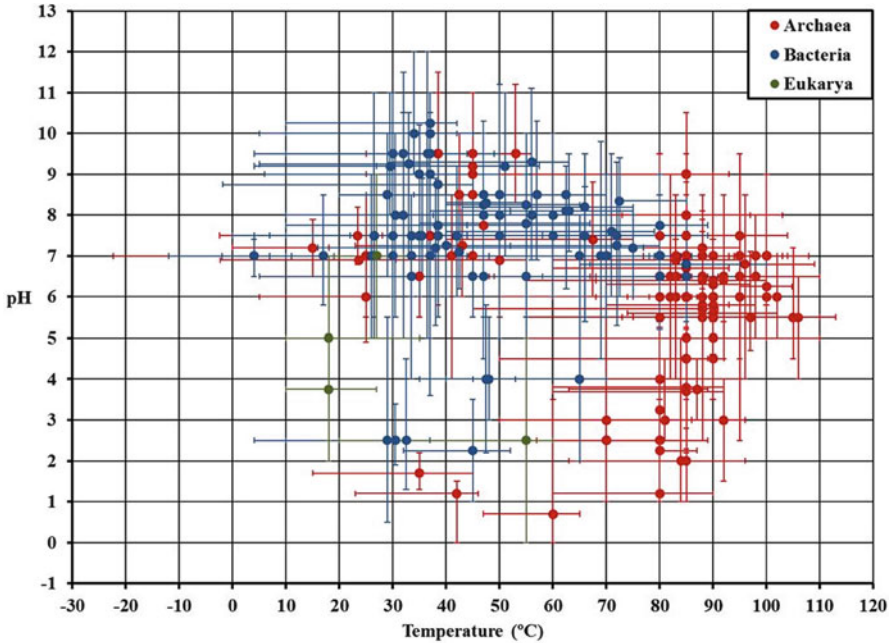


Figure 5. Distribution of archaeal, bacterial, and eukaryotic growth ranges as functions of environmental pH and temperature.

freezing point of water is paramount, and many environments in polar sea ice are cold brine solutions (Morgan-Kiss et al., 2006). *Psychromonas ingrahamii*, for example, has been found growing in temperatures as low as -12°C and salinities as high as 20 % NaCl (Auman et al., 2006). Thus, some degree of mild to moderate halophily is typical of psychrophiles, especially those that use the “high-salt-in” strategy for maintaining a hyperosmotic cytoplasm. In addition, the “compatible-solutes-in” approach to halophily and the development of freeze tolerance both include the production of the chaotropic osmolytes glycerol and glycine betaine. Thus, psychrophily and halophily have several natural synergistic mechanisms, although further investigations should be launched to test whether this correlation holds over a large selection of known psychrophiles and halophiles.

Thermophiles tend to be less successful at surviving high concentrations of salt, as their uncharged proteomes are extremely destabilized in hypersaline solutions (Zaccai, 2011). By burying charges, thermohalophiles not only lower the likelihood of denaturation by high-temperature solvent but also decrease the electrostatic interactions required to maintain native folding in a charged environment (Fukuchi et al., 2003). *Thermococcus waiotapuensis*, which grows at a maximum temperature of 90°C and maximum salinity of 13.9 % NaCl, is the most

halophilic hyperthermophile discovered to date, but the biochemical basis for this unusual adaptation is currently unclear (González et al., 1999).

3.3. TEMPERATURE AND PRESSURE

Temperature and pressure, while directly related thermodynamically, have opposite effects on a cell. This inverse physicochemical relationship stems from the impact each has on volume. Increases in volume are favored at high temperatures but disfavored at high pressures. The interplay between these environmental parameters has the most profound effect on membrane fluidity (Casadei et al., 2002). Due to the neutralization of temperature and pressure effects, membranes of some thermopiezophilic organisms, such as those found in deep-sea hydrothermal vents, are also capable of maintaining structural integrity at standard temperature and pressure (ZoBell, 1952). Proteomic adaptations are also synergistic, as both thermophilic and piezophilic proteins are composed of strongly hydrophobic cores with little surface charge. These trends make it intuitive that such thermopiezophiles as *Methanopyrus kandleri* and *Marinitoga piezophila* demonstrate the characteristic property of optimal growth temperature being directly proportional to the pressure (Miroshnichenko, 2006; Takai et al., 2008). One interesting biochemical response to high-pressure stress is the induction of both heat-shock and cold-shock response pathways, suggesting synergistic genomic, metabolomic, and proteomic effects between both temperature extremes and pressure (Vanlint et al., 2011).

Induction of the cold-shock pathway is particularly important to psychropiezophiles, which do not benefit from synergistic temperature-pressure thermodynamics. Instead, both of the extremes promote membrane crystallization, a fate that must be avoided with extensive incorporation of monosaturated and polyunsaturated fatty acids (Kato et al., 1998). The psychropiezophiles *Colwellia hadaliensis* and *Colwellia piezophila* produce polyunsaturated and monounsaturated fatty acids, respectively (Kato, 2011). While the underlying biochemical mechanism responsible for the distinction between monounsaturated and polyunsaturated fatty acids in piezophiles is currently unknown, all piezophiles have the characteristic of increased unsaturated fatty acid membrane content. The production of EPS has also been observed to be a common response to cold temperature and high pressure stresses (Marx et al., 2009).

3.4. TEMPERATURE AND RADIATION

Early organisms were exposed to extremely hot, anaerobic environments with terrestrial-based ionizing radiation levels ten times greater than those experienced today (Karam and Leslie, 1999). These conditions persist in contemporary deep sea basins. *Thermococcus gammatolerans* is an anaerobic organotroph found in

the Guaymas Basin 2.6 km below sea level that remains viable in temperatures up to 96 °C and more than 6 kGy of radiation (Jolivet et al, 2003a). Another hyperthermophile, *Pyrococcus abyssi*, survives exposure to doses of up to 2.5 kGy of ionizing radiation (Jolivet et al., 2003b; Toueille and Sommer, 2011). Jönsson and Schill (2007) have also reported evidence that heat-shock protein Hsp70 is induced by both high temperature and ionizing radiation stresses in the tardigrade *Richtersius coronifer*. In fact, 16S rRNA-based phylogeny clusters radioresistant and thermophilic bacteria into the *Deinococcus-Thermus* group, which suggests a strong evolutionary connection between these two environmental parameters (Toueille and Sommer, 2011). This biochemical connection between thermophily and radiation resistance may result from the genetic damage induced by high temperatures and radiation. High temperature promotes hydrolytic depurination, deamination, and single-stranded and double-stranded DNA breaks (Toueille and Sommer, 2011). Thus, thermophiles must upregulate DNA repair pathways in a similar fashion to radiation-exposed organisms. These repair pathways would confer resistance to all extremes that commonly cause DNA damage, leading to a synergistic relationship between thermophily and radioresistance.

3.5. pH AND SALINITY

Haloacidophiles and haloalkaliphiles are defined as organisms that require low or high pH, respectively, and high concentrations of salt for growth. The biochemical adaptations for life in high salt are both synergistic and antagonistic to those that allow life at the extremes of pH. For haloacidophiles, high extracellular salt concentrations allow more favorable efflux of H⁺ via Na⁺/H⁺ and K⁺/H⁺ antiporters. Since cytoplasmic acidification is lethal, this antiporter action is incredibly beneficial. On the other hand, anions, typically Cl⁻, are attracted to the positive Donnan potential maintained in the cytoplasm of acidophiles. Influx of anions would neutralize this potential, significantly impairing the ability of the membrane to restrict H⁺ influx and resulting in cytoplasmic acidification and cell death (Johnson, 2006; Krulwich et al., 2011b). The harm done by the latter effect is most likely more dramatic than the benefit of the former, leading to the conspicuous absence of terrestrial haloacidophiles.

The combination of high salinity and high pH, such as those conditions in the habitat of *Bacillus selenitireducens*, Mono Lake, CA, USA (Fig. 6), is much more common than haloacidophily (Blum et al., 1998). Many haloalkaliphiles, like *Natranaerobius thermophilus*, have been discovered, because monovalent cations of salts are essential for pH homeostasis and energetic coupling (Mesbah et al., 2007). However, high extracellular salt concentrations make Na⁺/H⁺ and K⁺/H⁺ antiporter action less favorable for H⁺ influx. This influx is required to prevent lethal alkylation of the cytoplasm; thus, at sufficiently high salt concentrations and pH, Na⁺ and K⁺ become cytotoxic (Padan et al., 2005). Recent work has found that haloalkaliphiles, unlike nonhalophilic alkaliphiles, maintain a constant gradient of about 1.0–1.5 pH units across the cell membrane, regardless



Figure 6. Mono Lake, CA, USA, is home to the haloalkaliphile *Bacillus selenitireducens*.

of extracellular pH variations (Mesbah et al. 2009). This finding indicates that the cytoplasmic pH is dynamic and increases with increasing extracellular pH. Cytoplasmic alkylation, therefore, becomes the limiting factor for the maximum growth pH of haloalkaliphiles (Mesbah and Wiegel, 2011).

3.6. pH AND PRESSURE

As discussed previously, pressure has a significant effect on reaction equilibria in a volume-dependent manner. The dissociation of acids and protonation of protein amine groups are characterized by negative volume changes and so are favored at high pressure (Abe et al., 1999). The dissociation of carbonic acid to bicarbonate, for example, has a change in volume of -26.0 ml/mol (Abe, 2011). At room temperature, this value corresponds to a K_a value at 100 MPa that is double that of the K_a at 0.1 MPa (Abe and Horikoshi, 2001). Since these reactions result in acidification of the solution, piezophiles may exhibit some acidotolerant or acidophilic characteristics, in which we dub the resultant polyextremophiles piezoacidophiles. The inverse pressure-pH relationship may also preclude the selection of the so-called terrestrial piezoalkaliphiles. The systematic identification and characterization of piezoacidophilic or piezoalkaliphilic organisms still needs to be investigated in order to acquire a full understanding of the interaction of these two extreme environmental conditions.

3.7. pH AND RADIATION

Alkaline environments have been shown to inhibit the redox cycling of Mn^{2+} , an essential defensive factor against protein damage due to radiation-induced superoxide

formation. In vitro studies of alkaline aqueous MnCl_2 solutions in an anaerobic atmosphere demonstrated the evolution of oxygen but not peroxide when exposed to ionizing radiation. Since superoxides are produced by the irradiation of water and Mn^{2+} is known to reduce superoxide to peroxide, the absence of peroxide indicates the inhibition of this reduction. Correspondingly, irradiation of *D. radiodurans* at pH 10.5 did not result in peroxide evolution and halved the survival rate compared to that of *D. radiodurans* at neutral pH (Daly, 2009). These results indicate that alkaliphiles, especially the haloalkaliphiles that allow cytoplasmic alkylation, are unlikely to demonstrate significant radioresistance. It is unknown how acidic conditions may affect the potential development of radioresistant acidophiles.

3.8. SALINITY AND PRESSURE

Hypersaline high-pressure environments are largely limited to deep anoxic basins, which can have pressures of 35 MPa and up to 47 % MgCl_2 . Although microorganisms have been identified in these basins, their physiologies remain largely uncharacterized. It is known that some samples from the Discovery basin in the Mediterranean Sea have DNA sequences with high similarity to the halophilic archaeon *Halorhabdus utahensis* (van der Wielen et al., 2005). Stock et al. (2012) also described the discovery of fungi and protists in the Thetis basin. The adaptations that allow life in high pressure and salinity may be centered around the compatible organic solutes that accumulate in halophiles and piezophiles. One molecule, in particular, β -hydroxybutyrate, has been observed to behave as both an osmolyte and a piezolyte (Martin et al., 2002). Thus, maintaining high intracellular concentrations of β -hydroxybutyrate would protect not only against salt-induced desiccation but also pressure-induced membrane, protein, and nucleic acid damage.

3.9. DESICCATION, TEMPERATURE, AND PRESSURE

Adaptations for survival at extremes in temperature, pressure, and water activity are highly synergistic. Psychrophiles, thermophiles, piezophiles, and xerophiles all accumulate compatible organic solutes, designated chaotropes, thermolytes, piezolytes, and lyoprotectants, respectively, to maintain the stability of lipids, proteins, and nucleic acids under harsh environmental conditions. While each organic molecule is not effective at all roles, many solutes can assist survival at multiple extremes. Furthermore, proteins with uncharged surfaces, like those in thermophilic and piezophilic microorganisms, are not destabilized in conditions of low water activity. The combination of these effects explains how anhydrobiotic tardigrades can be revived after being subjected to temperatures as low as $-271\text{ }^\circ\text{C}$ and as high as $151\text{ }^\circ\text{C}$ and pressures up to 600 MPa (Doyère, 1842; Rahm, 1923, 1937; Becquerel, 1950; Keilin, 1959; Seki and Toyoshima, 1998).

3.10. DESICCATION, pH, AND SALINITY

Although some osmolytes of “compatible-solutes-in” halophiles can also act as lyoprotectants, such as floridoside in red algae, the effects of low water activity and high salt concentration on proteomic pI are anticipated to be antagonistic (Reeb and Bhattacharya, 2010). Acidophiles, alkaliphiles, and halophiles express proteins with pI values far from neutral. These proteins have significant surface charges, which are stabilized by the charged solutions of acidic, alkaline, and hypersaline environments. In the absence of water, however, these charged proteins are more likely to denature and lead to cell death (Mesbah and Wiegel, 2011). This trend is evidenced in the loss of *Natronaerobius thermophilus* viability after 12 h of desiccation (Mesbah and Wiegel, 2011). The correlations between biochemical adaptations to low water activity and pH or salinity are not yet fully understood. Direct investigations into desiccation tolerance of microorganisms isolated from Mono Lake could answer this open question.

3.11. DESICCATION AND RADIATION

Genetic damage is common to both desiccation and radiation exposure. Desiccation- and radiation-induced damage must be repaired immediately upon rehydration and exposure, respectively (Cox and Battista, 2005). The similarities between these conditions have led to the hypothesis that adaptations to desiccation would also allow greater radiation resistance (Mattimore and Battista, 1996). There are many examples of xerophiles demonstrating radiation resistance, including the tardigrade *Macrobiotus areolatus*, which can tolerate exposure to 5.5 kGy of X-ray radiation, and anhydrobiotic larvae of *Polypedilum vanderplanki*, which are able to recover with 50 % viability after 9 kGy of ^{60}Co gamma rays (Watanabe et al., 2006). The opposite is also true, as *D. radiodurans*, the model bacterium for radioresistance, has exhibited xerophilic behavior of 85 % viability after 2 years at <5 % humidity. In fact, of the 26 known *Deinococcus* species, 14 have been isolated from arid environments. In addition to the upregulation of DNA repair pathways, both xerophilic and radioresistant microorganisms have been found to accumulate high concentrations of Mn^{2+} (Toueille and Sommer, 2011). Such a high degree of synergism between adaptations for radioresistance and xerophily indicates a direct correlation between these extremes. Due to the significance of radiation and desiccation resistance to panspermia and other origin of life theories, further investigations into this correlation is of great importance to astrobiology.

4. Synthetic Polyextremophiles and Space Exploration

As discussed in the previous sections, some combinations of polyextremophily have yet to be discovered on Earth. These biological gaps may be filled with polyextremophiles that have been engineered through synthetic biology. Synthetic

biology is one of the most promising and powerful emerging technologies today (Gutmann, 2010). NASA has shown interest in synthetic biology because of its promising applications to space exploration (Rothschild, 2010). One of the major challenges of space exploration is the limited payload mass that can be launched on a rocket and the difficulty of resupply mid-mission. Synthetically engineered organisms could be designed to perform many tasks useful for a space mission or settlement, including mining, heating, environmental sensing, and generation of food, fuel, and construction materials. Such biological tools have major advantages over classical tools due to their ability to self-replicate and regenerate. These advantages mitigate the need for large initial payloads as well as decrease the frequency of resupplying.

Given the harsh realities of space settlement, polyextremophilic characteristics could be beneficial to synthetic organisms engineered for use in such contexts. Severe environmental conditions, including low temperature, pressure, high radiation, and desiccation, are common on Mars and other planetary bodies favored for exploration. Organisms with psychrophilic characteristics have been synthetically engineered by introducing low-temperature resistance genes from extremophiles into non-psychrophilic bacteria. Ferrer et al. (2003) demonstrate this possibility through the incorporation of unique chaperonins, namely Cpn10 and Cpn60, of the bacterium *Oleispira antarctica* into *E. coli*. Cpn10 and Cpn60 assist protein folding at low temperatures in *O. antarctica* and successfully lowered the minimum growth temperature of *E. coli*. As the technology to create biological tools for space exploration develops, the artificial introduction and fine-tuning of other extremophilic characteristics will be an important and exciting avenue of research. While discussions about the possibility of planetary contamination will be necessary, such research could lead the way for future terraforming efforts.

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Biodata of **Joseph Seckbach**, author of “*Life on the Edge and Astrobiology: Who Is Who in the Polyextremophiles World?*”

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LIFE ON THE EDGE AND ASTROBIOLOGY: WHO IS WHO IN THE POLYEXTREMOPHILES WORLD?

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1. Introduction

Life exists in almost every ecological niche on Earth, and the majority of living organisms thrive in “normal” or “common” conditions. These are the environments that we are familiar with from our daily life. The organisms distributed under those conditions are at moderate temperature (5 to ~40 °C), 1 atm sea level pressure, with our known gas compositions, and oxygen rich atmosphere, close to neutral pH level. We consider these conditions as benign ambient habitats.

There are, however, also on land or below Earth’s surface organisms dwelling at the edge of the “normal” limits for life. These creatures exist under very severe (from our anthropocentric point of view) environmental conditions. We refer to these hardy organisms as extremophiles (Rothschild, 2007; Seckbach, 1999, 2000, 2007, 2012; Stojanovi et al., 2008). Among the extremophiles are unicellular and multicellular organisms. The many microbes thriving under multiple forms of stress are termed polyextremophiles (in this volume). These creatures include, among others, the hyperthermophiles at acidic (low pH) conditions or hypersalinity conditions with high UV radiation levels and high pressures at the bottom of the ocean with low/high temperatures. Harboring around the hydrothermal chimney vents are communities of microbial and multicellular organisms at elevated pressure, temperature, and acidic pH. Among the higher animals there are clams, mussels, tubeworms, and a variety of grazers (Islam and Schulze-Makuch, 2007). Let us look briefly at the organisms living in these severe conditions.

2. The Extremophiles

In this category, we include both autotrophic and heterotrophic microorganisms, prokaryotes, and to a lesser extent eukaryotic or multicellular representatives. A comparative table of oxygenic photosynthesis of prokaryotes and eukaryotes in extreme environments has recently been presented (Seckbach and Oren, 2007). Extreme environments exhibit a relatively lower biodiversity in which the organisms

show a high adaptation capacity. These microbes are living in a severe conditions of life, such as at high/low pH ranges or at high/low scales of temperature levels, high salinity (up to saturated salt solutions), in alkaline waters or soil (such as soda lakes), acidic sulfur-rich areas, and high pressures (at terrestrial subsurface or living in great depths of oceans). Some of the organisms live with low water activity, for example, osmophiles and xerophiles, while others may thrive with low nutrients or tolerate heavy and toxic metals. Among these organisms are bacteria, archaea, eukaryotes including algae, and other protists, unicellular and multicellular, lichens, fungi, plants, and invertebrate animals. Extreme environments are considered hostile and even deadly to “common” forms of life (mesophiles), while most extremophiles themselves are not able to live under mesophilic conditions.

Large amounts of biomass reside in the subsurface of the Earth, and communities of microorganisms have been observed under the surface. These organisms live without light, at elevated temperatures, and under high pressure. The extremophiles may well be models and analogues for extraterrestrial life (Seckbach and Chela-Flores, 2012).

2.1. CATEGORIES OF THE EXTREMOPHILES

Extremophilic organisms can tolerate very harsh conditions such as:

High temperature (thermophiles to hyperthermophiles, 40–115 °C and even higher up to 122 °C at the hydrothermal vents under high pressure (Stan-Lotter, 2012; Stetter, 2006; Takai et al., 2008)). An older publication by Baross and Deming (1983) reported that thermophilic marine bacteria isolated from the vicinity of a submarine hot spring at temperatures up to at least 250 °C at 265 atm. However, no other source could confirm that super heat value. On the other temperature scale, organisms grow at minus 20 °C (for cryophiles/psychrophiles).

Very low pH (−0.5 to 4 pH: acidophiles) or high pH levels (8–12: alkaliphiles).

High salt concentrations (halophiles) up to saturated brines (hyperhalophiles). For example, the green alga *Dunaliella salina* tolerates >5.5 M salt, and another species of *Dunaliella* survives in the Dead Sea, Israel (Giordano and Beardall, 2009).

High hydrostatic pressures (barophiles/piezophiles): maximum pressure reached ~1,100 atm on the ocean floor at a depth of 10,900 m. For every 10 m of water column (towards the deepness), there is a hydrostatic pressure increase of 1 atm. Similarly, barophilic organisms are also in the subsurface of dry land (such as in deep mines).

Environments rich in toxic chemicals: heavy toxic metals such as arsenic (Wolfe-Simon et al., 2011), Cu^{II} (Twiss, 1990), or in Zn^{II} and Cd^{II}.

Moreover, it is known that some bacteria are able to metabolize hydrocarbon compounds from oil spills at the sea surface. Further features of extremophiles are presented in the ensuing.

2.2. HABITATS AND LIVING CONDITIONS OF EXTREMOPHILES

Not only prokaryotes but also several eukaryotic microbes can live under anoxia (Altenbach et al., 2012) and utilize anaerobic metabolism. In addition, some algae are able to thrive under pure CO₂ gaseous stream and show higher growth rates and more oxygen release by photosynthesis than when air is bubbled through the microbial cultures (Seckbach, 1994; Seckbach et al., 1970).

One can observe extremophiles in various harsh habitats, such as in hot springs and other geothermal ecosystems (as found in Yellowstone National Park, USA; Rio Tinto, Spain; or in extreme hypersaline solution in the Dead Sea, Israel; sites in Iceland, south Italy, New Zealand, Japan; and on the floors of the hydrothermal vents in the oceans). The upper limit of (hyper) thermophiles has been determined at 113 °C and possibly up to 121 °C (Stan-Lotter, 2012; Stetter, 2006). There are microbes that live in severe cold areas such as those found in Antarctica and the northern hemisphere. There are microorganisms in the anoxic brine basins in the sea, and endolithic microbial life (Golubic et al., 1981) was observed in Antarctica (de los Ríos et al., 2003). Further information has been published by Stan-Lotter (2012) who presented tables of prokaryotic extremophiles with their tolerance to extreme factors.

2.3. PUBLICATION AND DISTRIBUTION OF EXTREMOPHILES

An early comprehensive survey of eukaryotic extremophiles was published 15 years ago (Roberts, 1998). Of late, some new journals devoted to extremophiles were established, and a number of books and reviews on the topic appeared (Horikoshi and Grant, 1998; Seckbach, 2000, 2012; Rothschild, 2007; Rothschild and Mancinelli, 2001; Rainey and Oren, 2006; Gerday and Glandorf, 2007; Seckbach and Walsh, 2009). International congresses have been organized around the topic of biological extremes.

3. The Polyextremophiles and Early Earth

Organisms that dwell in multiple harsh conditions are common in certain extreme environments. Some of their environments might resemble the conditions that probably occurred on early Earth. The early Earth has been assumed to have been warmer than today, anaerobic, and with higher concentration of CO₂, while the first prokaryotic microorganisms have been assumed to be thermophiles (or thermotolerant), or at least those that survived the late heavy bombardment. Several scientists believe that the origin of life was at the hydrothermal vents at high temperature level (Martin et al., 2008). It is interesting to note that the most deeply rooted microorganisms (in hydrothermal vents at the bottom of the ocean) are thermophiles, suggesting that the earliest common ancestor might have been a

thermophilic cell (Stojanovi et al., 2008). The early atmosphere supposedly contained only trace amounts of O₂, at least; the main gases were CO₂, water vapor, H₂S, N₂, methane, and CO (Kasting, 1993). Only with the appearance of the cyanobacteria was the atmosphere oxidized and “poisoned” with their O₂ release via PS_{II} (photosystem II is involved in the light reaction stage of the photosynthesis process of cyanobacteria, algae, and plants. The oxygen is released following the splitting of water). Finally, the oxygen level reached the present level of >20 % in the atmosphere. Hence, the early living organisms which were under the above described as well as post-biotic conditions (pressure, high temperature, anaerobic atmosphere) found niches to survive and thrive in similar environments.

3.1. BIODISTRIBUTION OF EXTREMOPHILES

Some extremophiles live in harsh niches like the Sahara Desert (northern Africa, with high temperature, radiation, and desiccation), the very arid Atacama Desert (Chile), and haloalkaline soda lakes, which represent a unique ecosystem with high pH (up to 11) and salinity, even up to saturation, due to the presence of high concentrations of sodium carbonate in brines. Despite these doubly extreme conditions, most of the lakes are highly productive and contain a fully functional microbial system. Such soda lakes are located in Nevada, California (Mono Lake, Searles Lake), and Egypt at Wadi Natrun (see chapter “Two Centuries of Microbiological Research in the Wadi Natrun, Egypt: A Model System for the Study of the Ecology, Physiology, and Taxonomy of Haloalkaliphilic Microorganisms” by Oren in this volume). Among the organisms living in these lakes are copepods, aquatic insects, unicellular eukaryotic algae, and brine shrimps. The microbial sulfur cycle takes place in most soda lakes. Other extremophiles live in geysers, hot springs, and deep-sea hydrothermal vents. The hypersaline microbes live in high saline places such as in the Dead Sea, Israel (see Oren and Seckbach, 2001), or in Great Salt Lake (Utah, USA). Halotolerant bacteria from Great Salt Plains (GSP) in Oklahoma (USA) grow in high concentrated MgSO₄, while others live in saturated salt solution. The barophilic organisms tolerate high pressure and thrive at the bottom of the oceans or in subterranean environments. Among these organisms living in harsh conditions, we find archaea, bacteria, and eukaryotes.

A novel ultramicrobacterium (*Herminiimonas glaciei*) was isolated from a 120,000-year-old Greenland glacial ice core, at a depth of 3,042 m, and successfully revived (Loveland-Curtze et al., 2009). The primitive type of cyanobacterium *Chroococcidiopsis* is capable of surviving in a large variety of extreme conditions, such as dryness, high and low temperature, exceptional aridity, salinity, and other harsh environments. It lives beneath translucent pebbles which act both as a moisture trap and a UV shield (Friedmann and Ocampo-Friedmann, 1995). Likewise, the eukaryotic unicellular acido-thermophilic alga *Cyanidium caldarium*, a red alga (Rhodophyceae), appears as green spherical cells (Seckbach, 1994, 2010;

Castenholz and McDermott, 2010) and thrives in pure CO₂ (Seckbach et al., 1970), at elevated temperatures (57 °C), and in very acidic solutions (pH 0–4). *Cyanidium* culture even tolerates rinsing in 1N H₂SO₄, which is a good method to obtain purified cultures. One genus of this family (Cyanidiaceae), *Galdieria sulphuraria*, thrives in autotrophic and even better in heterotrophic conditions with supply of carbohydrates (Seckbach, 1994). In the harsh conditions of Antarctica grow bacteria and 300 species of algae (such as *Chlamydomonas*, *Chlorella* and mosses- see Chela-Flores and Seckbach, 2011). In the Dry Valleys of Antarctica, cyanobacteria live inside rocks as endolithic layers. In the snow and ice, as in the Siberian permafrost, Antarctica, and the Arctic zones, are the cryophilic bacteria and algae. Such snow algae may appear with green, yellow, orange, or red coloration and have carotenoids during some periods.

3.2. LONG-LIVED BACTERIA

In 1998, NASA reported that the bacterium *Streptococcus mitis* survived on the surface of the Moon in a camera left almost for ~3 years and then was revived (Mitchell and Ellis, 1971). This demonstrates an additional feature of the ability of microorganisms to tolerate very severe conditions (such as extreme temperatures, UV radiation, and lack of nutrition). Among the UV radiation-resistant microorganisms are the *Deinococcus radiodurans* bacteria (Singh and Gabani, 2011). Cyanobacteria species can live endolithically under the surface, as, for example, the extremophile *Chroococcidiopsis*.

The above facts about organisms tolerating harsh conditions of life might not be too surprising since it is known that bacteria and some toxic microorganisms can be still vital after thousands of years in isolated dryness, such as the Egyptian mummies inside the pyramids. Furthermore, *Bacillus* sp. were revived after 25–30 million years from insects embedded inside amber, while Vreeland et al. (2000) claimed to have isolated and revived a 250 million-year-old halotolerant bacterium from a primary salt crystal (see below).

3.3. EUKARYOTIC LOWER AND HIGHER EXTREMOPHILIC ORGANISMS

Among the eukaryotic extremophiles are algae, fungi, mosses, and lichens. Each lichen is a symbiotic association between a fungus host and cyanobacterium or alga occurring as crusty patches grown on bare ground or tree trunks. They may survive in extreme environments on Earth and in the unprotected conditions of space. They were exposed to space under conditions of vacuum, ultraviolet radiation, and severe cold and survived. They have survived also under simulated conditions of space (de Vera et al., 2003, 2004; Raggio et al., 2011).

3.3.1. *Shrimp Beneath Ice and Pompeii Worm*

NASA found shrimp 200 m beneath the Antarctic ice where almost no advanced life should be. Among the polyextremophilic invertebrates is the Pompeii worm *Alvinella pompejana*, which is one of the most heat-tolerant animals—up to 105 °C—on Earth (Islam and Schulze-Makuch, 2007). It has been described as a deep-sea polychaete that resides in tubes near hydrothermal vents (black smokers) along the seafloor. This worm has symbiotic relations with chemolithotrophic bacteria that are in a layer on the dorsal body. The worms tolerate high hydrostatic pressure, high temperature, and other stress factors.

3.3.2. *Subsurface Nematodes*

Mephisto worms—*Halicephalobus mephisto*—are nematodes from the terrestrial deep subsurface of South Africa (Borgonie et al., 2011). These round worms live in gold mines' rocky, ca. 2 km underground. They are 0.5 mm long and exist in low oxygen, their body is soaked, and they live from 1.3 to 3.6 km down in the deepest mine. These nematodes' environment is estimated to be 2,000–12,000 years old. One species survived even the space shuttle Columbia breakup in 2003. They are adapted to tolerate hot temperatures that would kill most of its land-living species. Some were found in hot springs (at elevated temperature of ~55 °C), and they may colonize the most inhospitable habitats such as dry, frozen soils in Antarctic Dry Valleys.

3.3.3. *Tardigrades*

Tardigrades or water bears (or moss piglets) are even more extraordinary extremophilic organisms—the tardigrades meaning “slow walkers.” They are segmented, multicellular animals, mainly aqueous organisms with eight legs and of small size (mostly <1 mm long). They are found in damp pools, in water on lichen and mosses, in acidic solution of algal culture, on soil, and in marine or freshwater sediments. They are classical polyextremophiles and are able to survive in extreme environments. These creatures have the potential to survive travel to other planets because of their tolerance to extreme environmental conditions by means of a dry ametabolic state called cryptobiosis. Their survival capacities in severe conditions are stunning as they tolerate various extreme environments that would kill almost any other animals. Tardigrades tolerate very high and low temperature ranges (180 to –273 °C), 1,000 times more radiation than other animals (they must have a very efficient means of repairing DNA damage after such strong radiation), and survive prolonged periods of drought and almost a decade without water. These animals were exposed to weightlessness, space vacuum, and lashing of both cosmic and solar radiation for 10 days aboard a Russian satellite about 270 km above sea level, and upon return to Earth they were unharmed and continued to reproduce. They tolerate extreme dehydration, freezing temperature, and high pressures in cryptobiosis; they can be dormant for years while needing only a drop of water to revive them. Anhydrobiotic eggs of the tardigrade *Ramazzottius varieornatus* also have a broader temperature resistance compared to hydrated ones (Horikawa et al.,

2012). They may be considered as ideal analogues for candidacy for extraterrestrial living. For more sources on these water bears, see Chela-Flores (2011, p. 115) and the relevant references, such as Horikawa (2011), chapter “[Tardigrades: An Example of Multicellular Extremophiles](#)” by Schulze-Makuch and Seckbach in this volume.

3.3.4. *Ticks Inside the Electrons Stream*

The case of Ticks in SEM. Recently it was reported (Ishigaki et al., 2012) that living ticks (*Haemaphysalis flava*) were placed in a vacuum and bombarded by electrons in a SEM (scanning electron microscope). Upon release from the SAM chamber, these animals were still fully alive.

4. Astrobiology

4.1. THE POSSIBILITY FOR EXTREMOPHILES TO LIVE IN EXTRATERRESTRIAL PLACES

Astrobiology is a relatively new branch in the astronomical-biological sciences that seeks (among other tasks) to understand the origin of life and the interrelation of life with environments. It deals also with the question of how life could extend beyond our planet. Akin to Astrobiology is the SETI group (*Search for Extraterrestrial Intelligence*) which tries to find communication with extraterrestrial civilization. In the past, parallel study was termed Exobiology, Bioastronomy, Cosmobiology, and so on.

Astrobiology tries to answer whether life is common or rare in the universe. The Panspermia hypothesis (McNichol and Gordon, 2012; Wickramasinghe, 2012) claims that life in the universe has been spread by spores or bacterial cells from space and developed in a suitable environments as on Earth. One of the prerequisites and priorities for the search of life also beyond Earth is the availability of liquid water, sources of energy, and a supply of organic molecules. These factors are important for cellular metabolism. We know that wherever there is liquid water, there are good chances to find living organisms (Chela-Flores, 2011).

4.1.1. *Mars: Our Sister Planet*

The investigations about life (or lifelessness) of Mars, Europa (moon of Jupiter), and Enceladus (satellite of Saturn) and Titan (satellite of Saturn), to a lesser extent, are one of the main targets today in the search for extraterrestrial life. The frozen desert of Antarctica resembles the chilled dry world of the Martian surface (2 °C, -65 °C, and 20 to -126 °C) of today (McKay et al., 2012). Several photos by various flybys over Mars as well as rovers rambling over the Martian surface show that in the past (~3.5 billion years ago), this planet was warm and wet with plenty of water. The Red Planet appears to have been sculpted in part by flowing liquid, as by ancient rivers, winding channels, and lakes; this adds to the growing evidence

that Mars once, long ago, had large volumes of water on its surface. Some photos taken by Mars Express spacecraft reveal ancient rivers and winding channel series of “pit chains” on the sides of volcano, which may possibly be places of life.

Over three and a half decades ago, NASA concluded that 1976 Viking rovers did not discover any evidence or traces of life on Mars. New analyses led (by a few scientists) to the conclusion that the NASA results from the Viking Robots, which landed in summer 1976, were wrong and the probe actually did find microbial life on Mars (Levin, 2011; Houtkooper and Schulze-Makuch, 2007; Bianciardi et al., 2012; cf. also to Navarro-González et al., 2010). NASA has launched the MSL (Mars Science Laboratory) mission that released the Curiosity Rover at the Martian Gale Crater. Curiosity landed on 6 August 2012 (after 9 months of space travel); it will search for water and organic matter that eluded two Viking probes in 1976. Moreover, an additional spacecraft for a robotic mission (ExoMars—by ESA with Russian space agency Roscosmos), scheduled for the end of this decade, is to drill ~2 m in the soil of Mars and analyze the scooped samples and those drilled from rocks in an attempt to detect building blocks of life. Perhaps life survives in the subsurface, in permafrost, in hydrothermal areas, or at the polar caps on Mars.

In addition, the unstable compound methane has been discovered on Mars which might support some bio-sources from methanogenic microbial activities. Methane-eating bacteria survived in Canada in extreme northern areas (such as in Lost Hammer Spring “LHS”—similar to areas present on Mars). The conditions in LHS are subzero temperature and high salty areas with no consumable oxygen (Miller and Whyte, 2012).

The search for life beyond Earth is in fact essentially the search for habitability on other worlds. In addition, biomarkers on extraterrestrial places should assist in finding traces of life beyond Earth. There have been some attempts (McKay et al., 1996) to discover traces of fossil nano-bacteria from a Martian meteorite that fell in Antarctica (ALH84001). However, several opponents rejected these observations (and conclusions) and claimed that the illustrations are just mineral artifacts (see descriptions by Kargel, 2004, p. 410; Reitner, 2004). Carbonates of the same microstructure as the host rock of the “ALH84001” have been discovered in rocks from alkaline Lake van which is the largest lake in east Turkey; it is a saline and soda lake (Kazmierczak and Kempe, 2003). That finding suggested, at least, that the hydrous environment on Mars was alkaline. Other chemical biomarkers were also observed in archaean rocks and on carbonaceous meteorites. It seems that traces of life in meteorites need further proof of its existence. Only after the return of samples from Mars to Earth (an MSR mission which might take place before the end of next decade) will we know for sure about life on other planets. Following the terraforming by Sagan (1961, 1967) and others, Friedmann and Ocampo-Friedmann (1995) pointed out the cyanobacterium *Chroococcidiopsis* for “Greening the Red Planet.” For an older survey concerning life on Mars, see Brack and Pillinger (1998), while further information and recent photos of Mars have been presented by Kargel (2004).

4.1.2. *Europa: The Ocean Moon of Jupiter*

Among the extraterrestrial places within our solar system that are quite promising for being habitable sites are Mars, Enceladus (Saurian moon), and Jupiter's satellite Europa, which is one of the four Galilean moons of Jupiter. Europa is the smallest of the four Galilean moons of Jupiter. It is slightly smaller than The Earth's Moon and cracks and streaks crisscross its surface. This Jovian moon is the home of the solar system's largest subsurface ocean. Several moons of The nearby celestial bodies may carry subsurface oceans, and they may provide the greatest volume of living area in our solar system. Europa is considered one of the greatest potential places for microbial life habitability (Chyba and Phillips, 2001; Greenberg, 2010). Europa has under its 5–15 km ice sheet an internal salty ocean of ca. 100 km depth, under a hydrostatic pressure of 1,100–1,300 atm, which may contain living species. The geochemical conditions in this sub-icy ocean are assumed to be suitable for life, while the temperature in this ocean is estimated to be 4 °C (Chela-Flores, 2011) and under high hydrostatic pressure. Below the ice surface of Europa, the ocean is kept warm by tidal forces (Pappalardo et al., 1999) and perhaps by volcanic sources. For further information and photos of Europa, see Greenberg (2005) (cf. Pappalardo et al., 1999). A subsurface ocean predicted to be on Europa is located on Earth in Antarctica under the Vostok station; this might be the best analogue we have on Earth for Europa's ocean. It is located beneath 4 km of ice and contains salty liquid water, where microbes were observed deep in the icy layers. Microbial life likely should exist in Lake Vostok, but it is much more uncertain whether it might exist in Europa's subsurface ocean. The body of the Lake Vostok subsurface water is up to 1.2 km deep, and the temperature of the water is –3 °C, which does not freeze because of the heavily salted water and the icy pressure above its surface.

4.1.3. *“JUICE” Mission to the Jovian Moons Next Decade*

JUICE (Jupiter-ice-Explorer) is the next large ambitious space mission by ESA to visit the icy moons of Jupiter. The launch is planned to be realized in 2022, and after 8 years it will reach the target. Among the Jovian satellites to be covered are Callisto, Europa, and Ganymede (the largest moon in the solar system). The purpose of this mission is to investigate the possibility of habitability, to look for potential hosts for microbial life, and to measure the thickness of Europa's icy layer. All three moons are supposed to have a subsurface ocean, which means liquid water below their icy surface that might have environments conducive to simple biology. As we know, life requires a solvent (such as water), an energy source, and chemicals.

4.1.4. *Penetrator with a Drill Designed to Enter into Europa's Ice*

Lately there is a variety of instruments that aim to characterize the surficial properties of the Jovian satellite Europa. Proposals include landers or penetrators that carry a suite of instruments. Hard penetrator solutions have been proposed (Gowen et al., 2011), although so far this concept has to be demonstrated

in the actual conditions that would offer the European environment (Korablev et al., 2011). More ambitious solutions have been envisaged in the Russian Laplace-Europa Lander Mission (Zelenyi et al., 2011). Although a total mass of over 1 ton was suggested, the more feasible penetrator still packaging a significant suite of miniaturized instruments would still be possible within a concept that is currently supported by ESA: the EJSM mission (Europa Jupiter System Mission) has to be reformulated due to the lack of funds. Fortunately, the new ESA project JUICE (JUperiter ICy moon Explorer) has left an attractive alternative (Dougherty et al., 2011), briefly, a mole-like thermal penetrator with a drill designed to bore through the icy surface of Europa in the future mission (to be launched in 2020). The thermal drill should be “the nose” of a penetrator, using heat to melt the ice and rotating drill blades to clear away rocky material and burrow itself into Europa’s ice shell. There are several models designed for the penetrator body shapes. Some of them are micro-penetrators—lightweight (5–15 kg) probes delivered to enter Europa’s body surface. Penetrators could provide information about mineralogy, geophysics, astrobiology, interior body structure, chemistry, mechanical and electrical properties, radiation environment, and magnetic, thermal surface/subsurface material, electrical, and thermal properties. The drilling action and penetration into the ice layer with the penetrator is vital since any landing probe that searches for biosignatures on Europa must go deeper than 2 m into the surface ice—due to heavy radiation and particle bombardment which could have erased any biological traces in the top layer. The plan is that the drilling into the icy crust layer searching for bio-samples should be from 1 m (which is sufficient to get beneath the radiation reworked surface) up to 10 m. The European upper ice thickness is between a few km and tens of km, while the internal ocean could be 100 km deep. On Europa’s ocean floor (at a depth of 150 km from the upper surface), it is assumed that there is a warm temperature environment, due to hot water-rock interaction. Such conditions may synthesize complex organic chemicals and produce energetic compounds that could support the emergence of life. The case of Europa’s drilling might be a model of other large icy satellites that have vast global oceans of liquid water deep underground.

4.1.5. *Titan*

Titan is the largest moon of Saturn. Its surface is very cold ($-179\text{ }^{\circ}\text{C}$), and this satellite contains lakes and perhaps rivers of liquid methane and ethane. In this celestial body, there is no permanent surface body of liquid water or large quantities of CO_2 . Both its liquid lakes and seas consist of hydrocarbons (methane and ethane). A region on Titan has been found to be similar to the Etosha Pan in northern Namibia, Africa. Both are ephemeral lakes, large, shallow depressions that sometimes fill with liquid (in Titan it is covered with hydrocarbons). Although there might be a possibility that the surface (if liquid hydrocarbons can take the role as solvents for life) or subsurface is habitable, we cannot currently know for

certain. Titan does not have an atmosphere similar to Earth and is covered with hazy methane clouds which hide the surface. There is in its atmosphere CO_2 at the ppb level only. Its surface pressure is 1.5 bars. Environmental conditions on Titan and Earth were similar in many respects four billion years ago; thus, in various ways it is analogous to early Earth. Recently large tides have been observed by the Cassini probe on Saturn's moon Titan, which pointed out to a subsurface liquid ocean. This underground sea is mostly likely to be of water, located 100 km beneath Titan's icy surface and it swirling around below the surface (Kerr, 2012).

See comprehensive reviews of the Saturn system and especially Titan (and their references) by Raulin (2012), Raulin et al. (2004), and Simakov (2004).

4.1.6. *Enceladus: The Satellite of Saturn (The King of the Rings)*

Most of the information about Enceladus was obtained from flybys over this moon and not from rovers. There is a connection between internal liquid water reservoir and space. This moon has a great interest for astrobiology, due to the water-ice plumes on the surface; it has good chances for habitability. Enceladus has the tenth size of Titan (the largest satellite of Saturn); its temperature is -190°C and its diameter is 500 km. The active ice volcanism comes from the internal hot rocks to the pressurized liquid water accumulation, to water ice, which comes out as plumes to the atmosphere. The clouds at the South Pole are composed of 65 % water vapor (from the aqueous plumes) while also CO_2 , N_2 , and CO were detected in the atmosphere (Shapiro and Schulze-Makuch, 2009).

4.1.7. *Venus: Out of the Habitability Question*

Focus is on Mars, Enceladus, and Europa in the search for habitable zones, but one might be surprised to know how much Venus has been explored—from initial telescope observations and the early flyby missions to the landers and orbiters. We know quite a lot about Venus, but the planet surely did not give up its secrets easily. We now know the physical conditions on this “Earth twin planet” which contains mainly CO_2 in its atmosphere. Venus is the hottest place in the solar system with a temperature of 750°C , with a surface pressure of 90 bars. There is no possibility for finding any Cytherean life on the surface of the planet, but prior to the current data over Venus, there are some older proposals for Venusian microscopic life on its surface (Seckbach and Libby, 1970). Sagan (1961, 1967) and Morowitz and Sagan (1967) suggested terraforming Venus clouds by seeding the Venusian lower atmosphere with cyanobacteria in order to make this celestial land habitable. The rhodophyten *Cyanidium caldarium* (Seckbach, 1994) is able to tolerate severe condition close to Venus (such as CO_2 , elevated temperature, acidic environment) and could be pointed out as a pioneer candidate for the Cytherean cloud settler. More recently Schulze-Makuch and Irwin (2002) and Schulze-Makuch et al. (2004) published their “hypothetic papers” about the possibilities for Venusian life. For more information on Venus and life possibilities, see Lomb (2012).

4.2. THE EXTREMOPHILES AS ANALOGUES FOR EXTRATERRESTRIAL BODIES: MARS AND EUROPA

Moons of the outer solar system carry subsurface oceans, and some of them have suggested being habitats of microbial life (Seckbach and Chela-Flores, 2012). However, is there any chance for finding life on the surface? In the past decades, some effort has been invested in locating terrestrial life forms on other planets. The recent book *Astrobiology* (Chela-Flores, 2011) discusses the new interdisciplinary field's concern with all of these extremophiles, as they may be models or analogues for survivors in similar extraterrestrial environments in space. A substantial amount of data about extraterrestrial bodies, mainly from the solar system, has come from a variety of sources (telescopes, surface rovers, flyby space crafts, and other means).

In the solar system and beyond, there might be habitable niches for organisms to exist in *life as we know it*. Until now, neither organic matter nor life has been detected on the Martian surface. It is extremely saline, has cold temperatures, and is desiccated and exposed to radiation. Mars possibly contains liquid water in spite of its harsh surface temperature. Speculation points out that the discovery of complex life in the Earth subsurface and deep aqueous environments (with organisms such as Pompeii worms, tardigrades, or mephisto nematodes) could have implications for the search for life on Mars or other places in our solar system. After all, it is agreed that bacterial spores are strong enough to withstand the journey to Mars (see McKay et al., 2012). As mentioned above, this planet was a warm-water world, good chances that in the past life could have thrived on the Red Planet. Spores from ancient microorganisms may be waiting dormant under the surface of this planet until a favorable change in their environment. We could compare it to *Bacillaceae* that were found sealed on Earth after 250 millions of years of dormancy and then revived (Vreeland et al., 2000).

4.3. BLOOD FALLS, ANTARCTICA AS A MODEL FOR EXTRATERRESTRIAL LIFE

An ancient ecosystem discovered beneath Antarctic glaciers at “Blood Falls” may also show how alien organisms might live in icy worlds. Blood Falls bacteria have thrived for millions of years beneath a rusty Antarctic glacier. The blood red color comes from an underground saltwater lake trapped by the encroaching glacier at least 1.5 million years ago. The water temperature is $-5\text{ }^{\circ}\text{C}$ (salt prevents it from freezing). The subarea is rich in iron salts—hence the source of the red hue (rust glacier). This is an ecosystem of bacteria trapped in a condition that could hardly be more inhospitable to life. The bacteria exist there without light penetrating the thick ice of the glacier to the lake lying 400 m beneath it. The microbes have lived there for millions of years, and the conclusion is that similar conditions may exist on planets and satellites where microorganisms are still waiting to be woken up.

4.4. CONCLUSION FOR POSSIBILITY OF EXTRATERRESTRIAL LIFE

Considering that a number of the polyextremophiles are adapted to multi-stress conditions, one could propose to perform “terraforming” of some celestial bodies such as Mars and others. This idea is not new, having been suggested in the literature (Sagan, 1961, 1973; Morowitz and Sagan, 1967; McKay, 1982), but at present we have more information about our neighboring planets and satellites. The cyanobacterium *Chroococcidiopsis* was pointed out for “Greening the Red Planet” (Friedmann and Ocampo-Friedmann, 1995). Also, *Cyanidium caldarium* members (Seckbach, 1994) could be good candidates for such Venusian clouds’ terraforming. We have to remember that such extravagant planetary engineering of terraforming is far from the kind of experiment that we can control at the planetary scale.

If micro-life could stay dormant in our local planet for millions of years and then “wake up,” why could not the same phenomenon take place in other extraterrestrial bodies? There is hope that in the future, we will find biosignatures of life further away from the solar system in habitable extraterrestrial locations.

5. General Summary and Conclusions

The term extremophiles refers to hardy organisms mainly prokaryotes and to a lesser extent eukaryotes, aerobic and anaerobic, microbes, and lower animals. While polyextremophiles are organisms that live under multiple forms of stress; some of their characteristics were discussed above. These microorganisms and some higher forms tolerate, live, or thrive in severe environments, such as in extreme ranges of temperatures, pH, hypersaline solutions, high pressure, oxygen scarcity, and a variety of radiations. They exist in dryness and desiccation for a long period and can be revived after millions of years in a dormant stage. The reader could find relevant information on extremophiles in Rothschild (2007), Stojanovi et al. (2008), and in the Springer series of *Cellular Origin, Life in Extreme Habitats and Astrobiology* (editor by Seckbach J., volumes 1–25 and further).

Due to their ability to live and survive in such very harsh and enigmatic conditions, these organisms were indicated as models for extraterrestrial life. Conditions on Mars, Europa, and Enceladus (and in the future perhaps Titan) may fit several of these extremophiles. The discovery of life on extraterrestrial bodies such as Mars, Europa, or elsewhere in the outer solar system would have a colossal effect and impact on science and society. The question is: Are we too hopeful in our hunt for extraterrestrial life, within the solar system, exoplanets, super Earth, and Goldilocks zones? The probability of life elsewhere in the universe is still a moot point—assuming life *does* exist somehow, somewhere besides Earth, would it really be all that alien? On the other hand, are they reachable for us? Again, if microorganisms may live in such severe environments on Earth, why could they not exist in similar niches on Mars or on other celestial places?

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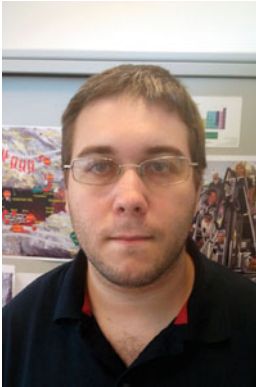
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THE DYNAMIC GENOMES OF ACIDOPHILES

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1. Introduction

Most acidophilic Bacteria live in extremely hostile environments where, in addition to acidity ($\text{pH} < 4.0$), they often must contend with high salinity, high metal content, or elevated temperatures. For example, the acidophilic microorganisms that populate the Tinto river in Southern Spain thrive not only in pHs as low as 1.8 but also at concentrations of sulfate of up to 128 g/L and high content in heavy metals (Parro et al., 2007), and those that live in the underground acid mines of Iron Mountain (California) can live at pH near 0, concentrations of Fe of up to 24 g/L, and high levels of zinc and arsenic (Ram et al., 2005). The dominant microorganisms in acid mine drainage environments belong to the genera *Leptospirillum*, *Acidithiobacillus*, and *Ferroplasma*.

On the other hand, the acidic environments generated by geothermal activity (also known as “mud pots”) are the preferred habitats of thermoacidophilic Archaea that could probably hold the record for polyextremophily, as *Picrophilus torridus* can live at pH 0 (1.2 M sulfuric acid) and temperatures of up to 65 °C (Fütterer et al., 2004) and *Acidianus infernus* tolerates pH 1 and temperatures of 95 °C. These environments are dominated by the Euryarchaeota class Thermoplasmata (*Picrophilus*, *Thermoplasma*, and *Ferroplasma*) and the Crenarchaeota order Sulfolobales (*Acidianus*, *Metallosphaera*, and *Sulfolobus*).

Acidophiles have been the target of many studies about adaptation to extreme environments (Auernik et al., 2008) but also, especially in the last decade, of studies about genome evolution with the objective of answering basic questions of microbial population genetics. They have been prime models in which to test hypothesis related to the extent of variation in natural environments, bacterial speciation, recombination, and ecology (Cadillo-Quiroz et al., 2012; Deneff et al., 2010). The main reason for the interest in acidophiles is the fact that they thrive in discrete, well-defined, and homogeneous microbial communities with low species diversity. This fact has allowed the use of culture-independent analysis by genomics, proteomics (reviewed in Wilmes et al., 2009), or transcriptomics (Moreno-Paz et al., 2010; Parro et al., 2007).

In addition to their interest in microbial evolution, acidophiles are highly relevant in biomining (bioleaching and bio-oxidation) (Cardénas et al., 2010;

Rawlings and Johnson, 2007), in the study of geochemical cycles, and as a source of enzymes of biotechnological interest. For example, there is a strong interest in the production of biocatalysts that are stable at high temperatures and low pH as tools for biofuel production (Hess, 2008). However, the use of these organisms has been hampered by the difficulty in understanding their complex communities, for example, in bio-oxidation tanks, or to manipulate them genetically, as when enzymes of interest are the main objective.

In this chapter we examine the knowledge gained in recent years from the study of acidophilic communities, with special emphasis on the mechanisms that contribute to gene flow and genomic plasticity under extreme conditions. Some of the relevant questions are the following: Does life in extreme acidic environments impose constraints in genome composition or plasticity? Do the genomes of extremophiles reflect a high rate of genetic exchange (HGT, horizontal gene transfer) or recombination? Which are the preferred routes of DNA exchange? Which are the roles of phage in promoting and maintaining diversity? Which are the genes that are exchanged and that contribute to adaptation? The answers to these questions are of relevance in bacterial evolution and ecology, but they also seem critical to develop and manipulate bacterial communities of industrial or biotechnological interest, such as those used in biomining. At this moment, given our limited knowledge of the extent and dynamics of genetic variation in the environment and the degree of genetic exchange, it is impossible to predict the evolution of communities in bioleaching operations or to improve its efficiency and rate (Cardénas et al., 2010). On the other hand, although our ability to genetically engineer archaeal acidophilic microorganisms is growing, especially in the model organism *Sulfolobus* (Aucelli et al., 2006; Leigh et al., 2011; Maezato et al., 2011; Wagner et al., 2009), the genetic tools to manipulate bacterial acidophiles are very limited (Glenn et al., 1992; Wang et al., 2012).

2. Genomic Variation in Natural Populations

The fact that acidic environments are geographically isolated and have low species diversity has made them excellent models for the study of the evolutionary processes acting on natural microbial populations. The last decade has been fruitful in studies aimed at dissecting the roles of recombination and mutation, and of selection versus neutral forces, in shaping the genomes of acidophilic organisms. These studies have been possible thanks to large-scale sequencing of coexisting strains and their genome-wide comparison, and focused mainly in natural populations of *Leptospirillum* and *Ferroplasma* (Allen et al., 2007; Deneff et al., 2010; Ram et al., 2005), and *Sulfolobus* (Cadillo-Quiroz et al., 2012; Reno et al., 2009). All these studies have highlighted the importance of genetic recombination as the main driving force of genomic evolution in Bacteria over short periods of time.

The decade-long studies by the Banfield laboratory of the bacterial communities of extreme acidophiles in Iron Mountain (California) using metagenomics and

proteomics have allowed the follow-up of specific bacterial lineages and a detailed view of their evolution (Denef and Banfield, 2012; Denef et al., 2010). These studies have revealed recent events of large horizontal gene flow between lineages, followed by periodic selective sweeps. In the case of *Leptospirillum*, the main sources of variation include recombination, and phage and transposon-mediated genomic rearrangements (Simmons et al., 2008). A mutation rate of 1.4×10^{-9} ($\pm 0.2 \times 10^{-9}$) substitutions per nucleotide per generation was determined recently (Denef and Banfield, 2012). The genomes of *Leptospirillum* Group II contain the required genes for DNA uptake, DNA secretion, and homologous recombination (Lo et al., 2007). Interestingly, Lo et al., using a proteomics approach to identify protein variants, reported large-scale recombination events of segments (10–100 kb) which generate hybrid genotypes (Denef and Banfield, 2012). For *Leptospirillum*, positive selection for specific genes is a significant evolutionary force, rather than neutral processes (e.g., drift generated by periodic bottlenecks). Genes that are global regulators of gene expression were often carriers of mutations, suggesting their importance in the selection of specific successful genotypes. This observation highlights the fact that just a few mutations could explain the predominance of particular phenotypes over others and opens the possibility that positive selection of recombinant variants could occur over short time scales (Denef and Banfield, 2012).

On the other hand, metagenomic analysis of *Ferroplasma* type II strains obtained from AMD biofilms showed a mosaic genome that was likely the result of extensive recent recombination. Recombination occurred frequently, about every 5 kb, within *Ferroplasma* type II (nucleotide sequence 98 % similar between individuals of the same population) but was rare between *Ferroplasma* type I and II (average nucleotide similarity of 33 %) (Tyson et al., 2004). No conjugation genes were observed in the genome, suggesting that any horizontal DNA transfer was due to phage transduction. Most heterogeneity within the *Ferroplasma* type I population was due to recent movement of insertion sequences (Allen et al., 2007; Eppley et al., 2007).

Sulfolobus islandicus populations also reveal a high rate of recombination (Reno et al., 2009; Whitaker et al., 2005), but this does not seem to be a barrier for ecological specialization within populations (Cadillo-Quiroz et al., 2012). Differences between ecotypes depend on large genomic regions with distinct gene content and point to ongoing processes of sympatric speciation (Cadillo-Quiroz et al., 2012). An average rate of nucleotide substitution per site per year of 4.66×10^{-9} ($\pm 6.76 \times 10^{-10}$) was obtained based on comparison among *Sulfolobus islandicus* strains and correlation with historical geological records (Reno et al., 2009).

Finally, it should be noted that genes of acidophilic organisms are shared among them much more easily than with organisms from other environments, or from phylogenetically closer relatives. For example, the genome of *Picrophilus torridus* (Euryarchaea) shares many genes with *Sulfolobus solfataricus* (Crenarchaeota), which were likely acquired by HGT (Angelov and Liebl, 2006).

An analysis of the genes not shared among *Sulfolobus islandicus* strains and probably acquired recently by HGT showed most similarity to genes from other *Sulfolobus* species (Reno et al., 2009). This finding highlights the fact that acidic environments could be fairly closed and restrictive to foreign genetic input. Most likely phage could be the dominant force for gene flow and dispersal, combined with the pervasive presence of repetitive sequences such as transposons.

3. Virus and CRISPR Loci

It is now widely acknowledged that phage predation is a powerful force in microbial evolution. Phage could help preserve metabolic diversity in bacterial populations by favoring the coexistence of multiple, suboptimal, strains rather than a dominant generalist one (Rodríguez-Valera et al., 2009). In addition to exerting a selective force, phage could play two further roles in bacterial populations: They could serve as intra- and interspecies DNA exchange vehicles, and they could contribute to the generation of variability by integrating and promoting recombination in genomes. Phages seem to be the main vehicles for gene distribution in microbial populations as they can very quickly mobilize large DNA blocks with dozens of genes and promote new gene combinations and ecological specialization (Coleman et al., 2006). Indeed, a very significant study of long-term (2-year) diversity of *Sulfolobus* virus in a Yellowstone acidic hot spring showed that this extreme environment was constantly being repopulated by virus from external biomes, probably by air (Snyder et al., 2007). The rate of virus immigration and colonization, followed by extensive recombination, was significantly higher than the rate of mutation, resulting in a high local diversity. This result indicates that virus could likely be a source of gene reshuffling and novelty at a global scale (Snyder et al., 2007).

Several viruses that infect *Sulfolobus* species have been characterized extensively, structurally and mechanistically, and reviewed recently (Pina et al., 2011; Snyder and Young, 2011). Specific virus from AMD environments has been less investigated, although a metagenomic study of AMD biofilms showed the presence of prophages with broad host ranges contributing to DNA transfer between the genomes of *Ferroplasma* and G-plasma and between the genomes of *Leptospirillum* Groups II and III (Ram et al., 2005; Tyson et al., 2004). Phage transduction could alter gene composition and give rise to phenotypic differences in short time scales in coexisting populations of *Ferroplasma acidarmanus* (Allen et al., 2007). No known phage can infect *Acidithiobacillus ferrooxidans*, although numerous remnants of phage genes (integrases, recombinases) are present in its genome (Valdés et al., 2008) and a CRISPR locus has been identified within a genomic island in strain ATCC23270 (Valdés et al., 2010).

The CRISPR loci of Bacteria and Archaea consists of hypervariable arrays of short segments (spacers) of phage, plasmid, or transposon DNA flanked by constant palindromic repeats, and they provide resistance to further infections

from those elements (Barrangou et al., 2007; Wiedenheft et al., 2010). These regions are transcribed as CRISPR antisense RNA to target and degrade incoming phage or plasmids, thus providing a line of defense against infection. The interest on these elements is high because they can provide insights on the dynamics of phage and their hosts, the evolutionary history of bacterial genomes, and the structure of bacterial populations (Banfield and Young, 2009). In addition, the CRISPR systems of *Sulfolobus* have attracted interest in recent years as a model to study the basic mechanism of action of these loci and the roles of the various proteins involved in targeting invading RNA or DNA (Garrett et al., 2011; Lintner et al., 2011).

CRISPR loci have been studied quite extensively in acidic ecosystems. Banfield and collaborators described extremely high diversity in CRISPR loci from two metagenomic datasets of *Leptospirillum* Group II (Tyson and Banfield, 2008). They found that the phage segments are added to the CRISPR array in a unidirectional fashion that therefore reveals the infection history of the different strains. Further, since the sequences in CRISPR loci derive directly from phage, they have been used to analyze viral genomes, indicating extensive recombination among them (Andersson and Banfield, 2008). Phage genome recombination could probably help evade CRISPR-directed degradation in these populations. CRISPR loci do not only target phage but also other mobile DNA elements, such as insertion sequences and plasmids (Goltsman et al., 2009).

It has been assumed that phage predation would purge variation in the bacterial population by periodic infection and removal of susceptible clones (selective sweeps), and the heterogeneous pattern of spacers observed in the *Leptospirillum* CRISPR loci suggested this general scenario (Tyson and Banfield, 2008). However, a recent study of *Sulfolobus* CRISPRs seems to indicate an alternative picture in which many different genotypes coexist, and no events of population-wide selection are evident (Held et al., 2010). To explain the patterns of the *Sulfolobus* population, the authors suggest clonal interference between virus-resistant strains (Held et al., 2010). As the critical importance of the large virus populations as engines of gene dispersal and storage becomes more evident, it seems that the study of virus populations in extreme environments will play a relevant source of insights into genome evolution. The recently characterized phage-like particles (Lang et al., 2012) or the uncertain ecological role of secreted phages such as STSV1 of *Sulfolobus tengchongensis* (Xiang et al., 2005) suggests that the gene-transfer potential of phage has only been tapped.

4. Genomic Islands and Plasmids

Genomic islands (GIs) are segments of the chromosome of up to 200 kb that have been incorporated in the genome by HGT in the recent evolutionary past (Juhas et al., 2009). For this reason they often have a nucleotide composition (G+C content or codon usage) that makes them different from the rest of the

genome and detectable in silico, although they often have also been identified by comparative genomics of closely related species or strains. GIs often contain genes that provide an advantage to the host, such as metabolic operons, DNA repair genes, or pathogenicity determinants. Some GIs could be considered very large transposons or integrative and conjugative elements (ICEs) that can mediate their own movement, but others contain insertion sequences and transposase genes that allow them to easily recombine between replicons. Most GIs contain at least an integrase which could be used to analyze the phylogenetic origins and evolutionary history of the element (Boyd et al., 2009). The analysis of GIs in many genomes has allowed the identification genes that form part of the mobilome of the species and that contribute to the formation of ecotypes.

Acidophiles live in geographically isolated ecosystems with limited gene flow among them. This effect has been studied by comparative genomic hybridization for *Sulfolobus solfataricus*, where large variations in gene content can be observed between geothermal sites across the world (Grogan et al., 2008), and also by direct complete genome sequencing of isolated strains of *S. islandicus* (Reno et al., 2009). On the other hand, coexisting populations of *S. islandicus* can also present distinct GIs containing distinct metabolic genes (toluene monooxygenase and nitrate reductase), suggesting that they could be the source of incipient ecological differentiation (Cadillo-Quiroz et al., 2012).

Ecological differentiation is often determined by GIs, as it has been observed recently with the sequencing of the genome of *Thiomonas*, a moderate acidophile that can grow at pH 3 and with high tolerance to heavy metals (Arsene-Ploetze et al., 2010). Comparative genomics with other, non-extremophilic *Thiomonas* species showed that adaptation to metal tolerance and acidity was obtained via incorporation of genes (arsenic-specific operons *ars2* and *aox*, heavy metal resistance, biofilm formation, and motility) within GIs (Arsene-Ploetze et al., 2010). An unusual high divergence in gene content was also observed in strains from the same AMD site. Interestingly, GIs of *Thiomonas* contained a high number of IS elements, suggesting that they contribute to the variability and plasticity of these regions.

The studies of *Leptospirillum* by Banfield and collaborators on AMD ecosystems seem to reveal a somewhat different picture regarding the effects of variable gene regions of genomes in overall fitness. Two dominant and recombining strains of *Leptospirillum* Group II showed a very high similarity at the rRNA level (0.3 % difference) but a notable divergence in gene content (20 % of the genome was unique for each type) (Lo et al., 2007; Simmons et al., 2008). However, proteomic studies showed that most proteins encoded in these variable regions were not expressed at high levels or at all (Mueller et al., 2010), suggesting that they could have a limited impact on fitness. Genes related to energy metabolism (NADH dehydrogenase, NADPH-quinone reductases, and cytochromes) and sulfate assimilation, among others, have been found in populations in *Leptospirillum* Groups II and III in putative GIs associated with phage genes (Goltsman et al., 2009). The genome of *Leptospirillum ferriphilum* ML-04,

recently sequenced, contains two GIs which contain various genes involved in DNA repair and recombination (Mi et al., 2011).

Comparative genomics of *Ferroplasma* also indicated sections of the genome with anomalous G+C content where up to 79 % of the non-shared genes were located. These GIs were probably the direct result of phage integration, as they often were associated with, for example, phage-like integrases, DNA primase, and methylase genes (Allen et al., 2007).

A putative genomic island in *Acidithiobacillus ferrooxidans* contains 69 genes that are absent in *A. thiooxidans* or *A. caldus*; 13 of them are directly related to iron and molybdate uptake and metabolism (FepA2-FecA4 TonB-dependent Fe III transport system) (Osorio et al., 2008). On the other hand, comparative genome analysis of two strains of *Acidithiobacillus ferrooxidans* has shown distinct GIs in each strain, probably recently acquired via HGT. The genomic compositions are different, but in both cases it is obvious the association of metabolic and metal resistance genes with mobilome genes (transposases, virus) (Valdés et al., 2010).

In summary, the discovery of GIs from acidophiles reveals a wealth of genes that could provide local metabolic advantages in these environments and also highlight the fact that ecological specialization or increased local fitness could be determined by a small set of genes acquired by lateral transfer.

Various plasmids from *Leptospirillum*, *Acidithiobacillus*, *Acidiphilium*, and *Sulfolobus* have been described (listed by Cardénas et al., 2010). Plasmids isolated from *Acidithiobacillus caldus* contained arsenic-resistant genes (van Zyl et al., 2008) and numerous transposons. *A. ferrooxidans*, however, did not contain any plasmids, although many plasmid maintenance and stability genes were present in its genome (Valdés et al., 2008). A large plasmid from *Leptospirillum* was described recently that contained a CRISPR locus, suggesting possible competition among mobile elements (Goltzman et al., 2009).

5. Insertion Sequences and Transposase Activity

Insertion sequences (ISs) are the most common and simple mobile genetic elements. They determine their own mobility because they encode an enzyme, the transposase, which binds to specific sequences at the ends of the element and catalyzes the transposition reaction (Siguier et al., 2006a). If ISs are in multiple copies in a replicon, they can promote recombination between the copies and in so doing generate deletions, duplications, and other genomic rearrangements. Most importantly, they can also promote the recombination of DNA between replicons, thus contributing to the horizontal transfer of genes within of between species, and generate the required plasticity in populations required for fast adaptation (Toussaint and Chandler, 2012). The relative abundance of ISs could therefore be a good indicator of recent ecological transitions (Moran and Plague, 2004). Transposase genes are the most abundant genes in nature (Aziz et al., 2010).

Table 1. Number of ISs in fully sequenced thermoacidophilic archaeal organisms.

Genomes	IS1	IS110	IS200	IS21	IS256	IS3	IS30	IS5 (IS903)	IS607	IS630	ISH3	ISL3	ISC1217	ISA1214	Total
<i>Metallosphaera sedula</i> DSM 5348	0	0	1	0	0	0	0	0	1	0	0	0	0	0	2
<i>Sulfolobus acidocaldarius</i> DSM 639	0	0	0	0	0	0	1	0	1	0	0	0	0	0	2
<i>Sulfolobus islandicus</i> L.D.8.5	0	19	6	0	6	0	0	0	7	3	16	0	4	0	61
<i>Sulfolobus islandicus</i> L.S.2.15	0	13	7	0	0	0	0	0	5	1	13	0	1	0	40
<i>Sulfolobus islandicus</i> M.14.25	0	9	2	0	1	0	0	0	1	3	5	0	10	0	31
<i>Sulfolobus islandicus</i> M.16.27	0	4	2	0	1	0	0	0	0	2	9	0	14	0	32
<i>Sulfolobus islandicus</i> M.16.4	0	3	4	0	4	0	0	0	1	2	2	0	7	0	23
<i>Sulfolobus islandicus</i> Y.G.57.14	13	24	0	0	14	0	0	0	13	3	30	0	3	0	100
<i>Sulfolobus islandicus</i> Y.N.15.51	11	30	0	0	9	0	0	0	16	1	19	0	1	0	87
<i>Sulfolobus solfataricus</i> P2	7	35	2	0	4	0	0	1	14	24	18	0	23	0	128
<i>Sulfolobus tokodaii</i> str. 7	5	7	7	0	0	0	0	0	4	0	1	0	9	0	33
<i>Picrophilus torridus</i> DSM 9790	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Thermoplasma acidophilum</i> DSM 1728	0	0	1	0	1	0	0	0	1	0	0	0	0	0	3
<i>Thermoplasma volcanium</i> GSS1	0	0	3	0	2	0	0	1	0	1	0	3	0	2	12

ISs are generally abundant in acidophiles, although comparative studies with related genomes of non-extremophiles would be required. Since the activity of ISs can be the basis for rapid adaptive change, transposase expression could be an indicator of stress. This could be the case of a recent study that has been able to show a relatively fast change in the expression of ISs in acidophilic bacterial populations as a function of environmental conditions. High abundance of transposases was detected by proteomics in “young” biofilms composed mostly of *Leptospirillum* Group II Bacteria, as compared to matured biofilms with higher diversity (Mueller et al., 2010). In the low-diversity biofilms, along with increased transposase activity, there was higher proportion of physical and chemical stress defense proteins (Mueller et al., 2010), which could indicate that transposases and the stress response share regulatory mechanisms, as has been observed (Twiss et al., 2005). Strong transposase activity could therefore be a way by which the genome gains the plasticity needed to explore and colonize new environments and could be quickly lost in mature populations.

In addition, transposase expression has been detected in proteomic studies of AMD biofilms dominated by *Leptospirillum* Group II (Mueller et al., 2010; Ram et al., 2005) and also by microarray hybridization of *Leptospirillum ferrooxidans* genes (Parro et al., 2007), especially of the IS200/IS605 family.

The estimated ISs present in acidophiles for which there are fully sequenced genomes are shown in Tables 1 (Archaea) and 2 (Bacteria), classified according to the ISfinder database (Siguier et al., 2006b). *Sulfolobus solfataricus* P2 is notable

for the abundance of ISs in its genome (>200 elements of 25 different types if including fragmented and degenerated sequences) and high genomic plasticity (Martusewitsch et al., 2000; Redder and Garrett, 2006), but *S. tokodaii* only has 34 ISs and *S. acidocaldarius* only has two (Brügger et al., 2004). Interestingly, a comparison of ISs present in the *S. islandicus* chromosomes seems to indicate that, despite differences in the relative frequency of the different families, the total number correlates with the location of isolation of the strain. Thus, *S. islandicus* from Yosemite National Park (strains Y.G.57.14 and Y.N.15.51) contains the highest number of ISs, the three strains from the Kamchatka Peninsula have the lowest number (strains M.14.25, M.16.27 and M.16.4), and those from Lassen National Park (L.D.8.5 and L.S.2.15) have intermediate numbers. Possibly the immediate evolutionary past of the strains determines the total number of insertion sequences in the genome, and these elements are lost and gained quickly in evolutionary terms. Detailed sequence analysis of the IS populations in various strains of *Sulfolobus* will be required to determine the dynamics of change of these elements.

Interestingly, no ISs can be found in the genome of *Picrophilus torridus*, the smallest of any free-living aerobic organisms (1.55 Mb), despite the fact that its genome shows extensive sharing of genes with *Sulfolobus solfataricus* and *Thermoplasma acidophilum* (Fütterer et al., 2004). The small size of its genome and its extreme gene density could explain its lack of ISs (Touchon and Rocha, 2007).

The available genomic sequence of three species of *Acidithiobacillus* shows a high diversity of ISs, and only five families are shared among them (IS110, IS21, IS3, IS630, and IS66). Interestingly, a recent fast proliferation seems to have occurred for the families IS5 and ISL3 in the chromosome of *A. caldus* (27 and 39 copies, respectively) and for the ISAs1 family in *A. ferrooxidans* ATCC53993 (24 copies). In this latter case, the 24 copies of ISAs1 are identical, reflecting a very recent expansion of this mobile element in this chromosome.

Similar differences in IS composition and proliferation of specific families have been observed for *Leptospirillum* species. For example, the IS profiles found in two closely related strains (“5-way” and “UBA”) of *Leptospirillum* Gr. II from Iron Mountain (California) are quite distinct: Strain “5-way” has three copies of IS1182, while strain “UBA” contains 20 copies. In *L. ferrooxidans* isolated from the Tinto river (Spain), a large proliferation of IS200/IS605 was observed, 95 copies, out of a total of 185 ISs in the chromosome (our unpublished observations). On the other hand, only 36 transposases have been detected in the genome of *L. ferriphilum* ML-04 (Mi et al., 2011). These data seem to suggest highly dynamic IS populations and corresponding high plasticity in the chromosomes that harbor them.

The potential for fast adaptation in acidophiles due to proliferation of IS has also been observed in the laboratory in relatively short time spans. For example,

changes in the ISs present in the genomes of various *A. ferrooxidans* strains were observed when these strains were forced to adapt to growth on elemental sulfur, suggesting that IS can be critical during rapid adaptation to shifting cultivation conditions (Kondrat'eva et al., 2005). Also, Tyson observed a larger number of ISs in cultivated *Ferroplasma acidarmanus* than in genomic DNA of the same species isolated directly from the environment, suggesting that the transposases proliferated during cultivation (Allen et al., 2007).

Finally, ISs can also be vehicles of transmission of specific phenotypic traits. An especially interesting case is that of a strain *Acidithiobacillus caldus* obtained in a plant dedicated to arsenopyrite bio-oxidation. This strain contains an IS21-derived transposon in which an entire operon consisting of nine genes conferring resistance to high levels of arsenic is inserted within the IS (Tuffin et al., 2005) in the chromosome. Sequence analysis revealed that this arsenic-resistant strain of *A. caldus* most likely acquired the transposon from a heterotrophic neutrophilic species via horizontal gene transfer. Indeed, the same transposon was also found in a strain of *Leptospirillum ferriphilum* isolated from the same bio-oxidation tank (Tuffin et al., 2006), and the presence of arsenic-resistant operons flanked by ISs has also been reported in large plasmids *A. caldus* (van Zyl et al., 2008) and in *Leptospirillum ferriphilum* isolated from acidic waters in a hot spring from China (Mi et al., 2011).

6. Conclusions

The study of acidophilic organisms has provided in recent years a wealth of information on microbial population genetics and evolutionary dynamics. The fact that acidophilic communities are isolated and dominated by a few species has often helped to collect and analyze the data. These studies have highlighted the high diversity found in natural environments and the major role that mobile elements play in shaping bacterial communities, which seem to be in constant motion via the exchange and recombination of genetic material. Phage seems to play a major role in gene flow within populations and across populations (Snyder et al., 2007), and CRISPR loci are the most variable regions in the chromosome, reflecting extremely fast dynamics between virus and hosts (Wilmes et al., 2009). There is also high variation in the class and abundance of insertion sequences within acidophiles, as evidenced by the sequencing of strains or closely related species. ISs are critical in providing dynamism to the chromosome, but their life cycles in genomes are unknown. It is possible that they infect chromosomes and expand until the host losses fitness and is extinguished (Wagner, 2009), but a stable equilibrium is also a possibility. ISs are likely responsible in part for the diversity in gene content in large sections of the “variable genome” which shape and reassemble the metabolic capacity of the population in response to environmental changes.

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PART II: HALOPHILES

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Banciu
Sorokin
Oremland
Minegishi**

**Stan-Lotter
Fendrihan
Webb
DiRuggiero**

Biodata of **Aharon Oren**, author of “*Two Centuries of Microbiological Research in the Wadi Natrun, Egypt: A Model System for the Study of the Ecology, Physiology, and Taxonomy of Haloalkaliphilic Microorganisms.*”

Aharon Oren obtained his M.Sc. from the University of Groningen and his Ph.D. from the Hebrew University of Jerusalem (1978). After a postdoctoral period at the University of Illinois at Urbana-Champaign, he joined the faculty of the Hebrew University of Jerusalem and was appointed full professor in 1996. He is secretary/treasurer and past chairman of the International Committee on Systematics of Prokaryotes, secretary and member of three of its taxonomic subcommittees, editor for *FEMS Microbiology Letters*, *Extremophiles*, and *International Journal of Systematic and Evolutionary Microbiology*, and president of the International Society for Salt Lake Research. Prof. Oren was elected Fellow of the American Academy of Microbiology in 2000 and received an honorary doctorate from the University of Osnabrück, Germany, in 2010.

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TWO CENTURIES OF MICROBIOLOGICAL RESEARCH IN THE WADI NATRUN, EGYPT: A MODEL SYSTEM FOR THE STUDY OF THE ECOLOGY, PHYSIOLOGY, AND TAXONOMY OF HALOALKALIPHILIC MICROORGANISMS

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1. Introduction

The alkaline hypersaline lakes of the Wadi Natrun, Egypt, recently became renowned for the discovery of a novel group of polyextremophilic bacteria: the genera *Natranaerobius* and *Natronovirga*, classified in a new order, the *Natranaerobiales*. These obligatory anaerobic fermentative prokaryotes combine the ability to live at near-saturated salt concentrations, very high pH values (up to 10.8), and high temperatures (optimum up to 66 °C). This intriguing group of extremophiles was discovered thanks to the work of Prof. Juergen Wiegel of the University of Georgia, Athens, GA, USA, and his Egyptian student Noha M. Mesbah (Mesbah and Wiegel, 2005, 2008, 2009; Mesbah et al., 2007a, b; Mesbah and Wiegel 2011; Wiegel, 2011).

This chapter is based on a lecture prepared in 2011 at the occasion of the retirement of Prof. Wiegel (Oren, 2011a). Realizing on one hand how many important microbiological discoveries in the past were based on the study of the Wadi Natrun and its microorganisms, but on the other hand how little most microbiologists are aware of this, I collected the early biological studies of these lakes to make these better known in the hope that younger scientists will continue the study of this exciting habitat.

The Wadi Natrun is located about 100 km northwest of Cairo and about 100 km southwest of Alexandria. The bottom of the Wadi Natrun depression is 24 m below sea level (Jannasch, 1957). It is assumed that it is part of the ancient Nile River bed (“Bahr-bela-ma,” translated as “the river without water” (Andéossy, 1800), or “Bahr-el-farigh,” translated as “the empty river”). The

The author dedicates this chapter to his friend and colleague Prof. Juergen K. W. Wiegel at the occasion of his retirement as a faculty member at the University of Georgia, Athens.

valley is about 140 km long and about 20 km wide, and contains up to 16 shallow lakes (Sickenberger, 1892). Based on Google Earth (see also Fig. 5), the number comes to 6–8, depending on the time of the year and not counting the temporary very small lakes. During the summer most lakes are not much deeper than half a meter. These lakes are fed by underground seepages from the Nile. The water height of the lakes correlates inversely with the water level of the Nile. It is assumed that the difference is due to the time the water needs to travel from the Nile delta to the lakes (Sickenberger, 1892). The distance between the lakes and the Rosetta branch of the Nile is between 70 and 125 km. Most of the springs and water input into the lakes are on the slightly higher elevated north-eastern side of the lakes. The highest level is observed around mid-January. The lakes become hypersaline by evaporative concentration, which is according to Stocker (1927) (as cited by Jannasch, 1957) about 40 million m³/year. A very interesting feature, already mentioned in the early descriptions, is that the lakes differ strongly in appearance and in their mineral content. They differ in total salt concentration from 92 g/L to over 350 g/L, and the smaller ones dry up in summer. The waters contain sodium, chloride, sulfate, and carbonate/bicarbonate as the main ions, and the dissolved salts in the different lakes can be described as mixtures of NaCl (36–78 % by weight), Na₂SO₄ (9–27 %), and Na₂CO₃ (3–37 %). The pH of the brines is around 11 (Javor, 1989; Oren, 2002, 2006; Taher, 1999). Jannasch (1957) observed temperatures around 40 °C during the day, down to 28 °C in the night. Wiegel (2007, personal communication) reported to have found temperatures in the brine up to 60 °C during a late afternoon in the summer of 2005.

Soda (Na₂CO₃·10H₂O) and related evaporites such as trona (Na₂CO₃·NaHCO₃·2H₂O) have been collected from the Wadi Natrun throughout the ages. The Greek geographer Strabo (64/63 BCE–ca. 24 CE) mentions that soda (“nitre,” νιτρον) was gained near Naucratis, located about 40 km NE of the Wadi Natrun (Strabo, translation 1967). Pliny (23–79 CE), in Book 31 of his *Natural History*, even mentions a red color sometimes associated with the soda (Pliny, translation 1963):

nitrariae Aegypti circa Naucrati et Memphim tantum solebant esse, circa Memphim deteriores. ... sunt ibi nitrariae in quibus et rufum exit a colore terrae.

The soda beds of Egypt used to be confined to the regions around Naucratis and Memphis, the beds around Memphis being inferior. ... In this region are soda-beds from which red soda also is taken owing to the color of the earth.

(Translation by W. H. S. Jones)

Different shades of red are prominent in the brines of the Wadi Natrun lakes. Figure 1 shows what may be the first color photograph of one of these lakes, as published in 1957 by Holger Jannasch. It is of course tempting to associate these red colors with the story of Exodus 7: 19–25, where the waters of Egypt turned into blood. Sections 3 and 4 below provide more information about the nature of these colors.



Figure 1. Salt lake in the Wadi Natrun, about 1 km north of Bîr Hooker, colored red by microbial communities: haloalkaliphilic Archaea and/or anoxygenic phototrophic sulfur bacteria (From Janasch, 1957; reproduced by permission from Schweizerbart'sche Verlagsbuchhandlung, Stuttgart).

2. Studies of the Microbiology of the Wadi Natrun Lakes in the Nineteenth and Early Twentieth Centuries

The best known early studies of the microbiology of the Wadi Natrun lakes date from the 1970s, when different types of haloalkaliphilic microorganisms – aerobic Archaea and anaerobic anoxygenic phototrophic bacteria – were isolated from the site and characterized. However, it is only seldom realized that there are detailed records of biological phenomena in the brines from the nineteenth century, and the first report even dates from the end of the eighteenth century. Figure 2 provides a schematic overview of the studies performed from in the past two centuries.

The red coloration of the brines by “a vegetal-animal substance” was mentioned in 1799 by General Antoine Andréossy of Napoleon Bonaparte’s army (1761–1828) (Fig. 3). The material studied was collected by General Andréossy during a survey performed on 4, 5, 6, and 7 of the month Pluviôse in the seventh year of the Revolution (or, according to the conventional calendar, January 23–28, 1799). Andréossy’s report (1800, 1801) also includes a map of the area (Fig. 4, upper panel). In his “Essay about the valley of the Natron lakes and the valley of the river without water,” Andréossy wrote that “The water of part of

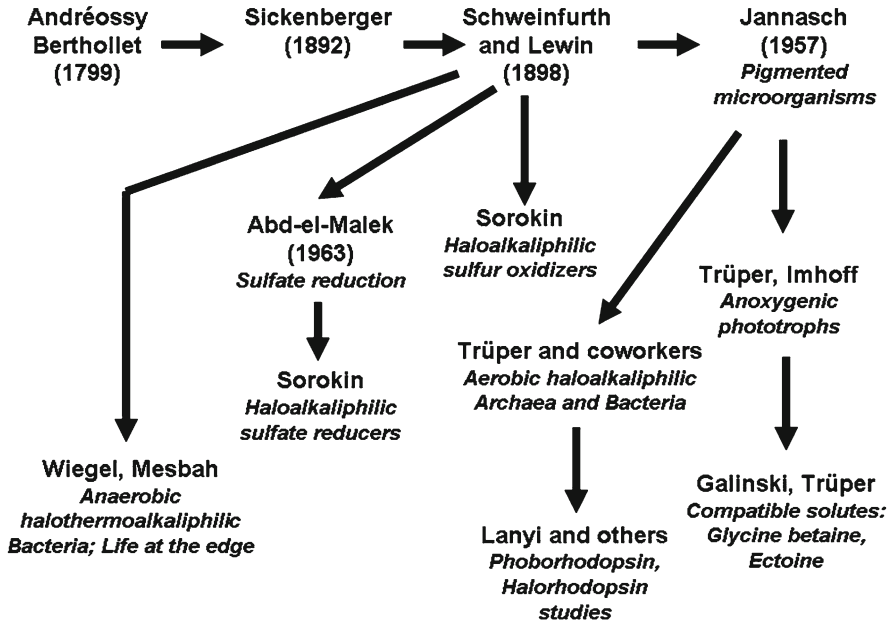


Figure 2. Outline of the history of microbiological research on the Wadi Natrun lakes and the organisms isolated from them.



Figure 3. Pioneers of early research on the Wadi Natrun lakes: General Antoine Andréossy, Claude Berthollet, Georg Schweinfurth, and Holger Jannasch.

lake no. 3 and the water of lake no. 4 is colored red by a vegetal-animal substance. When one lets the water evaporate, the crystallizing salt obtains a deep-red color, and obtains an agreeable smell of roses” (translations from German of this and of the following quotations in this section by the author). It is interesting to note that a similar smell was reported for the red (but neutral-pH) saltern brines from which Teodoresco (1905) isolated and described *Dunaliella salina*, a unicellular green alga also colored red by carotenoid pigments: “Cette eau, très concentrée, avait une couleur rougeâtre et répandait une odeur prononcée de violette.”

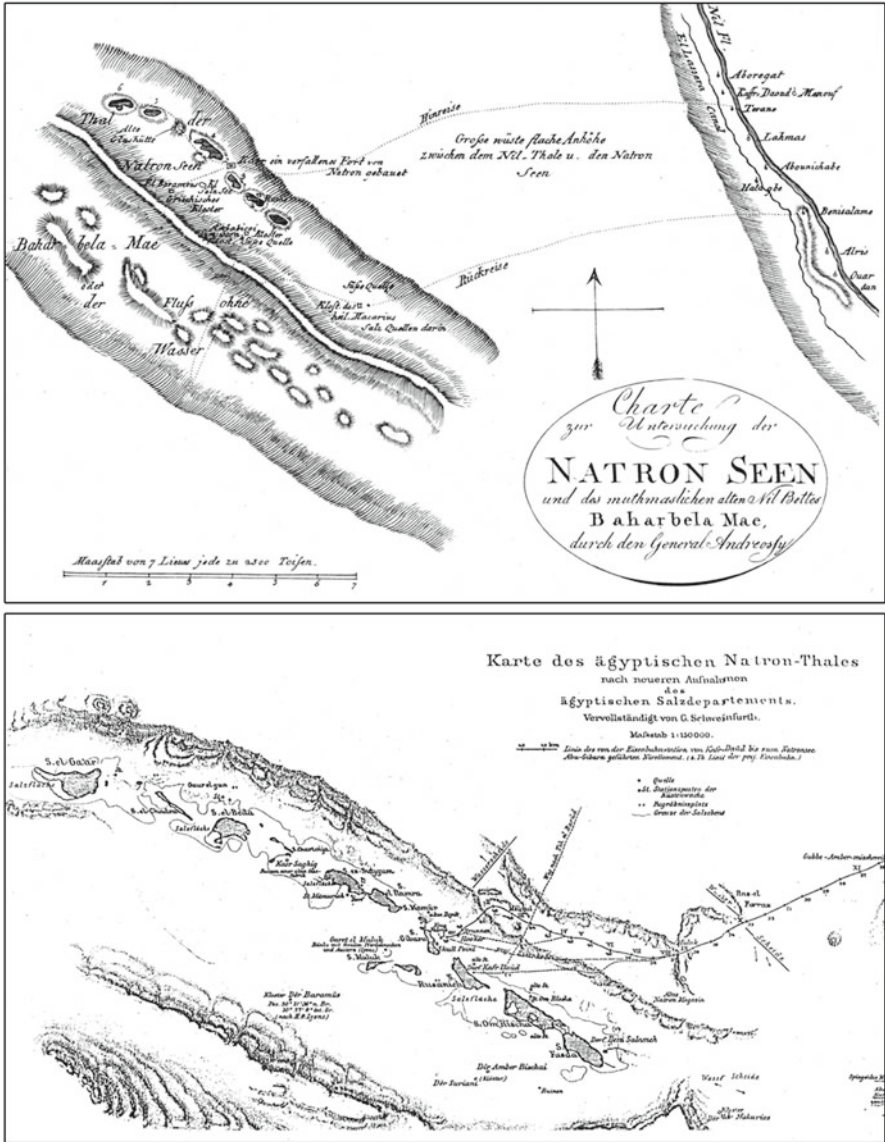


Figure 4. Maps of the Wadi Natrun and its lakes, as given by Andréossy (German translation 1801) and by Schweinfurth and Lewin (1898).

[“This very concentrated water had a reddish color and spread a pronounced smell of violets.”]

The report by General Andréossy contains an appendix by the chemist Count Claude Berthollet (1748–1822) who accompanied the expedition to the

Wadi Natrun lakes. Here, it is stated that “The water of part of lake no. 3 and the water of lake no. 4 looks red; the sodium chloride that crystallizes here is also red. This color is caused by a substance that is not of mineral nature, but that releases ammonia vapors upon combustion. This substance becomes black when deposited on the sodium carbonate.”

The author of this chapter found no records of renewed visits to the Wadi Natrun area by expert scientists until two short reports were published by the geologist Prof. Ernest Sickenberger from Cairo under the title “Letters from Egypt” (Sickenberger, 1892). His report of an excursion to the Wadi Natrun to examine the problems relating to the soda production from the lakes contains the following information:

As nowadays only one of the lakes is exploited for natron production, el hamrah, the red one, so called because of the color of its water, we pitched our tents in its neighborhood on pure sand that, like everything here, had a strongly alkaline reaction and taste. ... Under all conditions, but especially in the red, brown and black mud, a *Micrococcus* is found in large numbers. This organism appears to play the main role in the development of carbon dioxide, which is particularly strong in the red, brown and black mud; there the decay of the other alga is so far advanced that it can no longer grow. ... Direct experiments should determine the exact contribution of the microscopic alga and the *Micrococcus*. Unfortunately it was not possible to perform exact analyses here, and I only could take samples of the collected material with me to further investigate these after my return in October.

To the knowledge of the author, there are no records that such experiments were indeed performed.

The first dedicated study of microbiological phenomena in the Wadi Natrun lakes was published by Schweinfurth and Lewin in 1898. Georg August Schweinfurth (1836–1925; Fig. 3) was a well-known botanist and ethnologist, born in Riga, Latvia, and educated in Heidelberg, Munich, and Berlin, who spent much time exploring different parts of the African continent. In their essay on “Beiträge zur Topographie und Geochemie des ägyptischen Natron-Thals” (Contributions on the topography and geochemistry of the Natron Valley in Egypt), Schweinfurth and Lewin presented state-of-the-art information on biogeochemical processes in the lakes, including sulfate reduction (see also Fig. 6), other anaerobic degradation processes, oxygenic and anoxygenic photosynthesis, and the nature of the pigments of the microbial communities.

After a brief review of the earlier reports on the lakes, including those by Andréossy and Sickenberger, the authors presented a wealth of interesting observations, of which a few selections are given here, followed by comments by the author of this chapter:

The water that emerges from the springs develops hydrogen sulfide after a short distance. The green algae disappear, and by degradation of the green algae first a muddy red mass appears and then a black material. The latter is black iron sulfide. With the increase of the latter, the alkaline reaction of the water increases. The red and the black mud develop carbon dioxide because of the great masses of a *Micrococcus*.



Figure 5. Google Earth view of the Wadi Natrun lakes, showing lakes in different shades of red and green.

The sulfide, which also causes the black color of iron sulfide, is obviously derived from bacterial sulfate reduction. This process increases alkalinity, as shown much later in the Wadi Natrun by Abd-el-Malek and Rizk (1963). The studies on the connection between bacterial sulfate reduction and alkalinity by Yousef Abd-el-Malek in the 1960s remain relevant today. The anoxygenic phototrophic sulfur bacterium *Halorhodospira abdelmalekii* (basonym: *Ectothiorhodospira abdelmalekii*; see Sect. 3) was named to honor Abd-el-Malek's contributions. The muddy red mass may have derived its color from such carotenoid-rich bacteriochlorophyll *a*-containing phototrophs that oxidize sulfide to sulfate with elemental sulfur as intermediate product. Alternatively or in addition, the red material may consist of aerobic haloalkaliphilic Archaea. The nature of the CO₂-forming "Micrococcus" described here is less clear. It may be the sulfate-reducing bacteria that mineralize organic material. The nature of the "green algae" mentioned remains to be ascertained. Imhoff et al. (1979) report the occurrence of *Spirulina* in Lake Gabara whose waters had a green color, and mats of filamentous and unicellular cyanobacteria were often found on the wet mud surface near the lakes. One of the Wadi Natrun lakes is even called Khadra (= green). The Google Earth view of the Wadi Natrun shows some of lakes in red-purple and others in different shades of green (Fig. 5).

A fresh piece of the red salt from a soda lake – also near Alexandria in the bay of the saltern such red salt is found – showed according to our investigations the following properties: brighter and darker red, in part deep burgundy-colored areas alternated. The red, in water and alcohol soluble pigment disappeared upon heating of the salt.

Neither treatment with zinc and sulfuric acid, nor with strong bases, changed its intensity significantly. The microscopic examination showed dark bodies that aggregated in a characteristic way. It was impossible to establish their nature, but they may be fungal spores.

The dark bodies are probably bacteria rather than fungal spores, as fungi are not known to proliferate at a combination of such high salinity and high pH. During crystallization of salt, red microorganisms, especially halophilic Archaea, are often entrapped in liquid inclusions within the crystals, and unpurified salt that accumulates in saltern crystallizer ponds is often red. The dominant pigment present is carotenoids, in the case of halophilic Archaea of the family *Halobacteriaceae* mainly 50-carbon α -bacterioruberin and derivatives, which are soluble in alcohol and other organic solvents. The observation that the pigment is also soluble in water may at first sight be unexpected, as carotenoids show a strong hydrophobic behavior. However, it is well known that most members of the *Halobacteriaceae*, including alkaliphilic ones of the genera *Natronobacterium*, *Natronomonas*, and *Natrialba*, require high salt for structural stability and lyse in water in the absence of salts, leaving a clear solution colored red by the carotenoid pigments. The stability of this color in the presence of zinc + sulfuric acid or strong bases was easily confirmed.

The entire salt mass had a strong smell of trimethylamine, particularly at fresh fracture planes. A preformed presence of this base in the salt is unimaginable. It should rather be assumed that this compound is formed from choline. This alkaloid, which occurs e.g. in higher and lower fungi, can be formed during degradation of protein and lecithin-containing material, and yields trimethylamine upon treatment with bases.

One can safely guess that the identification of the smelly product as trimethylamine was correct, but its probable precursor is not choline but another quaternary ammonium compound: glycine betaine. Glycine betaine (*N,N,N*-trimethylglycine) is accumulated as an osmotic stabilizer by many halophilic prokaryotes, including members of the genus *Halorhodospira* and different halophilic cyanobacteria, organisms that abound in the Wadi Natrun lakes. In fact, glycine betaine as an osmotic solute in prokaryotes was first identified in *Halorhodospira halochloris* isolated from the Wadi Natrun (Galinski and Trüper, 1982; see also Sect. 3). One of the products formed during anaerobic degradation of glycine betaine is trimethylamine (Oren, 1990). Even small amounts of trimethylamine formed in the Wadi Natrun salt crust can probably be easily noticed due to the high pH of the system, well above the pK of 9.8 for protonation/deprotonation of trimethylamine, which is volatile in its unprotonated form. Theoretically another precursor for trimethylamine can be trimethylamine *N*-oxide, which can serve as an electron acceptor for anaerobic respiration by many halophilic Archaea (Oren and Trüper, 1990). However, there is no obvious way of formation of trimethylamine *N*-oxide in an environment such as the Wadi Natrun, and therefore, the option of glycine betaine as precursor for trimethylamine formation is much more plausible. No further studies have been devoted to the metabolism of methylated amines in the Wadi Natrun or in any other hypersaline alkaline environment, but the topic may prove to be of considerable interest.

Two groups of Schizophytes may primarily be involved here. First, the group of numerous, already long-known bacteria which degrade sulfate. ... There are numerous other bacteria, e.g. *Spirillum desulfuricans*, a strictly anaerobic species, that make sulfide from sulfate. Under conditions these may produce sodium sulfide from sodium sulfate or calcium sulfide from calcium sulfate.

Spirillum desulfuricans is now known as *Desulfovibrio desulfuricans*. At the time Schweinfurth and Lewin wrote their paper, the nature of the process of bacterial sulfate reduction was known only for a short time: *Spirillum desulfuricans*, the first sulfate-reducing bacterium isolated, was described by Beijerinck in 1895. The authors were well aware of the recent literature, and they cited studies Sergei Winogradsky, Martinus Beijerinck, and other contemporary pioneers of microbial biogeochemistry. As stated above, Abd-el-Malek and Rizk (1963) further looked into the process of sulfate reduction in the Wadi Natrun lakes, but the topic has been sadly neglected since. However, the recent characterization of a novel type of haloalkaliphilic sulfate reducer from the Wadi Natrun described as *Desulfonatrosipira delicata* (*Deltaproteobacteria*) (Sorokin et al., 2008a) suggests that more of novel types of sulfate-reducing microorganisms with unusual properties may be present.

3. The Anoxygenic Phototrophic Sulfur Bacteria and the Chemoautotrophic Sulfur Oxidizers of the Wadi Natrun Lakes

Nearly 60 years passed before the in-depth but little known 1898 study by Schweinfurth and Lewin was followed up and microbiologists had a closer look at the biota of the Wadi Natrun lakes. Except for a short monograph on the vegetation in the valley (Stocker, 1927), no biological publications on the area appear to have been published.

The first twentieth-century paper on the microbiology of the Wadi Natrun (Jannasch, 1957) deals with the nature of the red coloration of the brines and the surface sediments. The red color, which reminded Jannasch of one of the biblical seven [sic!] plagues of Egypt (“eine der ‘sieben Plagen Ägyptens’”), was attributed to the massive development of anoxygenic phototrophic purple bacteria. The color photograph reproduced in Fig. 1 was derived from Jannasch’s study. He also published micrographs of organisms collected from the microbial blooms, tentatively identified as *Chromatium*, *Rhabdochromatium*, *Thiospirillum*, and *Thiocystis* (all anoxygenic phototrophs) and *Desulfovibrio* on the basis of their morphologies.

Jannasch was not aware of the possibility that part of the red color may be due not to photosynthetic purple bacteria but to taxa of the family *Halobacteriaceae*, a group of red halophilic prokaryotes already known for a long time to be responsible for the color of saltern crystallizer ponds (Baas Becking, 1934) and recognized as members of the Archaea since the late 1970s. A more in-depth microbiological survey by Johannes Imhoff, Hans Trüper, and colleagues in 1976 documented a much larger microbial diversity (Imhoff et al., 1979). Attempts to culture photosynthetic bacteria from the samples yielded a number of hitherto

unknown types of polyextremophilic (extremely halophilic as well as alkaliphilic) bacteria (Imhoff et al., 1978). Four of these were described as novel species. Two are extremely halophilic: the red *Ectothiorhodospira abdelmalekii*, named in honor of Abd-el-Malek's contributions to the understanding of the sulfur cycle in the Wadi Natrun lakes, and containing bacteriochlorophyll *a* as photosynthetic pigment (Imhoff and Trüper, 1981), and the green *Ectothiorhodospira halochloris* which possesses bacteriochlorophyll *b* (Imhoff and Trüper, 1977). Both species were later reclassified in the genus *Halorhodospira*, established in 1996 to accommodate the extremely halophilic members of the genus *Ectothiorhodospira*, a change also supported by 16S rRNA sequence information (Imhoff and Süling, 1996). Two other bacteriochlorophyll *a*-containing isolates with less extreme salt requirements remain classified in the genus *Ectothiorhodospira*: *E. haloalkaliphila* (Imhoff and Süling, 1996) and *E. variabilis* (Gorlenko et al., 2009).

When *Halorhodospira halochloris* was first recovered from the Wadi Natrun lakes, nobody could predict how important this bacterium would later become in the studies of osmotic adaptation of bacteria. The role of glycine betaine as an osmotic "compatible" solute, and the first such osmotic solute ever detected in a prokaryote, was discovered in a study of *H. halochloris* (Galinski and Trüper, 1982). Even more important was the finding of another novel osmotic solute, ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) in this species (Galinski et al., 1985). It soon became obvious that ectoine is very widespread as an osmotic solute in the bacterial world. This interesting molecule also found a number of important biotechnological applications (Oren, 2010).

Anoxygenic phototrophs are not the only bacteria oxidizing sulfide and other sulfur compounds under the extremely alkaline and hypersaline conditions encountered in the Wadi Natrun lakes. Studies by Dimitry Sorokin and colleagues recently led to the isolation and characterization of a number of novel chemoautotrophic sulfur oxidizers adapted to life at high salt and high pH, all belonging to the *Gammaproteobacteria*. These include *Thioalkalivibrio paradoxus* (Sorokin et al., 2002), *Thioalkalivibrio nitratireducens* (Sorokin et al., 2003), and *Thiohalospira alkaliphila* (Sorokin et al., 2008b). *Thioalkalivibrio thiocyanodenitrificans* was grown from a mixed inoculum that contained sediment samples from the Wadi Natrun and from soda lakes in the Kulunda Steppe (Altai, Russia) (Sorokin et al., 2004); thus, it cannot be ascertained from which site the organism was obtained.

4. Haloalkaliphilic Archaea in the Wadi Natrun Lakes and Their Contribution to the Color of the Brines

Much of the color of the brines of the Wadi Natrun lakes, as shown in Figs. 1 and 5, is probably due to the presence of dense communities of aerobic haloalkaliphilic Archaea of the family *Halobacteriaceae*, which derive their color from bacterioruberin carotenoids, possibly accompanied by retinal pigments such as halorhodopsin; bacteriorhodopsin or the gene encoding its formation was not yet

found in any haloalkaliphilic member of the family. Imhoff et al. (1979) presented the absorption spectra of an extract of a culture of such haloalkaliphilic Archaea as well as that of a culture of a bacteriochlorophyll *a*-containing anoxygenic phototroph, displaying a prominent peak near 800 nm. No large peak in the infrared range was found in an extract of the brine examined. It was concluded that the Archaea abound mainly in the brine, the phototrophic sulfur bacteria in dense mats covering the sediments. The interplay between the aerobic Archaea and the anaerobic phototrophs on a spatial and a temporal scale in the Wadi Natrun lakes deserves a more in-depth investigation.

One novel species of haloalkaliphilic Archaea was described from the Wadi Natrun: *Halobacterium pharaonis* (“of pharaoh, title of the kings in ancient Egypt”) (Soliman and Trüper, 1982), later renamed *Natronomonas pharaonis*. *Natronomonas pharaonis* later became a popular model organism for the study of the primary light-driven retinal-containing chloride pump halorhodopsin (see, e.g., Lanyi et al., 1990; Kulcsár et al., 2000) and of the retinal-containing membrane-bound light sensor phoborhodopsin (see, e.g., Hirayama et al., 1992; Tomioka and Sasabe, 1995). Its genome sequence was released in 2005 as the second species of halophilic Archaea that was sequenced, 5 years after the publication of the complete genome sequence of *Halobacterium* NRC-1 (*salinarum*) (Falb et al., 2005).

Recently a new archaeon was isolated from the Wadi Natrun, as mentioned by Bowers and Wiegel (2011). This haloalkalithermophilic organism, to be described as *Natronolimnobius aegyptiacus*, is the most extremophilic among the halophilic Archaea with respect to salt, alkalinity, and temperature optima.

5. Alkaliphilic Aerobic and Anaerobic Endospore-Forming Bacteria from the Wadi Natrun Lakes

In addition to a new species of archaeal haloalkaliphiles, inocula from the Wadi Natrun yielded a novel type of endospore-forming halophilic and alkaliphilic aerobic bacteria. The organism, originally designated strain WN13, grows at salt concentrations up to 200 g/L and pH 8.5–10 (Weisser and Trüper, 1985). It was later formally described as a new species within the genus *Bacillus* as *Bacillus haloalkaliphilus* (Fritze, 1996) and later renamed as the type species of the newly formed genus *Alkalibacillus* as *Alkalibacillus haloalkaliphilus* (Jeon et al., 2005).

Originally it was assumed that *Alkalibacillus haloalkaliphilus* uses inorganic ions (Na^+ , K^+ , Cl^-) to establish osmotic equilibrium with its surrounding brines, but it was also reported that NaCl at concentrations above 0.5–1 M strongly inhibits the activity of key enzymes such as isocitrate dehydrogenase and malate dehydrogenase (Weisser and Trüper, 1985). At the time the role of organic compatible solutes in the osmotic adaptation of bacteria was not yet fully realized. Later reexamination of the chemical composition of the cells' cytoplasm showed presence of ectoine in cells grown in minimal medium. When grown in complex media, glycine betaine is taken up from the medium (Fritze, 1996).

Another interesting endospore-forming bacterium recovered from the Wadi Natrun lakes is *Natronobacillus azotifigens*. Strains of this strictly fermentative but oxygen-tolerant nitrogen-fixing bacterium, phylogenetically affiliated with the *Bacillus* group, were also isolated from alkaline soils in the Kulunda Steppe (Altai, Russia) and from northeastern Mongolia (Sorokin et al., 2008c).

6. The Wadi Natrun Lakes Revisited: Discovery of the Anaerobic Haloalkalithermophiles

A sampling expedition to the Wadi Natrun lakes in May 2005 (Fig. 6) led to the discovery of an entirely new group of polyextremophiles: the anaerobic halophilic alkalithermophiles described by Noha Mesbah, Juergen Wiegel, and coworkers. Thus far the names of three representatives of this group have been validly published: *Natranaerobius thermophilus*, *Natranaerobius trueperi*, and *Natronovirga wadinatrunensis*. They ferment organic compounds to products such as lactate, formate, and acetate. Phylogenetically these organisms form a novel lineage within the class *Clostridia*. They were classified in a novel family, the *Natranaerobiaceae*, belonging to a new order, the *Natranaerobiales* (Mesbah and Wiegel, 2005, 2009; Mesbah et al., 2007a, b).

These haloalkalithermophiles are the polyextremophiles par excellence. They grow optimally at Na^+ concentrations of 3–5 M, pH values around 9.5–10, and temperatures above 50 °C, with growth being possible at pH values up to 10.8 (*Natronovirga wadinatrunensis*) and 66 °C (“*Natranaerobius jonesii*”). Doubling times are in the order of 3 h under optimal conditions. Figure 7 compares the growth potential of these novel organisms with other (poly)extremophiles



Figure 6. Lake Um Risha as photographed during the May 2005 expedition, showing salt precipitation on the southwestern shore (left panel) and black sediment smelling strongly to H_2S taken on the northeastern side of the lake (Photographs by Juergen Wiegel and Noha M. Mesbah).

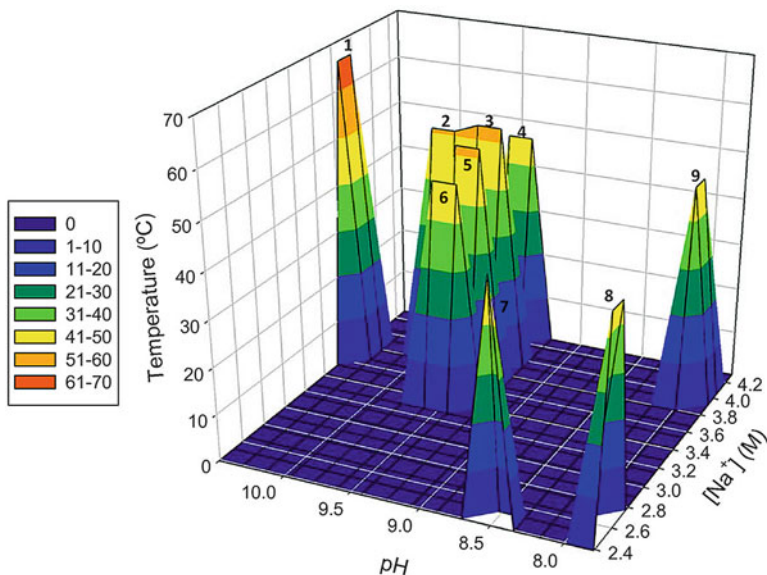


Figure 7. Clustering of polyextremophiles relative to other extreme halophiles. Representation of extremely halophilic *bacteria* for which both additionally considered growth conditions (pH and temperature) approach or exceed thermophilic and alkaliphilic levels. The optima of the discussed recently isolated polyextremophiles cluster in the upper range for each criterion, well separated from other representative microorganisms. The Z-axis color coding depicts the temperature optimum of each bacterium. Species represented: (1) “*Natranaerobius jonesii*,” (2) *Natronovirga wadinatrunensis*, (3) *Natranaerobius thermophilus*, (4) “*Natranaerobius grantii*,” (5) *Natranaerobius truperi*, (6) *Halorhodospira halochloris*, (7) *Dichotomicrobium thermohalophilum*, (8) *Halanaerobacter salinarius*, and (9) *Salinibacter ruber* (From Bowers et al., 2009, reproduced with permission of the authors).

within a three-dimensional space of Na^+ concentration, pH, and temperature (Bowers et al., 2009). All six organisms clustering in the upper left corner originate from the Wadi Natrun lakes: five fermentative anaerobic haloalkalithermophiles and the anoxygenic phototroph *Halorhodospira halochloris*. The other organisms featured in this graph are *Dichotomicrobium thermohalophilum* from Solar Lake, Sinai, a moderately halophilic slightly alkaliphilic bacterium; *Halanaerobacter salinarius*, a neutrophilic anaerobic fermentative halophile from a solar saltern in the south of France; and *Salinibacter ruber*, an aerobic extremely halophilic member of the *Bacteroidetes* from Spanish solar saltern brines.

7. Final Comments

The hypersaline and alkaline lakes of the Wadi Natrun have only occasionally been studied by microbiologists. However, every sampling expedition has yielded major discoveries, so that the impact of those few studies performed on microbiological sciences has been much greater than expected for such small and shallow bodies

of water. The remoteness of the place has probably prevented more extensive studies in the past.

Still, the Schweinfurth and Lewin (1898) paper presented state-of-the-art science for the period, and the data collected and ideas presented in that paper are even relevant today. This and other early studies of the lakes (e.g., Jannasch, 1957) are now little known, probably as they were written in German and no English translations are available.

Some of the polyextremophilic prokaryotes isolated from the Wadi Natrun lakes were studied in-depth, and these studies led to several important discoveries. The compatible solutes glycine betaine and ectoine were first detected in *Halorhodospira halochloris*. Ectoine later turned out to be a highly versatile molecule with important biotechnological applications today and more potential applications in the future. The recent discovery and characterization of the anaerobic haloalkalithermophiles of the order *Natranaerobiales* was another major breakthrough. The existence of these organisms raises some of the most basic questions about the limits tolerated by living cells and about the nature of life itself. How can these bacteria survive on a type of metabolism (fermentation) that yields only little energy (Oren, 1999, 2011b), have to maintain a reversed (acid-inside) proton gradient across the membrane at a high energetic cost, have to provide osmotic balance by pumping ions through the membrane and/or synthesize or accumulate organic osmotic solutes – all energy-requiring processes – and also be adapted to life at elevated temperatures. The study of these unique organisms will undoubtedly tell us much more about the adaptation of microbial life to extreme conditions in the years to come. The recently obtained genome sequence of a representative of this group (Zhao et al., 2011) will undoubtedly help in this task.

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ADAPTATION IN HALOALKALIPHILES AND NATRONOPHILIC BACTERIA

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1. Introduction

Studies of the past decades revealed an impressive diversity of organisms that inhabit highly saline and alkaline environments (Duckworth et al., 1996; Oren, 2002, 2012; Grant and Sorokin, 2011). Most of the prokaryotes and even a few eukaryotic species inhabiting saline–alkaline systems are well adapted to high salinity/alkalinity and high pH conditions. In general, obligate or true halophiles are defined as organisms that require at least 0.2 M or 15 g/l of sodium salts (specifically NaCl) in their milieu, with optimal growth between 0.2 and 5.2 M of total Na⁺ (Kushner and Kamekura, 1988; Ventosa et al., 1998). Obligate (true) alkaliphilic organisms *sensu lato* are those living optimally at pH values of at least 9, mostly growing best between pH 9.5 and 10.5 (Krulwich, 2006). Based on their minimal salt or pH requirements, both categories of extremophiles could be further divided into slight, moderate, and extreme halophiles or alkaliphiles, respectively (Table 1) (Grant et al., 1998; Hamamoto and Horikoshi, 1992; Ventosa et al., 1998; Mesbah and Wiegel, 2008). Interestingly, while most of true halophiles are flourishing at neutral or slightly acidic pH (6–7), many true alkaliphiles are also halophiles in their requirements, being termed haloalkaliphiles and qualifying as polyextremophiles. On the opposite, non-halophilic alkaliphiles require high pH and low salt in their medium (Hamamoto and Horikoshi, 1992; Horikoshi, 1999). The term “natronophile” (“natron-loving” organisms or “chloride-independent sodaphile”) has been proposed for the alkaliphilic organisms that have an absolute requirement for Na₂CO₃/NaHCO₃ (Banciu, 2004; Sorokin et al., 2011a; Muyzer et al., 2011a). However, some natronophiles still require low concentrations of chloride ions for the optimal growth in soda brines. Life at haloalkaline conditions is energetically costly, and therefore, extreme haloalkaliphiles could hardly exhibit other extremophilic features (Oren, 2011). Moderate halophilic and thermoalkaliphilic anaerobic bacteria with validly published names belong to the orders *Halanaerobiales* (e.g., *Halonatronum saccharophilum*) and *Natranaerobiales*

Table 1. Main types of extremophilic microorganisms living at high salt and/or high pH conditions.

Growth characteristics	Range of pH (optimum pH)	Salt range (optimum salinity)	Representative bacterial species
Obligate (true) extreme halophile	5.2–8.5 (7–7.5)	12–30 % (15–25 %) (w/v) NaCl	<i>Salinibacter ruber</i>
Moderate halophile	5.0–8.5 (6.8–7.5)	3–15 % (7–12 %) (w/v) NaCl	<i>Halomonas halophila</i>
Facultative halophile	6.0–11 (6.8–7.5)	0–35 % (6–12 %) (w/v) NaCl	<i>Planococcus halophilus</i>
Halotolerant	5.0–8.5 (6.8–7.5)	0–15 % (1–5 %) (w/v) NaCl	<i>Salinivibrio costicola</i>
Obligate (true) alkaliphile	9.0–11.5 (9.5–10.5)	0.05–2 M (0.05–0.2 M) of total Na ⁺	<i>Bacillus alcalophilus</i> ATCC 27647
Facultative alkaliphile	7.5–11 (7.5–10)	0.05–0.1 M of total Na ⁺	<i>Bacillus pseudofirmus</i> OF4
Alkalitolerant	7.5–11 (7.5–10)	0.6–16 % (w/v) NaCl	Alkalitolerant <i>Bacillus</i> species (e.g., <i>Bacillus horikoshii</i>)
True extremely haloalkaliphile/ natronophile	8–10.7 (9.5–10.2)	10–30 % (12–25 %) (w/v) NaCl or 1.5–4.4 M (2–3.5 M) of total Na ⁺	<i>Thioalkalivibrio versutus</i>
True moderately haloalkaliphile/ natronophile	8–10.5 (9.0–10.0)	3–20 % (5–12 %) (w/v) NaCl or 0.5–2 M (1 M) of total Na ⁺	<i>Ectothiorhodospira haloalkaliphila</i>
Low-salt-tolerant natronophile	8–10.5 (9.5–10.0)	0.3–1.5 M (0.6 M) of total Na ⁺	<i>Thioalkalimicrobium aerophilum</i> AL3 ^T

(e.g., *Natronaerobius* spp. and *Natronovirga* spp.). Triple extremophiles are apparently exceptions to the above-mentioned statement, but they are not extreme in any of their optimal growth requirements (Zhilina et al., 2001; Mesbah et al., 2007; Mesbah and Wiegel, 2009). A more frequent situation is that of moderate haloalkaliphiles that are either thermotolerant (e.g., strains of the sulfur-oxidizing bacterium *Thioalkalivibrio versutus*, some actinomycetes isolated from saline soils) (Sorokin and Kuenen, 2005; Ningthoujam et al., 2009) or psychrotolerant (e.g., *Planococcus* spp. strain ZD22 isolated from a saline–alkaline soil in the vicinity of an oil field) (Li et al., 2006).

Besides the main metabolic types listed in Table 1, there are different degrees of mixed halophily and alkaliphily observed in various microbes. For example, strains of *Bacillus halodurans* are facultative alkaliphiles and moderately halotolerants, with optimal growth at pH 9–10 and a salt tolerance ranging from 0 up to 12 % NaCl (Nielsen et al., 1995). *Oceanobacillus iheyensis* HTE831 is facultatively alkaliphilic and extremely halotolerant and is closely related to *Bacillus* spp. It grows optimally at pH between 7.5 and 9.5, tolerating up to 21 % NaCl at pH 7.5. Isolated from deep-sea sediment, *O. iheyensis* can withstand pressures up to 30 MPa (Lu et al., 2001).

Often, saline–alkaline environments hosting haloalkaliphilic prokaryotes are exposed not only to relatively elevated temperatures but also to intense sunlight irradiation and, consequently, to a high rate of water evaporation during dry seasons (Grant and Sorokin, 2011). In these harsh conditions, survival of salt- and alkaline-loving microorganisms depends on a palette of adaptive mechanisms that aim to remain alive during prolonged periods of low water activity. Acute and long-term adaptation as a response to changing salinity and/or alkalinity include modulation of membrane fluidity, acquisition of osmoprotectants, adjustments of energy metabolism concomitant with fine-tuning of stress-sensing, chemotaxis, and nutrients transport at the membrane level. Model halophilic and alkaliphilic organisms have for a long time been used to test for phenotypic and genotypic response to variation of external salinity and pH, respectively. A few examples of detailed physiological investigations concerning the double-stress adaptation at high salinity and alkalinity are known, and they are mostly focused on natronophilic lithoautotrophic bacteria and haloalkaliphilic archaea. In this chapter, we endeavor to discuss up-to-date relevant findings in the haloalkaliphilic adaptative strategies as they were directly observed or as extrapolation of well-known alkaliphilic and halophilic settings. Accumulating information on genome sequences aids to reveal even more insights in the mosaic of various adaptive strategies to the haloalkaline environment.

2. Habitats and Diversity of Haloalkaliphilic and Natronophilic Bacteria

The majority of known obligate haloalkaliphilic and natronophilic bacteria and archaea are isolated from soda lakes and saline–alkaline soils. In the past, detailed investigations on the biota living in soda systems have been carried out mainly in

soda lakes from the East African Rift Valley and western USA. Currently, Central Asian (Chinese, Mongolian, and Siberian) soda lakes are under intense screening for microbial diversity.

2.1. SALINE–ALKALINE HABITATS

Saline–alkaline environments are characterized by increased salinity (>60 g/l total salinity) and high actual (soluble) alkalinity (up to molar concentrations) and pH (pH >9.5 – 11). Because in such environments salinity and alkalinity are simultaneously provided by a high concentration of sodium carbonate and sodium bicarbonate, they are also known as soda systems. Saline–alkaline (soda) lakes or soils are located mostly in arid and semiarid regions where particular climate, geochemical, hydrological, and topological factors interact. Differing from neutral saline lakes (pH 6 – 8 , with low buffering capacity and high NaCl, Na_2SO_4 , K^+ , and Mg^{2+} concentration), soda lakes are strong natural buffering systems at pH >10 , containing high concentrations of Na_2CO_3 , NaHCO_3 , and, in some cases, a significant concentration of NaCl. One of the major chemical characteristics of soda lakes is the lack of solubilized divalent cations (Mg^{2+} , Ca^{2+}) that precipitate as carbonates under alkaline conditions. The early removal of divalent cation carbonates results in the accumulation of sodium or potassium carbonates, thus increasing the concentration of monovalent cations. By repeated cycles of concentration and evaporation, salts crystallize and brines are formed. In some parts of the world, the shallow lakes may end as a layer of solid rock termed trona (crystalline sodium sesquihydrate, $\text{Na}_2\text{CO}_3 \cdot \text{NaHCO}_3 \cdot 2\text{H}_2\text{O}$) by evaporation during dry seasons (Grant and Sorokin, 2011). A broad range of intermediate saline and/or alkaline lakes occurs by the mixing of the minerals in various ratios. Other important consequences of the soda brine chemistry, including the presence of high levels of soluble inorganic phosphorus, low toxicity of sulfide and nitrite, and high toxicity of ammonia, have important implications on the microbial system.

Salt lakes originating from sea are called thalassohaline, while those with land origin are termed athalassohaline (Oren, 2002). Most of neutral salt lakes and salterns (pH 7 – 8) are thalassohaline, while soda lakes are obviously of continental (athalassic) origin. Bodies of water with a significantly higher salinity (>80 g/l, w/v) than that of the seawater (35 g/l, w/v) are categorized as highly saline or hypersaline lakes (Poehls and Smith, 2009). Many soda lakes are also hypersaline, reaching a salinity of over 300 g/l. Well-known saline–alkaline lakes are located in eastern Africa (along the East African Rift Valley), the western and northern USA, the Middle East (Turkey, Armenia), and Central Asia (southern Siberia, northeastern and Inner Mongolia, China). Remote soda lakes are also located in other parts of the world, such as Australia, Central and South America (Chile, Venezuela), and southern Asia (India). Low-salt alkaline ponds and soils are described in Europe (Austria, Hungary, Serbia) (Tindall, 1988).

Saline–alkaline soils are either natural or man-made, formed upon intensive irrigation/evaporation cycles. Natural sodic (soda or soda solonchak) soils and deserts are formed in dry areas located in Europe (e.g., the Pannonian Basin), Asia (SW Siberia, NE Mongolia, Central China, India), Africa (Egypt), and North America. In comparison with soda lakes, the composition and diversity of microbial community living in soda soils is scarcely characterized (Lysak et al., 1994; Zenova et al., 2005; Sorokin et al., 2008e).

Saline–alkaline ecosystems have attracted the attention of investigators due to their unique chemical and biological features. Soda lakes are considered as models of ancient Martian or Archean terrestrial aquatic ecosystems (Kempe and Degens, 1985; Kempe and Kazmierczak, 1997; Zavarzin, 1993).

In spite of the apparently unfavorable physical and chemical conditions (high pH, high salinity, low water activity, paucity of divalent cations, high irradiation, elevated temperature, etc.), saline–alkaline environments display a full and balanced recycling of chemical elements (C, O, H, N, S, P, metals, etc.) and often a spectacularly high primary biomass production. In this category of extreme habitats, an almost complete trophic web assures the biological utilization and transfer of elements. Prokaryotes, represented by archaea and bacteria, as well as microscopic and macroscopic eukaryotes (protozoa, algae, brine shrimps, insect larvae) thrive in saline–alkaline lakes. Prokaryotes are the most diverse and numerous group of soda lake inhabitants. Recent advances in molecular biology have the potential to reveal complex metacommunities that play key roles in the ecological status of such habitats (Riesenfeld et al., 2004; Grant and Heaphy, 2010).

2.2. BIODIVERSITY OF SODA ENVIRONMENTS

As a general observation, in saline–alkaline environments, species diversity increases as the salinity and/or alkalinity decreases (Grant and Tindall, 1986). In diluted saline–alkaline lakes of the East African Rift, representatives of macrofauna, such as fishes (*Tilapia grahami*), are found. Brine shrimp (*Artemia* sp.), commonly found throughout the world's saline lakes, is documented in saline–alkaline Mono Lake (USA) (Dana and Lenz, 1986) as well as in hypersaline soda lakes in the Kulunda Steppe (Altai, Russia) (Sorokin et al., 2012d). In soda lakes with moderate salinity (<90 g/l) and pH <10, from East Africa and the western USA, zooplankton species including rotifers (e.g., *Brachionus plicatilis*), cladocerans (*Moina hutchinsoni*), and copepods (*Diaptomus sicilis*) were recorded (Grant and Tindall, 1986; Bozek, 1989). These microscopic invertebrates feed on phytoplankton such as microscopic algae and cyanobacteria. Haloalkaliphilic photoautotrophic cyanobacteria from the genera *Arthrospira* spp., *Cyanospira* spp., *Synechococcus* spp., and *Synechocystis* spp. consistently contribute to the high primary productivity in hyposaline and moderate soda lakes (Grant et al., 1990). Cyanobacteria are essential both for inorganic C fixation and O₂ production in the

ecosystem of saline–alkaline lakes. In addition to cyanobacterial populations, hypersaline soda lakes such as Lake Magadi in Kenya and Wadi Natrun haloalkaline lakes in Egypt, host significant communities of anoxygenic phototrophic purple bacteria belonging to *Ectothiorhodospira* and *Halorhodospira*, which also contribute to the primary biomass production (Imhoff et al., 1979; Grant and Tindall, 1986). Photolithoautotrophic haloalkaliphilic bacteria such as *Thiorhodospira sibirica* and *Thioalkalicoccus limnaeus* are the sole species of their genera that have been isolated from the low saline soda lakes from the Siberian steppe (Bryantseva et al., 1999a, 2000). Anoxygenic, photoheterotrophic heliobacteria such as alkaliphilic, low-salt-tolerant members of *Heliorestis* spp. were identified in low saline soda lakes from Siberia and Wadi Natrun (Egypt) (Bryantseva et al., 1999b; Asao et al., 2006). A relatively large nutritional and ecological spectrum of aerobic and anaerobic prokaryotes, bacteria and archaea, assures the natural cycling of the elements in the soda environments. Basically, all major metabolic groups are represented among the soda lakes inhabitants (Jones et al., 1998; Zavarzin et al., 1999; Grant and Sorokin, 2011).

Chemoheterotrophic aerobes thriving in soda lakes are well represented by archaea, as well as Gram-negative and Gram-positive bacteria. Haloalkaliphilic members of the family *Halomonadaceae* (e.g., *Halomonas magadiensis*, *H. kenyensis*, *H. mongoliensis*) have been isolated from soda lakes around the world, being an important part of the easily culturable aerobic chemoheterotrophic communities thriving in these ecosystems (Duckworth et al., 1996, 2000; Boltysanskaya et al., 2007; Shapovalova et al., 2008). Other haloalkalitolerant and haloalkaliphilic *Halomonas* spp. have been discovered in neutral saline lakes (*H. alkaliantarctica*), salt pools (*H. campaniensis*, *H. alkaliphila*), and saline soils (*H. campisalis*, *H. boliviensis*) (Poli et al., 2007; Romano et al., 2005a, 2006a; Mormile et al., 1999; Quillaguamán et al., 2004). Soda systems are the sources for many aerobic Gram-positive heterotrophs belonging to the phyla *Firmicutes* and *Actinobacteria*. Most *Bacillus* isolates from soda lakes or soils are alkaliphilic and moderately halophilic or halotolerant (*Bacillus alkalisediminis*, *B. aurantiacus*, *B. bogoriensis*, *B. daliensis*), and only a few of them have been categorized as true, although moderately, haloalkaliphiles, such as *B. localis* (Borsodi et al., 2008, 2011; Vargas et al., 2005a; Zhai et al., 2012; Márquez et al., 2011). A wealth of other *Bacillus*-related species with various degrees of mixed halo- and alkaliphily have been recently and continuously isolated from soda lakes: the halophilic and alkalitolerant *Salsuginibacillus kocurii* and *S. halophilus* (Carrasco et al., 2007; Cao et al., 2010), the moderately halophilic and alkalitolerant *Halolactibacillus alkaliphilus* (Cao et al., 2008), and the haloalkalitolerant *Amphibacillus jilinensis* (Wu et al., 2009).

The important question of polymer degradation at soda lake conditions has barely been touched. So far a possibility of anaerobic utilization of cellulose and pectin as growth substrates was demonstrated in pure cultures of fermentative haloalkaliphilic members of the *Clostridia* and the *Bacteroidetes* (Zhilina et al., 2005; Sorokin et al., 2011d, 2012c). Many fermentative and anaerobic respirers from soda lakes belong to the orders *Clostridiales*, *Halanaerobiales*,

and *Natronaerobiales*. From the *Halanaerobiales*, two natronophilic species of *Natroniella* have been described: the chemoorganotrophic homoacetogenic *Natroniella acetigena* (Zhilina et al., 1996) and the chemolithoautotrophic sulfidogenic *N. sulfidigena* (Sorokin et al., 2011b). Both have been isolated from anoxygenic sediments of soda lakes in Kenya and SW Siberia, respectively. *Natroniella sulfidigena* is capable of acetate-dependent sulfur respiration, being responsible of hydrogen sulfide formation at extremely haloalkaline conditions (pH >10; salinity >2 M Na⁺). Sulfate-reducing bacteria (SRB) thriving in the anoxic sediments of various soda lakes are represented by extremely or moderately natronophilic lithotrophs belonging to the order *Desulfovibrionales* (genera *Desulfonatronovibrio*, *Desulfonatronum*, and *Desulfonatronospira*) (Zhilina et al., 1997; Sorokin et al., 2008b, 2011c, 2012b; for a review, see Sorokin et al., 2011a). Moderately natronophilic heterotrophic SRB capable of utilizing propionate or volatile fatty acids as carbon and energy source have been recently assigned to the order *Desulfobacterales* and described as *Desulfonatronobacter acidivorans* and *Desulfobulbus alkaliphilus* (Sorokin et al., 2012a). The most recent example of an extraordinary sulfate reducer described as *Desulfohalophilus alkaliarsenatis* (Switzer Blum et al., 2012) was isolated from hypersaline–alkaline Searles Lake in California. This new member of the family *Desulfohalobiaceae* can grow by either sulfate or arsenate respiration at salt-saturating condition and high pH. The oxidative part of sulfur cycle in soda lakes is performed by natronophilic chemolithoautotrophic sulfur-oxidizing bacteria (SOB) belonging to four recognized genera: *Thioalkalimicrobium*, *Thioalkalivibrio*, *Thioalkalispira*, and *Thioalkalibacter*. Representatives of the former two genera are widely distributed in various soda lakes all over the world, while the latter two were only found occasionally (Sorokin and Kuenen, 2005; Banciu et al., 2008; Sorokin et al., 2011a). Haloalkaliphilic members of versatile fermentative spirochaetes (*Spirochaeta alkalica*, *S. africana*, *S. asiatica*, *S. americana*, and *S. dissipatitropha*) were isolated from brine sediments of Lake Magadi in Kenya, from sulfide-saturated mud of Lake Khatyn (Tuva, Siberia), and from sediments of the saline–alkaline Mono and Owens Lakes (California) (Zhilina et al., 1996; Hoover et al., 2003; Pikuta et al., 2009).

Examples of haloalkaliphilic and natronophilic Gram-positive anaerobes isolated from soda environments are the acetogenic *Natronoincola histidinovorans* (Zhilina et al., 1998) and members of the genus *Tindallia* (Pikuta et al., 2003; Alazard et al., 2007), halophilic and alkalithermophilic *Natronaerobius thermophilus*, *N. trueperi*, and *Natronovirga wadinatronensis* (Mesbah et al., 2007; Mesbah and Wiegel, 2009). Haloalkalitolerant and haloalkaliphilic methanotrophs such as *Methylomicrobium buryatense* (Kaluzhnaya et al., 2001), *M. alcaliphilum*, and *M. kenyense* (Kaluzhnaya et al., 2008; Sorokin et al., 2000) identified in the sediments of moderate or highly saline soda lakes are Gram-negative, aerobic bacteria responsible of methane oxidation and formation of CO₂ and various organic C₁ compounds. Methanol, formaldehyde, and formate formed as products of partial methane oxidation by aerobic methanotrophs, as well as methylamines produced by the breakdown of organic osmolytes, are used as carbon source by moderately

haloalkaliphilic aerobic methylobacteria such as *Methylophaga alcalica*, *M. natronica*, *M. lonarensis*, and *Methylnatronum kenyense* (Doronina et al., 2003a, b; Antony et al., 2012; Sorokin et al., 2007; for review, see Trotsenko et al., 2007).

In addition to bacterial populations, the prokaryotic communities of soda lakes comprise a variety of haloalkaliphilic archaea, many of them belonging to the *Halobacteriaceae* family. Haloalkaliphilic archaea isolated from soda lakes in East Africa, Central Asia, and the USA are found within the genera *Natrialba*, *Natronomonas*, *Natronococcus*, *Natronobacterium*, and *Natronorubrum* (Grant and Sorokin, 2011).

Other haloalkaliphilic bacteria possessing interesting metabolic characteristics or promising biotechnological applications have been isolated from saline-alkaline soils (e.g., the N_2 -fixing Gram-positive anaerobic *Natronobacillus azotifigens*) (Sorokin et al., 2008f), soda lakes sediments (*Nitriiliruptor alcaliphilus* capable of growing on nitriles) (Sorokin et al., 2009), soda lake water (arsenite-oxidizing *Alkalilimnicola ehrlichii*) (Hoeft et al., 2007), coastal lagoon mud (Gram-positive anaerobe fermentative bacterium *Halonatronum saccharophilum*) (Zhilina et al., 2001), etc.

3. Mechanisms of High-Salt and Alkaline Adaptation in Bacteria

3.1. ADAPTATION OF THE BACTERIAL CELL ENVELOPE TO HALOALKALINE CONDITIONS

Cell envelopes (cell wall and cytoplasmic membrane) are the first line of defense and, implicitly, of adaptation to changing of environmental factors. The main constituents of cell wall are polysaccharides and proteins, while membranes consist of mainly lipids and proteins. In bacteria, membrane lipids are typically represented by polar phospholipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and diphosphatidylglycerol or cardiolipin (CL) and nonpolar lipids such as isoprenoids (e.g., quinones, squalene, pigments). Ester phospholipids are constituted from a glycerophosphate backbone and two fatty acids esterified at the C2 and C3 positions. Therefore, the bulk of cell-extracted fatty acids originates from the plasma membrane. In this regard, it is not surprising that a significant part of the halophilic and alkaliphilic adaptation kit in bacteria might include more or less subtle changes of the membrane fatty acid structure and composition. As can be seen from the following evidences, besides particular ways of adapting the lipid composition, there are some general rules that apply in most high-salt and high-pH adaptation strategies.

3.1.1. Phospholipid and Fatty Acid Composition of Halophilic, Alkaliphilic, and Haloalkaliphilic Membranes

Based on their hydrocarbon chain structure, fatty acids can be divided into two main classes: straight-chain and branched-chain fatty acids (BFAs). Alicyclic (cyclopropane) fatty acids (CFAs) contain a cyclic structure within their molecule

and are characteristic of Gram-negative bacteria (e.g., C19:0 cyclo ω 7c). In general, many Gram-positive bacteria have significant amounts of BFAs (Kaneda, 1977). The most common straight-chained fatty acids found in bacteria are the saturated C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C16:1 (palmitoleic acid), and the monounsaturated C18:1 (oleic acid). Branching of fatty acids generally occurs as iso and anteiso positioning of methyl residue at the second or at the third to the last carbon in the chain. Examples of iso-branched and anteiso-branched fatty acids that are widespread in Gram-positives are iso-C17:0 (15-methylpalmitic acid) and anteiso-C17:0 (14-methylpalmitic acid). Fatty acids are presently used as important chemotaxonomic markers in differentiation of bacterial species (Oren, 2012).

The major functional difference between the straight and branched chains, as well as between saturated and unsaturated fatty acids, resides in their contribution to the membrane fluidity (Kaneda, 1991). Variations of saturation, length, and branching in fatty acids are, therefore, one of the key factors changing the membrane fluidity as a short- and long-term response to environmental stress. Nevertheless, adjustments of the fatty acid composition are adding value to other adaptive changes in membrane lipids, such as modification of the zwitterionic to anionic lipid ratios (i.e., PE to PG and/or CL) or varying the production of isoprenoid derivatives.

The effects of varying salt concentration on membrane lipid composition are relatively well documented in halophilic bacteria with a broad salt tolerance such as *Vibrio* spp., *Halomonas* spp., and *Chromohalobacter* spp. (Adams et al., 1990; Adams and Russell, 1992; Russell, 1993; Vreeland et al., 1984; Arahall et al., 2001; Vargas et al., 2005b). As general conclusions drawn from experiments of salt stress effect on lipid composition in bacteria, one can affirm that the membrane fluidity is finely modulated by a complex interplay between environmental factors (temperature and salinity) on one side and cell response in terms of different phospholipids and fatty acids production on the other side. In Gram-negative halophilic bacteria (e.g., *Halomonas* spp.), growth at elevated salt concentration is correlated with high contents of straight chain, more unsaturated and lesser branched-chain fatty acids in the membranes (Monteoliva-Sanchez et al., 1988; Adams et al., 1990). At increasing salinity or at close to optimal growth salinity, the relative amount of long-chain acids (with C16 < C18) increases, whereas that of short-chain acids (C14 and/or C15) decreases (Imhoff and Thiemann, 1991; Valderrama et al., 1998). At high salt (as NaCl) concentration, the anionic phospholipids (PG) predominate over zwitterionic ones (PE). Additionally, CL and CFA increase at high salt concentration as observed in various moderately halophilic bacteria (for a review, see Ventosa et al., 1998). In the extremely halophilic bacterium *Salinibacter ruber*, CL is making up to 20 % of total polar lipids, a significantly higher proportion than that found in other halophilic bacteria (Lattanzio et al., 2009). The role of CL in the energetic membranes is well known. Cardiolipin is a major membrane lipid that binds *c*-type cytochromes and cytochrome *c* oxidase (Choi and Swanson, 1995; Robinson, 1993; Schägger, 2002)

maintaining their optimal activity. In archaeal membranes CL derivatives are associated with terminal oxidases and are considered essential for haloarchaea adaptation at osmotic stress (Corcelli, 2009). Significant amounts of CL are correlated with high cytochrome *c* contents and cytochrome *c* oxidase activity in the membranes of alkaliphilic *Bacillus* strains (Clejan et al., 1986; Hicks and Krulwich, 1995) as well as in the natronophilic SOB *Thioalkalivibrio* spp. (Banciu et al., 2005).

Adaptation to high pH is similarly reflected in the membrane composition. In the alkali-tolerant *Listeria monocytogenes*, the cells develop a higher proportion of BFAs of the anteiso form at pH 9. It has been suggested that the balance between anteiso- and iso-fatty acids may be more important than changes in the amounts and/or degrees of saturation of fatty acids in pH adaptation (Giotis et al., 2007). The facultatively alkaliphilic *Bacillus pseudofirmus* OF4 proved an excellent and intensively studied model organism to follow the effects of varying the external pH at the phenotype and genotype level. Like many *Bacillus* species, *B. pseudofirmus* OF4 possesses branched-chain fatty acids as major acids (90 % of total fatty acids). The obligate alkaliphilic *Bacillus* strains contain unusually high proportions of unsaturated fatty acids (20 % of the total fatty acids and most common being C16:1). In addition, obligate alkaliphilic strains of *Bacillus* have appreciable amounts of PG, CL, long-chain fatty acids, squalene, and C40 isoprenoids as compared to facultative strains (Clejan et al., 1986). Interestingly, the mixture of a high concentration of BFAs and a substantial fraction of unsaturated fatty acids (UFAs) may make the membrane leaky at a suboptimal (neutral) pH in the obligate alkaliphiles, as implied from the experiments performed by Dunkley et al. (1991).

In many Gram-positive moderately halophilic, alkaliphilic, or alkali-tolerant species related to genus *Bacillus* (e.g., *Alkalibacillus* spp., *Halalkalibacillus* spp., *Oceanobacillus* spp.), the prevailing cell fatty acids are the BFAs (see also Table 2). However, dominance of BFA in cell membrane seems to be a general feature of Gram-positives (Kaneda, 1991) suggesting that the distinctive haloalkaline adaptations might be accommodated within the cell wall structure. It is considered that the branched anteiso-C15 fatty acid has a comparable function to that of UFAs in bacteria with a straight-chain lipid type, mainly because it has the lowest phase transition temperature ($T_m = -16.5$ °C) of all BFAs (Kaneda, 1991). The biosynthesis of UFAs occurs either aerobically or anaerobically (Aguilar and de Mendoza, 2006). UFAs, mostly as C16:1 ω 7 and C18:1 ω 7, are relatively common among anaerobes such as members of the family *Clostridiaceae* (Zhu et al., 2009). Representatives of haloalkaliphilic anaerobic bacteria (e.g., *Tindallia* spp., *Natroniella* spp., *Natronincola* spp.) contain significant amounts of UFAs. UFAs have a lower transition phase temperatures than their saturated counterparts, and consequently, they may profoundly influence the membrane fluidity (Silvius, 1982; Aguilar and de Mendoza, 2006).

Cyclopropane fatty acids (CFAs) are synthesized by the transfer of a methylene group from *S*-adenosyl-L-methionine to a double bond of unsaturated

Table 2. Fatty acid (FA) composition of several representative species of Gram-positive and Gram-negative bacteria with various degrees of halophily and alkaliphily.

Species	Physiological type ^a	Optimal pH/salinity	Dominant FA ^b	References
Gram-positive bacteria				
<i>Bacillus locisalis</i> CGI ^T	Moderately haloalkaliphilic aerobe	10/10 % (w/v) NaCl	anteiso-C15:0, iso-C15:0, anteiso-C17:0	Márquez et al. (2011)
<i>Oceanobacillus thelyensis</i> KCTC 3954 ^T	Extremely halotolerant and facultatively alkaliphilic aerobe	7.5–9.5/3 % (w/v) NaCl	iso-C15:0, anteiso-C15:0, C16:0	Lee et al. (2006)
<i>Nitriinuptor alkaliphilus</i> ANL-iso2 ⁽¹⁾	Moderately halotolerant and obligately alkaliphilic aerobe	9.0–9.5/0.2–0.3 M of total Na ⁺	C16:0, iso-C14, C16:1, C14:0, iso-C16	Sorokin et al. (2009)
<i>Alkaliphilus halophilus</i> E2R ^T	Moderately haloalkaliphilic anaerobe	8.0/7.5 % (w/v) NaCl	iso-C15:0, iso-C15:1, iso-C13:0	Wu et al. (2010)
<i>Natronaerobius thermophilus</i> JW/NM-WN-LFT	Halophilic alkalithermophilic anaerobe	pH ^{55 °C} 9.5/~12 % (w/v) NaCl	iso-C15:0, iso-C17:0, C16 :0DMA	Mesbah et al. (2007)
<i>Tindallia magadiensis</i> Z-7934	Obligate natronophilic anaerobe	8.5/3–6 % (w/v) NaHCO ₃	C16:1, aldehyde-C16	Zhilina et al. (1998)
Gram-negative bacteria				
<i>Indibacter alkaliphilus</i> LW1 ^T and <i>Nitritelexa halalkaliphila</i> LW7 ^T	Halotolerant and alkaliphilic aerobes	10/5 % (w/v) NaCl	iso-C15:0, iso-C17:0 3-OH	Anil Kumar et al. (2010a, b)
<i>Thioalkalimicrobium</i> <i>aerophilum</i> AL3 ^T	True natronophilic, low-salt-tolerant aerobe	9.8–10/0.6 M of total Na ⁺	C18:1, C16:1, C16:0	Banciu et al. (2005)
<i>Thioalkalivibrio versutus</i> ALJ15	True natronophilic, extremely salt-tolerant aerobe	10–10.2/0.6–1.2 M of (total Na ⁺)	C18:1, C16:0, eye-C19	Banciu et al. (2005)
<i>Halomonas campaniensis</i> 5AG ^T	Extremely halotolerant and facultatively alkaliphilic aerobe	9.0/10 % (w/v) NaCl	C18:1, C16:1, anteiso-C16:0, iso-C16:0	Romano et al. (2005a)

(continued)

Table 2. (continued)

Species	Physiological type ^a	Optimal pH/salinity	Dominant FA ^b	References
<i>Natronincola histidinovorans</i> Z-7940 ^T	Moderately haloalkaliphilic and natronophilic anaerobe	9.4/8–10 % (w/v) NaCl	C16:1, aldehyde-C16 C14:0; C16:0; C18:0	Zhilina et al. (1998)
<i>Natroniella acetigena</i> Z-7937 ^T	True haloalkaliphilic and natronophilic, anaerobe	9.7–10.0/12–15 % (w/v) NaCl	C14:0; C16:0, C18:0, C16:1, aldehyde-C16:1	Zhilina et al. (1996, 1998)
<i>Desulfonatronum thioautotrophicum</i> and <i>D. thiosulfatophilum</i>	Obligately moderate natronophilic anaerobes	9.5–10/0.4–0.6 M of total Na ⁺	iso-C17:1, iso-C15:0, C18:1, C16:1, C14:0	Sorokin et al. (2011c)

Taxa were chosen to reflect a broad variety of physiological types.

^aAll haloalkaliphilic species selected are mesophilic (optimal temperature 30–37 °C) with the exception of the halophilic alkalithermophile *Natronaerobius thermophilus*.

^bDominant fatty acids are listed in approximate order of decreasing concentration. In some situations, the summing of partial FA belonging to a structural type may exceed the dominant FA of other type.

fatty acid chains of membrane phospholipids. This conversion is catalyzed by cyclopropane fatty acid (CFA) synthase. CFA synthase is present in many bacteria and it is recognized to regulate the membrane lipid composition and fluidity, thus playing an essential part in the adaptation of bacteria in response to a drastic perturbation of the environment (To et al., 2011). The extremely salt-tolerant natronophilic *Thioalkalivibrio* strain ALJ15 has a very similar fatty acids composition to the closely related *Ectothiorhodospira* spp. and *Halorhodospira* spp. (Imhoff and Thiemann, 1991) and *Halomonas* spp. (Valderrama et al., 1998; Romano et al., 2001), in which C16:0, C18:1, and cyc-C19 are the dominant fatty acids. The concentration of monounsaturated C18:1 is, however, two times higher in haloalkaliphilic SOB than in the halophilic *Halomonas salina*. Moreover, the concentration of cyc-C19 is significantly (5 times) higher in the natronophilic *Thioalkalivibrio* spp. than that measured in *Ectothiorhodospira* and *Halomonas salina*. Increasing salt concentration induced the decrease of monounsaturated C18:1 and concomitant increase of cyc-C19 in *Thioalkalivibrio* spp. (Banciu et al., 2005), *Halomonas* spp. (*H. salina* and *H. halophila*) (Valderrama et al., 1998; Monteoliva-Sanchez et al., 1988), and *Lactobacillus* strains (Gilarova et al., 1994). This is consistent with the knowledge that cis-monounsaturated fatty acids like C18:1 can be converted into cyc-C19 and vice versa. This may suggest a salt-dependent activation of CFA synthase. The raised CFA content at the expense of unsaturated fatty acids would contribute to the increasing of membrane rigidity (Russell, 1993). A stimulation of CFA synthase activity by addition of organic compatible solutes (e.g., glycine betaine) was observed in the moderately halophilic bacterium *Pseudomonas halosaccarolytica* (Monteoliva-Sanchez et al., 1993). CFAs such as cyc-C19 are considered as biomarkers for Gram-negative bacteria in a similar way as the BFAs for the Gram-positives. They are common in the membrane of anaerobic SRB and have been associated with the low redox potential in soils (Dowling et al., 1986; Zelles, 1999).

The less common aldehydes and dimethylacetal (DMA) fatty acids are specifically obtained during extraction of cellular fatty acids from anaerobes, and they are often used to differentiate between species. Aldehydes and 1,1-dimethylacetals result from plasmalogens by chemical lysis during lipid analysis procedures (Mayberry and Lane, 1993). Bacterial plasmalogens are proven to have a substantial effect on membrane fluidity. In the plasmalogen-deficient mutants of *Megasphaera elsdenii*, Kaufman et al. (1990) have observed the prevalence of a more stable lamellar phase. Studies on the psychrophilic actinobacteria *Subtercola boreus* and *Subtercola frigoramans*, with high contents of BFAs and DMA, have revealed that lowering of the growth temperature favored the production of DMAs with shorter carbon chain lengths and the increase of anteiso-branched DMA at the expense of iso-branched congeners (Männistö et al., 2000). Generally it is believed that the presence of plasmalogens and their glycerol acetals could be responsible for substantial plasticity of bacterial membranes, especially for cells growing at a wide range of temperatures, salinity, and the presence of solutes such as hydrocarbons and solvents that have the

potential of perturbing the bilayer arrangement of the cell membrane. Another possible role of plasmalogens in anaerobic bacteria is to protect the cell against oxidative stress (Goldfine, 2010).

Polyunsaturated fatty acids (PUFAs) are typical for cyanobacterial membranes where they are believed to play important roles in growth, respiration, and photosynthesis. The γ -linolenic acid (GLA, γ C18:3) has an extremely low phase transition temperature (-60 °C) and abounds in membranes of halotolerant *Arthrospira* (*Spirulina*) spp. Its occurrence in *Arthrospira* may be important in protecting the photosynthetic machinery from photoinhibition at low temperatures in a similar fashion as shown in *Synechocystis* strains (Mühling et al., 2005; Tasaka et al., 1996).

Squalene (C30 isoprenoid) is a nonpolar lipid, likely to be located between the lipid monolayers, in the hydrophobic core of the membranes (Hausse et al., 2002). Squalene can act as a barrier decreasing the membrane permeability for ions (Clejan and Krulwich, 1988). In the eukaryotic membranes, squalenes are precursors of cholesterol synthesis, a lipid that increases the membrane rigidity.

The alkaliphilic bacteria were shown to contain high concentrations of squalene and anionic phospholipids, especially cardiolipin (Clejan et al., 1986). Squalene and its synthesizing enzyme have been detected in natronophilic SOB *Thioalkalivibrio* spp., suggesting a reinforced structure of cell membrane (Banciu et al., 2005; Muyzer et al., 2011a).

Recently, a surprisingly high amount of the triterpene compound lanosterol has been detected in *Thioalkalivibrio paradoxus*, comprising 50 % from the total membrane lipids (D. Sorokin, unpublished data). This finding indicates a possible important structural function of sterols in the alkaliphily and warrants the necessity of closer attention. One of the possibilities is that these compounds may help the cell membrane to lower the membrane permeability to ions (i.e., protons, sodium ions) and also to cope with water availability in the surroundings. In this regard, the change of cell surface composition should also involve a change in hydrophilicity (and hydrophobicity) in order to control the water flux across cell envelopes. High salt is usually associated with low water activity and danger of desiccation, while lower salt than the optimal concentration correlates with higher water activity and excessive water inflow that may result in cell lysis. In this aspect it has been demonstrated that the cell surface of Gram-negative halophilic *Halomonas elongata* increased its hydrophilic nature with the elevation of external NaCl concentration (Hart and Vreeland, 1988).

3.1.2. The Acidic Cell Wall of Halo- and Alkaliphiles

In Gram-positive alkaliphilic and halophilic bacteria, the cell wall is clearly playing an important role in protecting the cell against the stress caused by high alkalinity and salinity.

The cell wall of facultatively alkaliphilic *Bacillus halodurans* C-125 is built from peptidoglycan and two acidic polymers: teichuronic acid (TUA) and teichuronopeptide (TUP). TUA consists of galacturonic acid, glucuronic acid,

and *N*-acetylglucosamine, while TUP is a complex of polyglucuronic acid and poly- γ -L-glutamic acid (Aono, 1990). Cell wall-defective mutants of *B. halodurans* C-125 lost their ability to grow at alkaline pH, indicating the essential role of the cell wall in survival at high pH (Aono and Ohtani, 1990). Although not obligatory, the presence of negatively charged polymers in the structure of the alkaliphilic cell wall may favor the attachment of cations (such as sodium and hydronium ions) and repel hydroxide ions (Aono, 1990; Horikoshi, 1999). The presence of TUP seems restricted to cell wall of a few alkaliphilic *Bacillus* species. Only recently, the comparative genome analysis of *Oceanobacillus iheyensis* has revealed the presence of a putative protein showing significant similarity to the *tupA* gene product involved in TUP biosynthesis in *B. halodurans* (Takami et al., 2002). Proteomic and genomic analyses in the extreme facultative alkaliphile *Bacillus pseudofirmus* OF4 have shown that TUP is lacking and is substituted by an S-layer of protein nature (e.g., SlpA) (Gilmour et al., 2000; Janto et al., 2011). Additionally, a capsule made of negatively charged poly- γ -D-glutamate is anchored to the outer surface of the S-layer. Poly- γ -D-glutamate (PGA) occurs in the cell envelope of several non-extremophilic (e.g., *Bacillus amyloliquefaciens*) and facultative halophilic bacteria such as *Planococcus halophilus* and the moderately halophilic *Halobacillus halophilus* (Kandler et al., 1983; Geng et al., 2011). An α -linked L-glutamate polypeptide is present as complex exopolymers in some representatives of the archaea, including the haloalkaliphilic archaeon *Natronococcus occultus* and the extreme halophilic *Natrialba aegyptiaca* (Niemetz et al., 1997; Hezayen et al., 2001).

3.2. OSMOADAPTATION IN HALOALKALIPHILIC AND NATRONOPHILIC BACTERIA

Presence of high sodium chloride and/or sodium (bi)carbonate concentration in the external milieu of living cells results in a high osmotic pressure and low water activity. This situation, often called osmotic stress, is also analogous to a desiccation stress. Cells that are not capable of response to such unusual physical and chemical environmental parameters will lose cytoplasmic water which tends to diffuse outward the cell. Non-halophilic organisms shrink and perish in highly saline conditions. On the other hand, halotolerants and halophiles developed several strategies to counterbalance the high external osmotic pressure. Extreme halophiles are narrowly specialized to live exclusively at high osmolarity and they are harmed and lysed in hypotonic conditions, the presence of too much water becoming a “water stress.” Besides the mechanical support of the cell wall (in Gram-positive bacteria) or a strengthened cell membrane (in archaea and Gram-negative bacteria), adaptation to high salinity/high osmotic pressure (or to variations of such parameters) is aiming to an isosmotic cytoplasm. Osmoadaptation of all living cells has same principle: synthesis and/or accumulation of osmotic compatible solutes (osmoprotectants or osmolytes). Based on their

chemical nature, two main categories of osmolytes are met in nature: inorganic and organic osmolytes. Inorganic osmolytes (i.e., KCl) are imported and accumulated in molar concentrations mainly by neutrophilic halophilic and natronophilic archaea as the so-called “salt-in” strategy of osmoadaptation. Some bacteria within the orders *Natranaerobiales* and *Halanaerobiales* and the renowned example of halophilic bacterium *Salinibacter ruber*, a member of the *Bacteroidetes*, also use KCl as prevailing osmolyte (Oren et al., 2002). Interestingly, short-term osmoadaptation in *Escherichia coli* and *Salmonella typhimurium* involves an initial quick import of potassium ions followed by synthesis of glutamate as counterion. This response is enough only to assure the survival of the bacterial cell. The long-term osmoadaptation in such halotolerants, however, is based on the accumulation and/or synthesis of organic compatible solutes (e.g., trehalose, proline, or glycine betaine) which are less harmful to the cytoplasmic constituents (Oren, 1998).

Almost all known haloalkaliphilic or natronophilic bacteria have adapted to withstand a high osmotic pressure either by taking up or by de novo synthesis of organic compatible solutes (“salt-out” strategy). Unlike inorganic osmolytes, the presence of organic compatible solutes in the cytoplasm is more “friendly” to cell components, especially lipids and proteins. The most preferred organic osmolytes of bacteria are ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) and glycine betaine (*N,N,N*-trimethylglycine), followed by certain amino acids (glutamate, proline) and disaccharides (sucrose, trehalose). Accumulation of osmolytes is part of salt stress adaptation in many known halotolerant nonpathogenic and pathogenic bacteria (e.g., strains of *Escherichia coli*, *Bacillus* spp., *Corynebacterium glutamicum*, *Listeria monocytogenes*). They also may act as thermoprotectants in thermophilic prokaryotes. Organic osmolytes have a wide occurrence in algae (e.g., glycerol in *Dunaliella* spp.) and invertebrates (e.g., trehalose in the brine shrimp *Artemia salina*) living in saline ecosystems.

3.2.1. The Universal Compatible Solute, Glycine Betaine

Glycine betaine, or simply betaine, is a trimethylated derivative of glycine: a highly polar, low molecular weight, and chemically inert molecule. Betaine is broadly used as osmoprotectant in all three domains of life: bacteria, archaea, and eukaryotes (Oren, 2008; Tuteja, 2007; Lim et al., 2007). In bacteria, glycine betaine is the main osmoticum of high-salt-tolerant cyanobacteria, of halophilic anoxygenic phototrophic bacteria, and of salt-tolerant non-halophilic and halophilic heterotrophic bacteria (Imhoff and Rodriguez-Valera, 1984; Oren, 2002).

In halophilic archaea, de novo biosynthesis of glycine betaine is a rarity; it was assigned to one representative of halophilic methanogens, *Methanohalophilus portucalensis* both by physiological and genetic evidences (Robertson et al., 1990; Lai and Lai, 2011). Additionally, osmoadaptation in *M. portucalensis* involves betaine import as well as accumulation of potassium ions up to 1.1 M concentration (Lai et al., 1991). The analysis of the haloarchaeal genomes known by 2011, however, has revealed that 9 out of 10 genomes encode members of the

betaine–choline–carnitine (BCC) transporter family (Anderson et al., 2011). Glycine betaine accumulation is a more preferred strategy in many halophilic bacteria and in some halophilic archaea (e.g., *Methanosarcina mazei*, *Halococcus hamelinensis*) (Mackay et al., 1984; Spanheimer and Müller, 2008; Burns et al., 2012). Several moderately halophilic/halotolerant and alkaliphilic/alkalitolerant Gram-positive bacteria such as *Alkalibacillus filiformis* and *Oceanobacillus oncorhynchi* accumulate glycine betaine and glutamate (Romano et al., 2005b, 2006b). In the halotolerant alkaliphilic *O. iheyensis*, a large number of solute transport proteins have been inferred from genome analysis. Several genes encode OpuD-like ABC transporters that facilitate betaine and choline uptake, as well as putative BCC transporters and Na⁺/proline or pantothenate symporters (OpuE transporters). It seems that the organism, which can grow between 0 and 18 % (3.6 M) NaCl, is employing an unusual high number of osmoprotectant transporters, especially when it must cope with a salinity of at least 3 M NaCl (Takami et al., 2002). Similar genes, although in a lesser number, are also present in genomes of the facultatively alkaliphilic and slightly halotolerant *Bacillus halodurans* C-125 and in the facultatively alkaliphilic *B. pseudofirmus* OF4 (Takami et al., 2000; Janto et al., 2011). Overall it seems that Gram-positive bacteria respond to osmotic stress by activating and using an important number of ABC and BCC transporters and proteins from the sodium/solute symporter (SSS) family. They possess the ability to import betaine when available in the growth medium or to import betaine precursors (i.e., choline) for cytoplasmic synthesis of this compatible solute.

De novo synthesis of betaine in halophiles is restricted to several autotrophic members of the cyanobacteria and the *Ectothiorhodospiraceae* (Oren, 2008). *Halorhodospira halochloris*, *Ectothiorhodospira marismortui*, and *Ect. haloalkaliphila* are well-known examples of glycine betaine accumulation in anoxygenic halophilic and haloalkaliphilic phototrophs (Galinski and Trüper, 1982; Imhoff, 1993). Unusually, in the haloalkalitolerant cyanobacterium *Aphanothece halophytica*, Laloknam et al. (2006) have detected a Na⁺/betaine transporter, BetT_{A. halophytica}. This transporter is a member of BCC transporter family and is related to OpuD in *B. subtilis* and ProU in *E. coli*. It has a high affinity for betaine and differs from Gram-positive OpuD by its low isoelectric point (4.58, in comparison with the basic value pI 9.54 of OpuD from *B. subtilis*). This is the first and only description of a betaine transport system in cyanobacteria to date. Computational analysis of the *Halorhodospira halophila* (strain DSM 244/SL1) genome has revealed the existence of putative BCC transporters (Hhal_2364, Hhal_1851, Hhal_1384) as well as of a glycine betaine/L-proline ABC transporter (Hhal_0233).

Obligately chemolithoautotrophic sulfur-oxidizing bacteria (SOB) of the genus *Thioalkalivibrio* are closely related to *Ectothiorhodospira* spp. and *Halorhodospira* spp., being the dominant sulfur-oxidizing bacteria in soda lakes. Most of the *Thioalkalivibrio* species are true natronophiles, with optimal growth at pH 10 and at 2 M of total Na⁺. Experimental evidence has shown that in

Thioalkalivibrio versutus strain ALJ15 grown in substrate-limited continuous culture at optimal salinity and pH, glycine betaine was the main organic compatible solute. When grown at different salt concentrations, strain ALJ15 accumulated betaine in a salt-dependent fashion: 1.5, 7.5, and 9 % of total dry weight at 0.6, 2, and 4 M Na⁺, respectively. Sucrose was produced as a minor secondary organic compatible solute (0.3–2.5 % of the total dry weight) in this organism, and its concentration was highest in cells grown at 2 M Na⁺. It was clear that *Thioalkalivibrio* spp. grown exclusively on organic-free medium is capable of de novo synthesis of glycine betaine as osmoadaptation strategy (Banciu et al., 2004a, b, 2005). Genome analysis in the *Thioalkalivibrio* spp. K90mix (phenotypically similar to strain ALJ15) and *Thioalkalivibrio sulfidophilus* HL-EbGr7 (phenotypically different from strain ALJ15) has indicated the presence of the genes for glycine sarcosine N-methyltransferase and sarcosine dimethylglycine methyltransferase. In addition, *Thioalkalivibrio* spp. genomes contain the gene for sucrose phosphate synthase needed for the production of sucrose as a minor compatible solute (Muyzer et al., 2011a, b). Biosynthesis of glycine betaine starting from glycine is a three-step methylation process: glycine sarcosine dimethylglycine betaine, and is an energetically expensive pathway impairing growth at extreme salinity conditions (Nyssölä et al., 2001; Oren, 1999). Other chemolithoautotrophic SOB that produce glycine betaine as main compatible solute include the extremely halophilic *Thiohalorhabdus denitrificans* and *Thiohalospira halophila* and the moderately halophilic and facultatively alkaliphilic *Thiohalospira alkaliphila*, all gammaproteobacteria. The first two species are among the most extreme halophilic lithoautotrophs known, with optimal salinity at 18 % w/v NaCl. They were isolated from surface sediments of inland hypersaline lakes in Russia, Crimea, and Central Asia and from a sea saltern at the shore of the Adriatic Sea. *Thiohalospira alkaliphila* was isolated from a hypersaline–alkaline lake in the Wadi Natrun and grows up to pH 10 and within a range of 0.5–4 M NaCl (optimum at 2 M NaCl). All these obligately chemolithoautotrophic SOB were grown on mineral media at 4 M and accumulated betaine up to one third of the total cell mass which was clearly much higher than in their natronophilic counterpart *Thioalkalivibrio* ALJ15 (Sorokin et al., 2008a, c). The reason for this is explained below.

Chemolithoautotrophic SOB such as *Thioalkalivibrio halophilus* HL17^T and *Thioalkalibacter halophilus* ALCO1^T grow well both at high NaCl and Na₂CO₃/NaHCO₃ concentration (up to 4 M of total Na⁺), equally at pH 7.5 and 10. Such versatile, facultatively alkaliphilic high-salt-tolerant halophilic and facultatively natronophilic strains are ideal candidates for a direct study of the physiological effects of chloride versus carbonate/bicarbonate anions at same (high) sodium concentration, as well as at of variable pH. The NaCl-grown biomass of *Tv. halophilus* contained 19.8 % (w/w) glycine betaine, while the soda-grown biomass contained 12.4 % glycine betaine at the same concentration (4 M) of total Na⁺ (Banciu et al., 2004b). A comparable trend has been observed in haloalkaliphilic *Thioalkalibacter halophilus*. Unlike *Tv. halophilus*, strain ALCO1 is producing

ectoine as the main compatible solute and betaine as a minor one. The specific concentration of osmolytes (ectoine and glycine betaine) measured in strain ALCO1 cells grown at 3 M NaCl (pH 7.5) was approximately two times higher than in cells grown at 3 M Na-soda, pH 10 (Banciu et al., 2008). Dissimilarities in the production of compatible solutes in NaCl- and soda-grown cells could be at least partially explained by physicochemical differences between NaCl and $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ solutions. A decisive parameter that may influence the accumulation of osmolytes in halophiles and natronophiles could be the osmotic pressure of the chloride- and soda-rich media which is determined primarily by the electrolytic features of two types of sodium salts (see Table 3 in Banciu et al., 2004b). The calculated osmotic pressure in the 4 M NaCl solution was 2.8 times higher than that of 2 M Na_2CO_3 (= 4 M Na^+). This result is reasonably close to the experimentally measured osmotic pressure in growth media with 4 M NaCl and 4 M Na^+ soda (as a mixture of $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$), at 30 °C, a value which is 1.8 times higher in chloride medium than in carbonate medium. This clearly indicates that apart from the many negative effects of high alkalinity pH, the life in soda brines also has some benefits as compared to the life in NaCl-rich environment. Such physiological experiments on model organisms, complemented by theoretical simulations, could add more hard evidence to our suggestion of using the term natronophily as a special, yet different, form of halophily. As sodium sulfate brines have essentially the same electrolytic properties as sodium carbonate, one may expect that, except for the high pH, the osmoadaptation pattern of natronophiles put into Na_2SO_4 brines might also be similar.

3.2.2. Ectoine Is a Multivalent Compatible Solute

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is a derivative of aspartate. It was discovered in the phototrophic bacterium *Halorhodospira halochloris* (Galinski et al., 1985). In a similar manner as betaine, ectoine is accumulated by import and/or synthesis in salt-stressed cells. Unlike glycine betaine which is ubiquitous among bacteria, ectoine is the preferred compatible solute in many aerobic chemoheterotrophic eubacteria. The hydroxylated derivative of ectoine, β - (or 5)-hydroxyectoine, is more frequently found among halophilic and halotolerant Gram-positive bacteria (Severin et al., 1992; Detkova and Boltyanskaya, 2007). Ectoine alone is a minor compatible solute in the anoxygenic phototrophic *Halorhodospira* spp., while together with hydroxyectoine, ectoine is the dominant osmolyte in *Halomonas* spp., *Salinivibrio* spp. among the *Proteobacteria*, and in *Marinococcus* spp. and *Virgibacillus* spp., members of the *Firmicutes* (Severin et al., 1992; Grant, 2004).

By using ^{13}C -NMR spectroscopy and HPLC analysis, Kuhlmann and Bremer (2002) have proven de novo synthesis of ectoine in a variety of *Bacillus* and *Bacillus*-related species grown under salt stress conditions. The obligate alkaliphilic *B. alcalophilus* synthesized ectoine, the halophilic and alkalitolerant *Virgibacillus salexigens* produced both ectoine and hydroxyectoine, whereas *Virgibacillus pantothenicus* synthesized both ectoine and proline. The

ability to synthesize ectoine from L-aspartate-semialdehyde in a three-step pathway is widespread within the genus *Bacillus* and closely related taxa. In these Gram-positive microbes, ectoine biosynthetic genes, *ectABC*, encode diaminobutyric acid (DABA) acetyltransferase (EctA), DABA aminotransferase (EctB), and ectoine synthase (EctC). Expression of *ect* genes is controlled by salinity or osmotic pressure outside the cell (Louis and Galinski, 1997). The *ectABC* genes and the proteins for ectoine synthesis are highly conserved among the *Proteobacteria* and the *Actinobacteria* that synthesize ectoine (Lo et al., 2009).

In certain species (e.g., *Halomonas elongata*), stress conditions such as elevated temperature trigger hydroxylation of some ectoine to form hydroxyectoine. In species capable of hydroxyectoine formation, two different genes, *ectD* and *ectE*, coding for ectoine hydroxylase have been identified (*ectD* in *Virgibacillus salexigens* and *ectDlectE* in the halotolerant gammaproteobacterium *Chromohalobacter salexigens*) (Bursy et al., 2007; García-Estepa et al., 2006).

Most ectoine synthesizing species have also developed ectoine import systems. In *V. pantothenicus*, another gene, *ectT*, encodes a protein (EctT) that is a member of the BCC carriers. The EctT transporter specifically mediates the import of ectoine and hydroxyectoine but also possesses minor uptake activities for proline and glycine betaine (Kuhlmann et al., 2011).

In the extremely halotolerant *Halomonas elongata* DSM 2581^T, Grammann et al. (2002) discovered a novel solute transporter, termed TeaABC, that belongs to the TRAP (tripartite ATP-independent periplasmic) transporters family. The osmoregulated transporter TeaABC is encoded by three genes (*teaABC*), and it mediates the uptake of ectoine and hydroxyectoine. Its assumed physiological role in *H. elongata* would be the recovery of ectoine that is excreted or lost in the surroundings of the cell by a yet unknown mechanism. Active recovery of ectoine (and/or other organic osmolytes) after solute excretion upon osmotic downshock might be an energy-saving strategy for salt tolerance in organisms that must cope with shifts of salinity in their environment (Oren, 1999). The amino acid sequences of the small, TeaB (YP_003899344.1) and large, TeaC (YP_003899345.1) transmembrane components of TeaABC carrier from *H. elongata* are significantly similar (40 and 56 %, respectively) to those of the small (NP_244258.1) and large (NP_244257.1) subunits of TRAP transporter deduced from the *B. halodurans* genome.

In aerobic halophilic and halotolerant methylobacteria from soda lakes, the main compatible solute is ectoine, followed by glutamate and sucrose. The total pool of compatible solutes is enlarged by increasing NaCl concentration in *Methylobaculum alcaliphilum*, *Methylophaga natronica*, *M. alcalica*, and *M. lonarensis* (Trotsenko and Khmelenina, 2002; Doronina et al., 2003a, b; Trotsenko et al., 2007; Antony et al., 2012). The gene cluster *ectABC* has been identified in *Methylobacter marinus* and the natronophilic *Methylobaculum kenyense* AMO1^T. In other C₁-utilizing bacteria (e.g., the haloalkaliphilic *Methylobaculum alcaliphilum* ML1 and *Methylophaga alcalica* M8, and the

moderate halophilic *Methylophaga thalassica* 33146^T and *Methylarcula marina* h1^T) the *ectABC-ask* operon is additionally present, encoding aspartate kinase (Ask). Upstream the gene cluster *ectABC-ask*, another gene, *ectR*, encodes the MarR-like transcriptional regulator named EctR. In methano/methylotrophic bacteria hydroxyectoine is lacking, a fact proven by the absence of *ectD*-like genes (Reshetnikov et al., 2011).

Chemolithoautotrophic natronophilic and haloalkaliphilic SOB from the genera *Thioalkalimicrobium* and *Thioalkalibacter* also produce ectoine as the prevailing compatible solute (Banciu et al., 2005, 2008). The production of ectoine in *Thioalkalimicrobium aerophilum* AL3^T is clearly stimulated by increasing the total Na⁺ concentration from 0.2 to 1.2 M in the growth medium. At the highest salinity tolerated by the organism (1.2 M of total Na⁺), ectoine accounted for 8.7 % of the total dry weight. Another organic osmolyte detected in strain AL3^T in minor amounts was glutamate. The glutamate production decreased with increasing salt concentration (Banciu et al., 2005). Apparently, species of *Thioalkalimicrobium* are also capable of ectoine uptake by the TRAP system. A BLASTP search for the 388 amino acid sequence of a putative extracellular solute-binding protein, family 7, from *Thioalkalimicrobium aerophilum* AL3^T (Accession number ZP_08933362.1) has retrieved 71 and 57 % identity with the TRAP dicarboxylate transporter – DctP subunit from *Thiomicrospira crunogena* XCL-2 (YP_392275.1) and *Marinomonas* sp. MWYL1 (YP_001343266.1), respectively, and 50 % identity score with the TRAP transporter substrate-binding protein from *Halomonas elongata* DSM 2581 (YP_003899494.1). The phylogenetic relationships of the DctM subunit of TRAP-like proteins found in several alkaliphilic, haloalkaliphilic, and halophilic bacteria are presented in Fig. 1a. Moreover, genome sequences of *Thioalkalimicrobium* spp. have also indicated the possible presence of BCC transporters in these SOB.

Although ectoine and betaine require comparable energy investments from the cell metabolism, there are a number of advantages of using ectoine over glycine betaine. Ectoine and hydroxyectoine are not only involved in osmoprotection of salt-stressed cells, but they are required for heat- and cold-shock adaptation of bacterial cells (Bursy et al., 2008; Kuhlmann et al., 2011). Hydroxyectoine has protective and stabilizing effects on proteins, including enzymes, when cells are exposed to thermal stress or desiccation, apparently playing same role as mannosylglycerate compounds in archaea (Lippert and Galinski, 1992; Borges et al., 2002). Ectoines might enhance the fluidity of the cell membrane through increasing the hydration of the surface and the mobility of the lipid head groups (Harishchandra et al., 2010).

Besides its prevalent osmoprotective role, ectoine can act as a nitrogen storage compound, as carbon and/or energy source (Galinski and Herzog, 1990; Khmelenina et al., 1999; Vargas et al., 2006). It can be degraded and reused by the cells. Ectoine degradation proceeds via hydrolysis to N α -acetyl-L-2,4-diaminobutyric acid, followed by deacetylation to diaminobutyric acid. In *Halomonas elongata*, diaminobutyric acid can either generate aspartate or

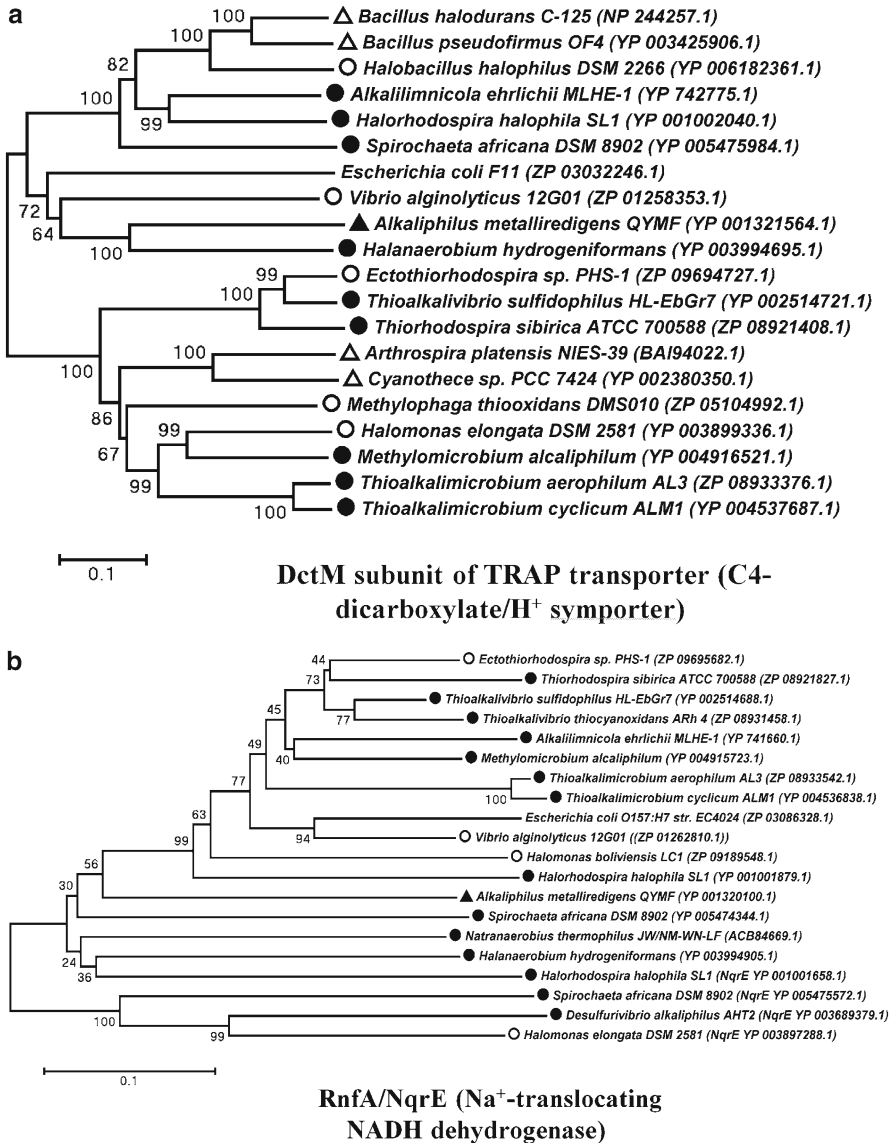
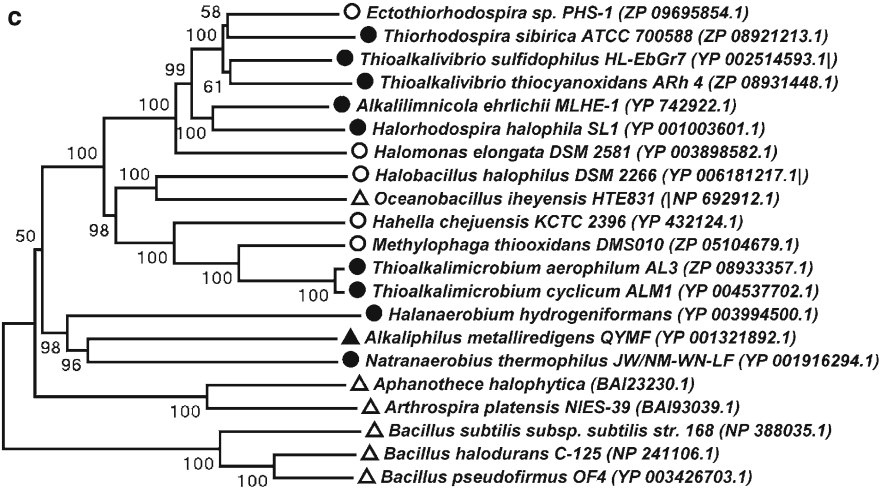
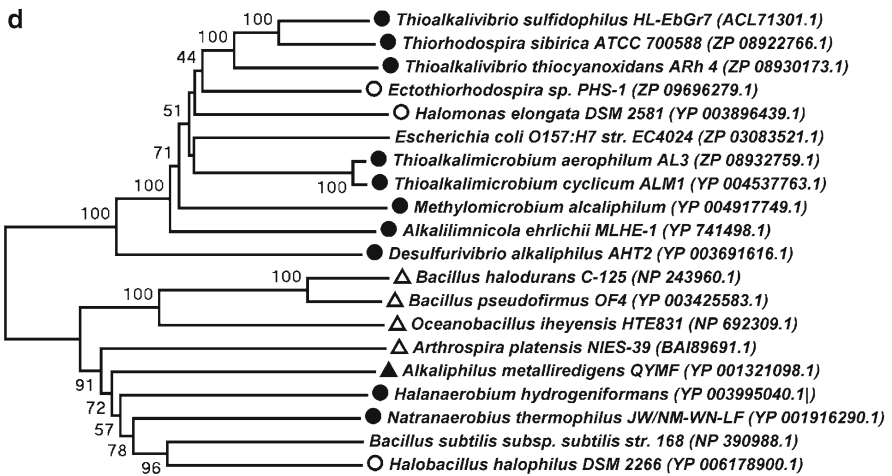


Figure 1. Phylogeny of four transporters essential for haloalkaliphilic adaptation: DctM (a), RnfA/NqrE (b), MrpA/MnhA (c), and TrkH/TrkH-like proteins (d) from different haloalkaliphilic (filled circles), halophilic or halotolerant (open circles), and alkaliphilic/alkalitolerant bacteria (filled triangles/open triangles). Proteins of non-extremophilic bacterial species were used as reference sequences. Gene products or proteins were selected after BLASTP analysis. Subsequently, the selected protein sequences were aligned by ClustalW. Evolutionary analyses were conducted in MEGA5 by using the neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The bar indicates 10 % sequence difference. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in units of the number of amino acid differences per site. All ambiguous positions in amino acid sequences were removed for each sequence pair.



MrpA/MnhA subunit of Mrp or Mrp-like complex (Na⁺/H⁺ antiporter, CPA3 family)



TrkH/TrkH-like subunit of Trk system (K⁺/H⁺ antiporter)

Figure 1. (continued)

reenter the ectoine synthesis pathway, forming a cycle of ectoine synthesis and degradation. However, a comparison of the available bacterial genomes indicated that the ectoine degradation pathway exists predominantly in non-halophilic bacteria unable to synthesize ectoine. Energetic considerations indicated that ectoine turnover might be fast and it could be finely tuned by the cell's necessity to respond promptly to changing conditions (Schwibbert et al., 2011). On the other hand, occurrence of ectoine as the main compatible solute in halotolerant and halophilic alkaliphilic aerobic methane-oxidizing bacteria as well as in low-salt-tolerant natronophilic *Thioalkalimicrobium* spp. is correlated with the limitation of their tolerance to high salt (<15 % w/v NaCl). At the same time, most of haloalkaliphilic phototrophic SOB (e.g., *Halorhodospira* spp.), natronophilic SOB (e.g., *Thioalkalivibrio* spp.) and SRB (e.g., *Desulfonatronospira* spp.), and extremely halophilic SOB (i.e., *Thiohalorhabdus* spp., *Thiohalospira* spp.) thrive under a broad range of salinity up to extreme salinity (>20 % w/v NaCl), and this ability is often associated with the use of betaine as the main compatible solute (Oren, 1999; Banciu et al., 2005; Sorokin et al., 2008b; Muyzer et al., 2011a, b). These observations allow us to conclude that the glycine betaine is a more efficient osmolyte at extreme salt concentration.

3.2.3. Glutamate as an Additional Anionic Osmolyte

Glutamate is an acidic compatible solute usually associated with low-salt response in halotolerant non-halophilic marine bacteria, moderately halophilic bacteria from the *Firmicutes* and the *Gammaproteobacteria*, and methanogenic archaea. In most cases, glutamate is accompanied by various other compatible solutes (potassium ions, glycine betaine, glutamine, ectoine, proline). As already stated, some aerobic methylotrophic and chemolithoautotrophic SOB bacteria from soda lakes are capable of salt-dependent glutamate production.

In the moderately haloalkaliphilic *O. iheyensis* and *B. halodurans*, glutamate synthesis from the branched-chain amino acids (i.e., leucine, isoleucine, and valine) is thought to play a notable role in alkaline adaptation. The putative ABC transporters for branched-chain amino acids identified in their genomes could facilitate the uptake of glutamate precursors. Since L-glutamic acid is negatively charged at pH values above its pKa (3.9 or 4.3), the converted L-glutamic acid and its accompanying proton could contribute to the cytoplasmic pH which is about two units lower than the external pH of around 10.5 (Krulwich et al., 2007; Takami, 2011).

3.2.4. Sucrose and Trehalose: Minor Osmolytes with Stabilizing Roles

Nonreducing disaccharides such as sucrose and trehalose are energy costly compounds involved in osmotic stress adaptation of many freshwater and low-salt-tolerant cyanobacteria (e.g., *Synechococcus* spp., *Phormidium* spp.) and non-halophilic and slightly halophilic bacteria when they need to adapt to elevated salt concentrations (Mackay et al., 1984; Oren, 1999). Unlike polyols and amino acid derivatives used as osmolytes, sucrose and trehalose have a lesser stabilizing

effect on enzymes at high salt and have lower water solubility. Disaccharides and their derivatives are the most energetically expensive compatible solutes, and therefore, their synthesis should be carefully managed in salt-stressed cells. As main compatible solute (5 % w/w, at 2.5 M Na⁺), sucrose is present in the moderately halophilic SRB *Desulfonatronovibrio*, together with the rare osmolyte compound *N*-acetylglutaminylglutamine amide (N-AGGN) found at an approximate concentration of 2 % w/w (Sorokin et al., 2011c). Sucrose, in a mixture with the amino acid glutamate, is used as a secondary compatible solute in aerobic methylotrophs from soda lakes, where sucrose increased significantly at the higher growth limit of salinity (Doronina et al., 2003a, b). As a minor osmolyte, sucrose is also present in chemolithoautotrophic SOB of the genus *Thioalkalivibrio*, where it reaches a maximum concentration (2.5 % of cell dry weight) at the optimal total sodium concentration (2 M) (Banciu et al., 2005).

Trehalose has multiple uses in osmoprotection, as thermolyte and as anti-drying agent in low-salt-tolerant cyanobacteria, halophilic actinomycetes, moderately halophiles, and thermophilic bacteria (Mackay et al., 1984; Alarico et al., 2005; Roberts, 2000). In the extremely halophilic actinomycete *Actinopolyspora halophila* grown at 24 % NaCl, Nyssölä and Leisola (2001) showed that main compatible solute was betaine (33 % of the cellular dry weight), followed by a significant amount of trehalose (9.7 % w/w). In the moderately halophilic sulfate-reducer *Desulfovibrio halophilus* grown at 15 % NaCl, trehalose is accumulated as principal osmolyte in the absence of exogenous betaine (Welsh et al., 1996). In combination with ectoine, trehalose is implicated in the thermal tolerance of halophilic *Chromohalobacter salexigens* (Reina-Bueno et al., 2012). Recent progress on deciphering prokaryotic genomes has allowed identification of genes for trehalose synthesis in halotolerant cyanobacteria and in halophilic and haloalkaliphilic bacteria and archaea. The widely distributed pathway of trehalose synthesis that involves trehalose-6-phosphate synthase (TPS) and trehalose-phosphatase (TPP) could be inferred from genomes of *Synechococcus* spp., *Synechocystis* spp., *Halalkalicoccus jeotgali*, *Haladaptatus paucihalophilus*, *Haloterrigena turkmenica*, *Natronobacterium gregoryi*, *Natrialba magadii*, etc., as well as from those of the halophilic bacterium *Salinibacter ruber* and the haloalkaliphilic “Halanaerobium hydrogenoformans.” Another pathway converts maltodextrins (maltooligosaccharides, glycogen, and starch) to trehalose in two enzymatic steps catalyzed by maltooligosyl trehalose synthase (TreY) and maltooligosyl trehalose trehalohydrolase (TreZ), respectively (Elbein et al., 2003). Trehalose synthesis by TreY and TreZ has been observed in archaea belonging to *Sulfolobus* but can also be assumed from the genome sequence of the haloalkaliphilic methanotrophic bacterium *Methylomicrobium alcaliphilum* (Avonce et al., 2006; personal BLASTP search). It is worth mentioning that exogenous trehalose can be taken up by specialized import systems (e.g., phosphotransferase system – PTS – trehalose-specific enzyme II, and trehalose/maltose binding protein, TMBP) found in a large number of heterotrophic bacteria and archaea, as concluded from a BLASTP analysis of available amino acid sequences.

Following its uptake, trehalose is phosphorylated to trehalose-6-phosphate which can be further degraded by trehalose-6-phosphate hydrolase (TreA in *B. subtilis* and TreC in *E. coli*) to provide the cells with glucose (Helfert et al., 1995; Rimmele and Boos, 1994). TreA-like proteins are present in halophilic and alkaliphilic *Bacillus* species (*Bacillus halodurans*, *B. clausii*, *B. selenitireducens*, and *B. pseudofirmus* OF4), as well as in *Oceanobacillus iheyensis* and *Halobacillus halophilus*.

3.2.5. K^+ Is the Main Osmolyte in Anaerobic Extreme Haloalkaliphiles

The natronophilic acetogenic *Natroniella acetigena* from the order *Halanaerobiales* as well as halophilic thermoalkaliphiles belonging to the *Natranaerobiales* (*Natranaerobius* spp. and *Natronovirga* spp.) have adopted the salt-in strategy to withstand high salinity conditions. In *N. acetigena*, the measured intracellular concentrations of K^+ (ca. 0.9 M) were almost two orders of magnitude higher than the K^+ level in the medium. At the same time, intracellular Na^+ and Cl^- concentrations were close to the extracellular values (Detkova and Boltyanskaya, 2007). Intracellular K^+ concentration in representatives of the *Natranaerobiales* is reliably kept at constant values of 0.2–0.3 M over a wide range of external K^+ (8–400 mM), at optimal or suboptimal pH and salinity. Apparently, in halophilic alkalithermophiles of the order *Natranaerobiales*, potassium homeostasis is tightly regulated. Accumulation of K^+ is accompanied by that of Cl^- ions, which reach molar concentration. However, the sum of both ions does not equilibrate the cytoplasm osmolarity with that of the outer environment. The genome analysis of *Natranaerobius thermophilus* has revealed the ability of this organism to import and even to synthesize glycine betaine. Apparently, this finding was confirmed experimentally. Intracellular concentrations of glycine betaine and glutamate have been detected in molar concentrations in *Natranaerobius thermophilus*. Such an observation, if confirmed by a detailed experimental study, would constitute a first known case of a fermentative extreme halophile capable of producing organic compatible solutes and use the “salt-in” strategy concomitantly (Mesbah and Wiegel, 2012).

3.3. ADAPTATION OF THE OXIDATIVE PHOSPHORYLATION (OXPHOS) MACHINERY TO OVERCOME LOW *PMF*

In highly saline and alkaline environments, the burden due to elevated osmotic pressure is doubled by an extremely low level of H^+ concentration. The bulk medium where haloalkaliphiles and natronophiles live is weakly supporting a favorable proton-motive force (*pmf*) to drive ATP synthesis by oxidative phosphorylation pathway as postulated by Peter Mitchell. Despite the apparently adverse pH gradient, alkaliphilic and, implicitly, the haloalkaliphilic prokaryotes flourish at alkaline conditions. Moreover, haloalkaliphiles are splendidly facing the challenge of expensive life in a hyperosmotic medium by synthesizing energetically costly compatible solutes. To respond to the multiple extreme factors, which additionally include high irradiation and, sometimes, relatively high temperatures

(45–60 °C), true haloalkaliphilic and natronophilic microbes must have an efficient energy metabolism. The adaptative modifications are met not only in the core of the ATP synthesis process but are also found in the respiratory chain components and in the overall organization of the cytoplasmic enzyme machinery. In the following we will briefly discuss the alkaliphilic adaptations of the respiratory chain components as it is best known from several model organisms.

3.3.1. Features of Alkaliphilic F-Type ATP Synthase

Three major ways of generating the ATP are known in various haloalkaliphiles and natronophiles: (i) membrane-bound oxidative phosphorylation in aerobic chemotrophs, (ii) photophosphorylation linked to photosynthetic inner membranes of aerobic and anaerobic phototrophs, and (iii) substrate-level phosphorylation of anaerobic (or fermentative) bacteria. Mixed sources of ATP could be found in non-obligately metabolic situations such as facultatively aerobes and in anaerobic phototrophs that rely on either (i) and (iii) or (ii) and (iii). One of the most intriguing types of energy metabolism occurs in obligately aerobic chemolithoautotrophic alkaliphilic bacteria, where anabolic maintenance and active transport processes rely almost exclusively on oxidative phosphorylation which is directly affected by the apparently suboptimal proton electrochemical gradient.

Oxidative phosphorylation (OXPHOS) is a membrane-localized metabolic pathway that couples the generation of an electrochemical gradient with ADP phosphorylation to form ATP. In prokaryotes the transmembrane electrochemical gradient, usually based on protons, is the result of the respiration process. Respiratory chain components perform simultaneous electron transfer from an internal or external donor to an oxygen molecule as the final electron acceptor, coupled with proton pumping across the membrane toward the outer or positive (P) side of the cell. The free Gibbs energy stored in the electrochemical gradient is transferred to chemical bonds within the ATP molecule by the ATP synthase activity of F-type primary pump. Universally found in all three domains of life, F-ATP synthase is a reversible enzyme that catalyzes both ATP synthesis and hydrolysis. The hydrolytic activity of the enzyme is mainly observed in anaerobes, while in many other bacteria, this function is low or latent. The equilibrium between the hydrolytic and the synthetic function of F-type ATP synthase is carefully controlled to maintain appropriate cytoplasmic ATP and ADP concentrations. The catalytic domain of the ATP synthase complex (also termed F_1) is located in the cytoplasm. The F_1 portion of ATP synthase consists of $\alpha_3\beta_3\gamma\delta\epsilon$ subunits and is relatively well conserved among all organisms. The transporting part of the F-ATP synthase (F_0) is a transmembrane complex made of a variable number of subunits (ab_2c_{10-15} in bacteria) facing the external milieu of the cell, as well as the hydrophobic membrane. The F_0 factor of ATP synthase is highly variable among different prokaryotic species. The critical issue that functioning of ATP synthase toward ATP synthesis must address is the apparently low electrochemical gradient. Therefore, it is expected that specific structural adaptations of this key enzyme to be found in the *a*-, *b*-, and *c*-subunits of the F_0 part.

The alkaliphilic adaptations of the F-ATP synthase and OXPHOS both at the physiological and the genetic levels have been documented in-depth in the aerobic, facultatively alkaliphilic bacterium *Bacillus pseudofirmus* OF4 (for review, see Hicks et al., 2010). As previously mentioned in this review, the facultative alkaliphilic and/or halophilic organisms are appropriate models to study the changes and adaptations at the genotype and phenotype levels.

There are certain key features of the alkaliphilic F_1F_0 -ATP synthase from *B. pseudofirmus* OF4 that allow ATP synthesis under conditions of low *pmf*, and they are summarized below together with general and particular considerations regarding other alkaliphiles.

1. *The functioning of the alkaliphilic F-ATP synthase toward ATP synthesis is H⁺-coupled.* So far, all F-type ATP synthases with a high rate of ATP synthesis from true and facultative alkaliphiles are proton-coupled. The recent examples of proton-driven F₁F_o-ATP synthase deduced from genome analysis are the aerobic lithoautotrophic extreme natronophilic *Thioalkalivibrio* spp. that demands high sodium concentration for optimal respiratory activity and growth (Banciu et al., 2004a; Muyzer et al., 2011a, b) and the anaerobic natronophiles with respiratory metabolism *Desulfurivibrio alkaliphilus* and *Desulfonatrosospira thiodismutans* (Sorokin et al., 2008b, d; Hicks et al., 2010). In anaerobic alkaliphilic, alkalitolerant, or haloalkaliphilic bacteria, ATP is almost entirely formed by substrate-level phosphorylation. However, Na⁺-driven F-type ATP synthase is present in several species with various degrees of alkaliphily (e.g., the alkalithermophilic *Clostridium paradoxum* and the halophilic thermoalkaliphilic *Natranaerobius thermophilus*) (Ferguson et al., 2006; Mesbah and Wiegel, 2011). The only cyanobacterium with Na⁺-translocating ATPase activity reported so far is the alkaliphilic and halotolerant *Aphanothece halophytica* (Soontharapirakkul and Incharoensakdi, 2010). In these bacteria, a membrane-bound V-type ATPase works at the expense of ATP to translocate Na⁺ outwardly, thus contributing to sodium homeostasis in cytoplasm but not to the energy generation. Same type of the sodium-coupled ATPase has been detected in the genomes of a natronophilic clostridium *Dethiobacter alkaliphilus* (Sorokin et al., 2008d; Hicks et al., 2010) and a fermentative low G+C firmicutes *Amphibacillus* (Kaieda et al., 1998; Satoh and Koyama, 2005).
2. *The intimate construction of alkaliphilic F-ATP synthase favors the catalysis of ATP synthesis and blocks the hydrolytic activity.* The regulation of bacterial F-ATP synthase activity is achieved by two major mechanisms. Tight but reversible binding of ADP as magnesium salt is an effective inhibitory process that slows the ATPase activity in all known F-ATP synthases (Minkov et al., 1979; Feldman and Boyer, 1985; Konno et al., 2011). In bacterial and chloroplast F-ATP synthase, the ϵ subunit is an “endogenous inhibitor.” Fine-tuning of conformational changes in the ϵ subunit is controlled by the *pmf* and the ATP/ADP ratio (Kato-Yamada et al., 1999; Suzuki et al., 2003). It is very likely that haloalkaliphilic ATP synthase is not exceptional in this regard.

For the Na⁺-translocating ATP synthase from *Natranaerobius thermophilus*, with a high hydrolytic activity and a low capacity for ATP synthesis, Mesbah and Wiegel (2011) have proposed a regulatory mechanism mediated by the ϵ subunit.

3. Most of adaptive modifications (as compared with the neutralophilic ATP synthase) have been identified in the F_0 factor.

In the alkaliphilic *Bacillus* or *Bacillus*-related species (i.e., *B. alcalophilus*, *B. halodurans*, *B. pseudofirmus* OF4, *B. selenitireducens*, and *Caldalkalibacillus thermarum* TA2.A1), the c -ring is made up of 13 subunits, a slightly higher number than that of decameric c -ring of most neutrophilic cells. Accordingly, the H⁺ to ATP ratio in these alkaliphiles would be 4.3, higher than the more usual 3.3 proton equivalents for one mol of synthesized ATP as calculated for neutrophilic ATP synthase (Hicks et al., 2010). Comparative analysis of genomic sequences revealed alkaliphilic-specific sequences in the genes coding for a - and c -subunits as well as a different pattern of alkaliphilic motifs in the Gram-positive alkaliphiles as compared with Gram-negative ones. Moreover, different levels of alkaliphily are accordingly reflected in these sequences (Hicks et al., 2010). Two specific amino acid sequences related to alkaliphily in *Bacillus* species were observed in the primary structure of the c -subunit: AxAxAXA – or at least three alanine residues, and PxxExxP – two proline residues flanking the glutamic acid with a critical carboxylate residue. The first motif is apparently contributing to spatial ordination of the c -ring, while the second is responsible for ion binding (Wang et al., 2004; Liu et al., 2009).

Another peculiarity of the primary structure of alkaliphilic c -subunits is the presence of a threonine residue (Thr³³) near the loop edge of the hairpin-like structure, which may influence the dynamics of the rotor part of the ATP synthase edifice. Recently, a high resolution crystal structure of the rotor ring in *B. pseudofirmus* OF4 unveiled a rather “tulip beer glass” shape of the c -subunit ring, different from the “hour-glass” shape of the c -rotors in other bacteria. At the same time, the alkaliphilic motifs in c -subunits promote tight proton binding in the ion-binding site in the form of a tetra-coordinated hydronium ion (H₃O⁺) (Preiss et al., 2010).

A brief comparative analysis by multiple alignments of available sequences for ATP synthase c -subunits from several haloalkaliphilic species (the aerobic Gram-negative *Thioalkalivibrio* spp., *Thioalkalimicrobium* spp., and *Methylo-microbium alcaliphilum*; anaerobic Gram-negative *Thiorhodospira sibirica* and *Ectothiorhodospira* spp.; anaerobic Gram-positive *Alkaliphilus* spp.) indicated the presence of “non-alkaliphilic” motif GxA/GxGxG, the same motif as found in c -subunit of *Escherichia coli* or *Oceanobacillus iheyensis* (see also Hicks et al., 2010). When searching for the second motif of c -subunit, analogous to alkaliphilic PxxExxP, same observation applied: non-alkaliphilic GxxD/ExxP/T motif has been detected in all surveyed haloalkaliphiles. Thus, it seems that minute changes in the last amino acid of the N-terminus motif would influence the

kinetics of ion binding at the nearby glutamate/aspartate residue. Thr³³ was not found, but all analyzed extremophiles contain the nonpolar Ala³³ residue instead.

In the stator part of F₁F₀-ATP synthase, the *a*-subunit of true alkaliphilic *Bacillus* spp. contains at least two crucial amino acids, Lys¹⁸⁰ and Gly²¹², that are not found in the neutrophilic counterpart. Situated at the interface between the *a*- and the *c*-ring, Lys¹⁸⁰ and Gly²¹² are essential for ATP synthesis at elevated pH, being probably implicated in proton capturing and transferring to the rotor ring of ATP synthase (Fujisawa et al., 2010). Our brief search within the sequence databases has indicated the substitution of Lys¹⁸⁰ and Gly²¹² in the haloalkaliphilic *Thioalkalivibrio* spp., *Thioalkalimicrobium* spp., *Methylochromobium alcaliphilum*, and *Thiorhodospira sibirica* in an *E. coli*-like pattern (Lys¹⁸⁰/Gly²¹² being replaced with Gly/His or Lys). These substitutions in places that face each other in trans-membrane helices 4 and 5, respectively, could ensure similar microenvironment for transferring the protons as it does in the *a*-subunit of alkaliphilic *Bacillus* spp. Additionally, the external surface of the *a*-subunit in alkaliphilic *Bacillus* spp. is somewhat more polar than the analogous region of non-alkaliphilic *Bacillus* species or *E. coli*. This region may have an enhanced capacity of retaining H⁺ on the outer surface of the membrane (Wang et al., 2004).

The molecular adaptations of ATP synthase from alkaliphilic, aerobic, and Gram-positive species only partially explain the ability of such organism to withstand a broad range of pH and/or extreme alkalinity. In particular, the phosphorylation potential (ΔG_p), the parameter that reflects the energy required to sustain the [ATP]/[ADP][Pi] ratio, is unexpectedly high under conditions in which the *pmf* is relatively low. As discussed by Hicks et al. (2010), in *B. pseudofirmus* OF4 grown at pH 10.5, the measured *pmf* is -50 mV, while ΔG_p is 478 mV. Similarly, in the thermophilic *Caldalkalibacillus thermarum* grown at pH 10, the observed *pmf* was -78 mV, while ΔG_p was around 430 mV. Close values (*pmf* = -58 to -89 mV, and ΔG_p = 477–491 mV) were measured in a sucrose-energized cell suspension of *Natranaerobius thermophilus* (Mesbah et al., 2009). In all situations, the observed ΔG_p is close to that measured in the neutrophiles. However, in the obligately anaerobic fermentative haloalkaliphile, the measured ΔG_p does not necessarily reflect the capacity of ATP synthesis by OXPHOS. Based on the relation $[H^+]/[ATP] = \Delta G_p / pmf$ and assuming the value of 3.3 for $[H^+]/[ATP]$, the theoretical *pmf* value that supports the observed ΔG_p would be around two times higher. A slightly increased $[H^+]$ to $[ATP]$ ratio of 4.3, corresponding to tridecameric *c*-rings of *B. pseudofirmus* and *C. thermarum*, does not suffice to obtain such a phosphorylation potential (Hicks et al., 2010). In this light, the hypotheses of localized proton gradients or local proton microcircuits that boost the efficiency of proton transfer, sequestration, and transport from respiratory chain components to the ATP synthase complex have been now revived since Williams proposed the proton circuits theory in 1961 (Williams, 1988; Guffanti and Krulwich, 1992; Hicks et al., 2010). In support of this theory, some evidence has risen. Localized microcircuits of protons and fast proton diffusion alongside the membrane surface have been directly measured in liposomes

by using a fluorescence correlation spectroscopy (FCS)-based approach (Brändén et al., 2006). Several other reports exist on the possibility of faster diffusion of protons along the lipid and/or protein surface of membranes than the expelling of protons out into the bulk phase (Heberle et al., 1994). Protons extruded during respiration were apparently retained close to the outer surface of the cell membrane in alkaliphilic *B. clarkii* K24-1U, but this observation needs further confirmation (Yoshimune et al., 2010).

3.3.2. Adaptation of Respiratory Chain Components at Alkaline pH

The aerobic facultatively alkaliphilic *Bacillus pseudofirmus* OF4 as well as *B. halodurans* C-125 and *Oceanobacillus iheyensis* have a primary H⁺-extrusion respiratory chain with *caa3*- and *bd*-type terminal oxidases (Krulwich et al., 1998; Takami et al., 2000, 2002). Additionally, cytochrome *bo3* oxidase is inferred from the *O. iheyensis* and *B. halodurans* genomes (Takami et al., 2000, 2002). Cytochrome *caa3* oxidase is induced during growth at alkaline pH. Liu et al. (2007) observed close physical interaction between *caa3* oxidase and F₁F₀-ATP synthase that might increase the efficiency of proton transfer to the F-ATP synthase. Cytochrome *bd* is a non-proton-pumping quinol oxidase that is upregulated during the alkaline pH adaptation in *E. coli* K-12 concomitantly with a decrease in expression of proton-extruding complexes (cytochrome *o* and types I and II NADH dehydrogenases) of the respiratory chain. Such a concerted response would probably diminish the export of protons from the cytoplasm during generation of *pmf* (Maurer et al., 2005).

The midpoint redox potential of cytochrome *c*, one of the main components of the respiratory chain, is considerably lower (< +100 mV) in alkaliphilic than in neutrophilic bacteria (approx. +220 mV). At the same time, the cytochrome *caa3* oxidase (complex IV) that accepts electrons from cytochrome *c* has an apparently normal redox potential (approx. +250 mV) (Muntyan and Bloch, 2008). Moreover, cytochrome *c* is highly abundant in alkaliphilic *Bacillus* when grown at alkaline pH (for reviews, see Hicks and Krulwich, 1995; Krulwich et al., 1998). It is suggested that the overall construction of the respiratory chain permits a quicker and more efficient H⁺ translocation and electron flow along the membrane in alkaliphiles than in neutralophiles (Goto et al., 2005).

In the aerobic natronophilic SOB, dominant cytochromes are both of the *c*- and *b*-type, in *Thioalkalivibrio* spp., and *c*-type only in *Thioalkalimicrobium* spp. Terminal oxidases have been identified as *caa3*-, *ccb3*- and *o*-type in the first genus, and cytochrome *ccb3* type in the latter. Presence of cytochrome *ccb3* oxidase, a low efficient proton pump (H⁺/e⁻ = 0.2–0.75) with high oxygen affinity ($K_m \sim 7$ nM) and a relatively low midpoint potential of its active site heme ($E_{m,7} \sim -60$ mV), is indicative of microaerobic adaptation (Pitcher et al., 2002; Rauhamäki et al., 2009). Microoxic conditions are likely in the saline environment where oxygen solubility is affected by presence of salts. In *E. coli* cytochrome *o* oxidase is produced under oxygen-rich conditions, does not extrude protons, and has a relatively low oxygen affinity ($K_m = 1.4$ – 2.9 μ M) (Tseng et al., 1996). Cytochrome *o* oxidase oxidizes ubiquinol but not cytochrome *c*, representing an

alternative route for electron transfer. Its presence in *Thioalkalivibrio* spp. with two altogether different terminal oxidases and low-potential cytochrome *b* may suggest a highly controlled, multiple-way electron flow and versatility of growing under variable oxic/microoxic conditions.

The high sodium concentration in the external medium of haloalkaliphilic bacteria would have a dramatic effect on their internal milieu unless they possess extruding systems to adjust their internal sodium level. One of such regulators of Na^+ -homeostasis could be the Na^+ -dependent NADH:quinone oxidoreductase (Nqr or type III NAD dehydrogenase). Nqr is a respiration-dependent primary sodium pump that couples sodium extrusion with electron transfer from NADH to ubiquinone to generate *smf*. Nqr is present in many Gram-negative marine bacteria such as *Vibrio* spp. and *Shewanella* spp. where it is related to the absolute sodium requirement for growth (Oh et al., 1991). Coexistence of proton-translocating NADH dehydrogenase (NDH-1) and Nqr is predicted from the genomes of pathogens (e.g., *Pseudomonas* spp., *Neisseria* spp., *Yersinia* spp.), obligate chemolithoautotrophs (*Nitrosomonas europaea*), and euryarchaeota (*Methanosarcina acetivorans*) (Melo et al., 2004). Homologous *nqr* genes (*rnfABCDE*) that code for the Rnf complex were recently identified in the genome of the natronophilic *Thioalkalivibrio* spp. and *Thioalkalimicrobium* spp., as well as in other soda-loving bacteria (Fig. 1b). In *Thioalkalivibrio* spp., the *rnf* operon coexists with the *nuo* operon (*nuoABCDEFGHIJKLMN*) encoding type I proton-pumping NADH dehydrogenase (NDH-1). Rnf are bacterial redox-driven ion pumps with flavin-binding subunits (D and G) homologous to Na^+ -Nqr B and C subunits (Steuber, 2001; Backiel et al., 2008). Genes coding for Nqr as well as for NDH-1 (*nuo* genes) were also discovered in the genome of haloalkaliphilic SRB *Desulfurivibrio alkaliphilus* (Pereira et al., 2011). Coexistence of both types of NADH dehydrogenases suggests a finely tuned redox metabolism in association with sodium extrusion from the cytoplasm.

Type II NADH dehydrogenase (energy-uncoupled NADH dehydrogenase or NDH-2) capable of sodium export as well as of Na^+/H^+ antiport (NapA) activity was described in the moderate halophile *Halobacillus dabanensis* D-8 (Yang et al., 2006). Genes for NDH-2 exist in the Gram-positive alkaliphilic *B. halodurans* C-125, *B. clausii*, and *B. pseudofirmus* OF4. Their predicted products are two types of NHA-2. NHA-2A found to have high sequence similarity (>70 %) among alkaliphilic *Bacillus*, while the gene for NHA-2B of *Bacillus pseudofirmus* OF is 56 % similar to that of putative “ Na^+ -NADH dehydrogenase (NapA)” from *Halobacillus dabanensis*. None of the NHA-2 expressed proteins has Na^+/H^+ antiport activity in *B. pseudofirmus* OF4, and they are supposed to play a gating role for electron entry in the respiratory chain (Liu et al., 2008). Interestingly, the facultatively alkaliphilic and halotolerant marine bacterium *Oceanobacillus iheyensis* seems to rely only on NDH-2, lacking other types of respiratory NADH dehydrogenases (Melo et al., 2004).

Isoprenoid (respiratory) quinones function as electron and proton carriers in respiratory and photosynthetic chains. Quinones are also known as antioxidants.

Ubiquinones (high-potential quinones, $E_m = +70$ to $+112$ mV) are found in eukaryotes and are widespread in aerobic alpha-, beta-, and gammaproteobacteria. Menaquinones (low-potential quinones, with $E_{m7.5}$ between -110 and -70 mV) are typical for prokaryotes and are often related to anaerobic metabolism (Nowicka and Kruk, 2010). In many haloalkaliphilic, alkalitolerant halophilic, and moderately halophilic Gram-positive bacteria, the major respiratory quinone is menaquinone (MK)-7 (e.g., in *Bacillus locisalis*, *B. qingdaonensis*, *B. chagannorensis*, *Salsuginibacillus kocurii*) (Márquez et al., 2011; Wang et al., 2007; Carrasco et al., 2007). MK-7 is also dominant in the membranes of alkali-tolerant halophilic, moderately halophilic, and alkaliphilic halotolerant Gram-negative bacteria belonging to *Virgibacillus* spp., *Mongoliococcus* spp., and *Mongoliitalea* spp. (Chen et al., 2009; Liu et al., 2012; Yang et al., 2012). Haloalkaliphilic species of *Halomonas* (*H. campaniensis*, *H. desiderata*, *H. campisalis*) contain MK-9 and MK-8 as dominant quinones. In aerobic natronophilic *Thioalkalivibrio* spp. and *Thioalkalimicrobium* spp. that belong to the *Gammaproteobacteria*, the dominant respiratory quinone is the high midpoint potential ubiquinone-8 (UQ-8) (Sorokin and Kuenen, 2005). A combination of ubiquinones and menaquinones is characteristic of phototrophic sulfur bacteria of the genera *Ectothiorhodospira* and *Halorhodospira*. MK-7 and UQ-7 are major respiratory quinones in *Ect. magna*, *Ect. shaposhnikovii*, and *Ect. vacuolata* (Bryantseva et al., 2010). The closely related haloalkaliphilic *Halorhodospira halophila* distinctively utilizes two menaquinones, MK-8 and MK-7, for the photosynthetic electron transfer reaction and UQ-8 for the respiratory reaction (Schoepp-Cothenet et al., 2009).

3.4. Na^+ -DEPENDENT FLAGELLAR MOVEMENT

Naturally, the concentration of sodium ions is higher in the surrounding medium than within the cell. When speaking of alkaliphilic, halophilic, and haloalkaliphilic bacteria, the favorable (inwardly oriented) sodium gradient in the form of an electrochemical sodium gradient or sodium-motive force (*smf*) is exploited in several ways. Flagellar movement driven by *smf* has been well documented in haloalkalitolerant *Vibrio* spp. and in alkaliphilic *Bacillus* spp. and haloalkaliphilic bacteria (Atsumi et al., 1992; Fujinami et al., 2009; Krulwich et al., 2007). A Na^+ -dependent flagellar motor, similar to PomAB of *Vibrio* spp. and MotPS of *Bacillus* spp., was predicted from the genomes of *Thioalkalivibrio sulfidophilus* HL-EbGr7 and *Thioalkalivibrio* K90mix. These motor proteins are also closely related to a similar protein of *Halorhodospira* spp. Remarkably, the sodium-dependent movement coexists with proton-driven MotB-like flagellar motors in *Thioalkalivibrio* spp., suggesting that the chemotaxis of these bacteria is finely regulated through a complex relationship between osmo- and pH-sensing (Muyzer et al., 2011b). In low-salt-tolerant natronophilic *Thioalkalimicrobium* spp. and *Methylomicrobium alcaliphilum*, the genome

sequences indicated the presence of H⁺-dependent flagellar motility, both putative OmpA/MotB complex and MotA-like flagellar stator protein being present. In *M. alcaliphilum*, however, MotA (YP_004918404.1) and PomA (YP_004917195.1) gene products coexist.

3.5. pH AND ION HOMEOSTASIS IN THE CYTOPLASM OF HALOALKALIPHILES

The most important strategy of regulating cytoplasmic pH in bacteria depends on the activity of primary and secondary proton transporters. Primary H⁺-pumps comprise proton-extruding complexes of the respiratory chain and H⁺-driven F-ATP synthase, while secondary proton transporters are antiporters that exchange monovalent cations for protons (Padan et al., 2005; Krulwich et al., 2011). The key antiport system of halo- and alkaliphiles introduces H⁺ while extruding Na⁺ that inherently accumulates within the cell living at high salinity and/or alkalinity. The alkaliphilic and/or haloalkaliphilic Na⁺/H⁺ antiporters are part of a constellation of many types of transporting proteins that seems to have minute particular adaptations in almost every species. The concerted result of membrane transport activity is the accomplishment of ionic (i.e., H⁺, Na⁺) and volume homeostasis.

3.5.1. Na⁺/H⁺ Antiporters

Presently, Na⁺/H⁺ antiporters are classified into five families (NhaA to NhaE) in the monovalent cation–proton antiporter (CPA) superfamily (<http://www.tcdb.org/>). Nha's differ in their structure, topology, substrate affinity, stoichiometry, and kinetics of ion exchange.

Members of the Na⁺/H⁺ antiporter NhaA family are commonly found among all living cells and make an essential contribution to the Na⁺ and pH homeostasis during alkaline adaptation of non-extremophilic and extremophilic organisms (Padan et al., 2001). The activity of electrogenic NhaA is regulated by the cytoplasmic pH, and it catalyzes the inward translocation of 2H⁺ in exchange with 1Na⁺ (or Li⁺) that is extruded, resulting in a net positive charge accumulation inside the cytoplasm. The secondary transport achieved by NhaA is energized by the electric potential ($\Delta\psi$) component of *pmf*. The prototype member of this family is NhaA from *E. coli* (Ec-NhaA). Ec-NhaA has a cytoplasmic pH-sensing domain in loop VIII-IX. It has a high affinity for sodium with apparent $K_m = 0.2$ mM Na⁺ at pH 8 (Gerchman et al., 1993; Tzuberly et al., 2004). Expression of the *nhaA* gene is modulated by Na⁺ at the transcriptional level, while the pH-dependent activity is an intrinsic function of the translated protein. The growth of *E. coli* at alkaline pH supported by the combined action of two transporters, NhaA and a multidrug transporter MdfA. Apparently, NhaA is aiding non-alkaliphilic cells to withstand alkalinity up to pH 9, while MdfA, which has Na⁺/H⁺ as well as K⁺/H⁺ antiport activity in addition to efflux of multiple drug substrates

in exchange for H^+ , takes over at higher pH (Padan et al., 2005). Ec-NhaA has maximal activity at pH 8.5, and growth of *E. coli* cells at pH >9 would raise the cytoplasmic pH at inhibitory value for NhaA activity. Ec-NhaA has many homologs and paralogs in bacteria and archaea (for review, see Padan, 2008). A survey of the database (BLASTP) for available protein sequences or gene products closely related to NhaA has revealed few homologs in halophilic *Halomonas* spp. and *Chromohalobacter salexigens*.

Two members of the NhaC family of Na^+/H^+ antiporters have been originally uncovered in *Bacillus pseudofirmus* OF4 and *Bacillus subtilis*, respectively. One member (also designed as Bp-NhaC) is a relatively high-affinity, electrogenic Na^+/H^+ antiporter with a minor role in alkaliphily (Ito et al., 1997); the other is a paralog described as mediating an electroneutral malate- H^+/Na^+ -lactate antiport important in malate uptake at low *pmf* (Wei et al., 2000). Bp-NhaC of *B. pseudofirmus* remains the only member of the NhaC family that is functionally characterized. Genes coding for NhaCs exist in both Gram-negative bacteria and Gram-positive bacteria as well as archaea (i.e., *Haloferax* spp.). An NhaC homolog is predicted from the genome of the halophilic thermoalkaliphilic *Natranaerobius thermophilus* JW/NM-WN-LF (Zhao et al., 2011). The *nhaC* gene product of *N. thermophilus* showed high amino acid identity (42–44 %) with similar products in the genomes of the alkaliphilic *Bacillus clausii* KSM-K16, *B. halodurans* C-125, the obligately anaerobic, non-halophilic and moderately alkaliphilic *Alkaliphilus metalliredigens* QYMF and *A. oremlandii* OhILAs, as well as with a partial protein sequence from *Halomonas boliviensis* LC1 (acc. no. ZP_09188066).

Since the first reports on Na^+/H^+ antiport activity in the moderately halophilic *Salinivibrio costicola* and the slightly halophilic *V. parahaemolyticus* (Hamaide et al., 1983; MacLeod et al., 1988), evidence arose for the simultaneous presence of NhaA-, NhaB-, and NhaD-type antiporters in halophilic bacteria of *Vibrio* spp. Genes coding for NhaA and NhaB from *V. parahaemolyticus* complemented mutants unable to grow at neutral pH and high salinity (0.5 M LiCl) or at alkaline pH (8.5) in the presence of low salt concentration (0.05 M LiCl). Expression of *nhaA* and *nhaB* was proposed to be pH-regulated (Kuroda et al., 2005). Genes for the latter antiporter, NhaD (or Vp-NhaD), identified for the first time in *V. parahaemolyticus* by Nozaki et al. (1998), were also isolated and their product functionally characterized in *Vibrio cholerae* (Vc-NhaD) and the haloalkaliphilic *Alkalimonas amylolytica* (Aa-NhaD) (Dzioba et al., 2002; Ostroumov et al., 2002; Liu et al., 2005).

NhaD proteins catalyze $Na^+(Li^+)/H^+$ antiport at a yet unknown stoichiometry. Homologs of *nhaD* gene products are found among many proteobacteria, including truly halophilic *Halomonas* spp. (Kurz et al., 2006). Activity of NhaD is optimal at alkaline pH ($pH_{opt} = 8$ for Vc-NhaD; 8.5–9 for Vp-NhaD, and 9.5 for Aa-NhaD). The activity of NhaD from *Alkalimonas amylolytica* has a relatively low Na^+ affinity ($K_m = 0.5$ mM Na^+) and it is optimal at a concentration of 600 mM Na^+ (Liu et al., 2005). These characteristics of haloalkaliphilic NhaD (Aa-NhaD) seem in accordance with the supposed chemical parameters

of the cytoplasm during growth at saline and alkaline conditions. However, pH homeostasis in *Alkalimonas amylolytica*, capable of growing optimally at pH 10–10.5 and 2–3 % (w/v) NaCl, is achieved upon combined activities of at least three other ion transporters: the K^+/H^+ antiporter (Aa-NhaP), the K^+ efflux pump (Aa-KefB, homolog of glutathione-regulated K^+ efflux protein B, KefB from *E. coli*), and, to a lesser extent, the calcium/cation antiporter (Aa-CaxA) (Wei et al., 2007).

3.5.2. Mrp and Mrp-Like Antiporters

In aerobic alkaliphilic *Bacillus* strains, growth at alkaline pH is strictly dependent on the presence of external Na^+ . Therefore, in these bacteria, pH homeostasis is mainly accomplished by a variety of Na^+/H^+ antiporters (Krulwich et al., 2011). A large hetero-oligomeric Mrp antiporter that extrudes Na^+ (K^+ or Li^+) in exchange for H^+ has a crucial role for survival at high pH (Ito et al., 2001; Morino et al., 2008, 2010). Unlike Ec-NhaA that is a single-gene product, the multidrug resistance efflux pump Mrp from *Bacillus* spp. is a seven-component system (MrpA–MrpG) encoded by an operon containing seven genes. Originally described in *Bacillus halodurans* C-125 by Hamamoto et al. (1994), it turned out that Mrp genes are virtually spread in many Gram-negative and Gram-positive bacteria and archaea, with vital contribution to cytoplasmic pH regulation and general physiology of microorganisms (Swartz et al., 2005). Mrp-like clusters from cyanobacteria have been demonstrated to be involved in salt stress tolerance and CO_2 deficiency-induced expression (Blanco-Rivero et al., 2005). A full Mrp-like complex (Ap–MrpA–G) with Na^+/H^+ and Li^+/H^+ antiport activity was functionally described in the halophilic and alkalitolerant cyanobacterium *Aphanothece halophytica* (Fukaya et al., 2009). An interesting tripartite clustering of the genes *bicA*, *napA1-2*, and an *mrp*-like gene was described in *A. halophytica*. The first gene codes for Na^+ -dependent HCO_3^- uptake, the second for a pH-regulated Na^+/H^+ antiporter, active at alkaline pH (Price, 2011; Wutipraditkul et al., 2005). A model of cooperation between the three transporters was proposed in *A. halophytica*. At alkaline and saline conditions, when CO_2 becomes limiting, the Na^+ extruded by Na^+/H^+ antiport catalyzed by NapA1-2 and the Mrp-like complex drives the inward flux of HCO_3^- . The pH of the cytoplasm is maintained at a tolerable level by simultaneous proton import (Fukaya et al., 2009). Genes of the *mrp*-like operon (*mhA-G*), together with those for other cation/proton antiporters (*nhaP*), have been reported in the natronophilic *Thioalkalivibrio* spp. (Muyzer et al., 2011a, b) as well as in the genomes of the arsenite-oxidizing *Alkalilimnicola ehrlichii* and *Halorhodospira halophila* (see also Fig. 1c).

3.5.3. K^+ Transport and Intracellular Homeostasis

It is expected that the alkaliphiles retain an outwardly directed K^+ gradient. Therefore, K^+/H^+ antiporters might be energized by the electrochemical potassium gradient to drive protons inside the cell. However, K^+/H^+ antiporters are not expected to play central roles in cytoplasmic acidification in alkaliphiles as the

removal of intracellular K^+ is not desirable. Accumulation of (monovalent) cations in the halo/alkaliphilic cytoplasm and particularly of K^+ is a strategy either to neutralize the charge of anionic osmolytes (i.e., glutamate, chloride ions) or to compensate for the risk of Na^+ toxicity at high intracellular pH (Padan and Krulwich, 2000).

Potassium transporters participate in the overall pH, K^+ , and volume homeostasis in non-halophilic or slightly halophilic bacteria (e.g., *E. coli*, *Vibrio alginolyticus*) (Radchenko et al., 2006; for a review, see Epstein, 2003), as well as in osmoregulation of *Halanaerobiales* and haloarchaea (Oren, 2002). K^+ transporters that contribute to pH and K^+ homeostasis of halo- and/or alkaliphiles are NhaPs belonging to the monovalent cation/proton antiporter-1 (CPA1) family and members of the K^+ transporter (Trk) family. The Aa-NhaP – $K^+(NH_4^+)/H^+$ antiporter was characterized in *Alkalimonas amylolytica* (see above) (Wei et al., 2007). The Ap-NhaP – $Na^+(Ca^{2+})/H^+$ antiporter has been functionally described in the halotolerant cyanobacterium *Aphanothece halophytica*. It exhibits Na^+/H^+ antiporter activity over a wide pH range (5–9) and shows high Ca^{2+}/H^+ antiporter activity at alkaline pH (Waditee et al., 2001).

The K^+ uptake protein TrkH could be inferred from the genome sequences of natronophilic and haloalkaliphilic *Thioalkalimicrobium* spp., *Thioalkalivibrio* spp., *Methylomicrobium alcaliphilum*, and *Desulfovibrio alkaliphilus* (Fig. 1d). It was also characterized in *Alkalimonas amylolytica* (Guo et al., 2009). Kinetics experiments revealed that the Aa-TrkAH complex has an optimal activity at pH >8.5 and it is salt tolerant. The TrkAH complex has a channel-like shape and a large acidic extracellular region. It catalyzes NAD^+ -regulated K^+ uptake possibly coupled with H^+ import (Domene and Furini, 2012).

3.5.4. Sodium Import Closes the Sodium Cycle in Salt- and Alkaline-Adapted Cells

Two major routes for Na^+ entry in prokaryotic cells are known: Na^+ /solute symport and Na^+ diffusion mediated by channels (including the flagellar Na^+ channel).

Na^+ -dependent solute uptake is essential in providing sodium for antiporter activity as well as nutrients (i.e., sugars, amino acids) or precursors (such as choline) for the purpose of general metabolism including osmoadaptation. Na^+ /solute symporters are ubiquitously spread in many bacteria and archaea as well as in eukaryotes. They belong to the sodium/solute symporter (SSS) family according to the Transporter Classification system (Saier et al., 2009). Activity of Na^+ /solute symporters is energized by the electrochemical Na^+ gradient (*smf*) (Jung, 2001). Na^+ -mediated import of L-proline by homologs of the PutP symporter from *E. coli* and *Vibrio parahaemolyticus*, respectively, is largely found in pathogens, slightly halophilic marine bacteria, extreme halophilic bacteria (*Salinibacter ruber*, *Halomonas elongata*), and archaea (*Halobacterium* spp., *Haloferax* spp.) (Jung et al., 2012).

Na^+ -coupled import of a nonmetabolizable solute (α -aminoisobutyric acid) has been reported in alkaliphilic *Bacillus* as early as 1985 by Krulwich

et al. Glutamate and sucrose uptake are dependent on the sodium gradient in *Caldalkalibacillus thermarum* TA2.A1. Apparently the electrical component of the *smf* is the driving force for solute uptake in this thermoalkaliphile (Peddie et al., 1999, 2000). Among the anaerobic halophilic and thermoalkaliphilic bacteria, Na⁺-coupled solute symport was detected in *Natranaerobius thermophilus* (Zhao et al., 2011). In comparative physiological experiments with anaerobic alkaliphilic bacteria from soda lakes, Pitriuk et al. (2004) concluded that metabolism and substrate-level energy formation during growth at alkaline pH in the saccharolytic fermenters *Spirochaeta alkalica*, *Amphibacillus tropicus*, and the *Halanaerobiales* representative *Halonatronum saccharophilum* heavily depend on transmembrane H⁺ and Na⁺ gradients.

The bacterial Na⁺-selective channels Na_vBac belong to the voltage-gated ion channel (VIC) superfamily. The prototype protein of Na_vBac is the NaChBac channel discovered in the alkaliphilic *Bacillus halodurans* C-125 (Ren et al., 2001). The sodium-selective NaChBac is a channel protein with a high specificity for Na⁺, although its activation and inactivation kinetics are slower than that of Na_vs (Ren et al., 2001; for a review, see Charalambous and Wallace, 2011). NaChBac homologues are encountered in diverse marine bacteria and/or bacteria living in alkaline niches (Koishi et al., 2004; Ito et al., 2004). A recent BLASTP search reported by Irie et al. (2010) yielded 26 homologs of NaChBAC, including those from *Oceanobacillus iheyensis*, the anaerobic *Alkalilimnicola ehrlichii*, and the haloalkaliphilic *Halorhodospira halophila*. However, our updated BLASTP evaluation of NaChBAC homologs indicated their presence in *Halomonas* spp. (37 % amino acid identity), *Ectothiorhodospira* spp. (39 %), and *Thioalkalivibrio* spp. (40 %).

A Na⁺-channel potentiated at elevated pH has been described as playing roles in pH homeostasis and pH-sensing in the facultatively alkaliphilic *Bacillus pseudofirmus* OF4. Deletion of the gene (*ncbA*) coding for the voltage-gated Na⁺ channel Na_vBP impaired growth at alkaline pH, indicating its significant contribution to the cytoplasmic ion homeostasis. Moreover, Na_vBP plays an active role in the motility and chemotaxis only during growth at pH 10 (Ito et al., 2004; Fujinami et al., 2007).

3.5.5. Bicarbonate Transport in Lithotrophic Haloalkaliphiles and Natronophiles

Uptake of carbon dioxide (CO₂) and/or bicarbonate (HCO₃⁻) by obligately autotrophic organisms is essential for providing the inorganic carbon needed in assimilation. Soda lakes are virtually unlimited sources for carbonate (CO₃²⁻), but, since it is not transported, the main source of inorganic carbon in soda lakes seems to be HCO₃⁻ which becomes less accessible at very high pH (>10). Therefore, the soda lake autotrophs have to rely on active transport of bicarbonate ion. However, hypersaline soda lakes host a high abundance and diversity of photo- and chemolithoautotrophic SOB from the family *Ectothiorhodospiraceae*

(*Thioalkalivibrio* spp., *Halorhodospira* spp.) (Sorokin and Kuenen, 2005; Kovaleva et al., 2011). Low to moderately saline soda lakes, instead, have an impressive seasonal biomass production by oxygenic phototrophic cyanobacteria.

In cyanobacteria, the CO₂-concentrating mechanism (CCM) involving the uptake of CO₂ and HCO₃⁻ is driven by light energy (Kaplan et al., 2008). To date, at least two CO₂ uptake systems bound to thylakoids, and three HCO₃⁻ transporters localized in plasma membrane covering the entire range of inorganic carbon availability are known in cyanobacteria. By this CCM, inorganic carbon is captured in the cytoplasm and delivered as HCO₃⁻ through thylakoid membranes to the RuBisCO active site (Price, 2011). Two membrane transporters for bicarbonate are sodium-dependent: the inducible, high-affinity HCO₃⁻/SO₄²⁻ transporter, SbtA (Shibata et al., 2002), and the low-affinity, high-flux HCO₃⁻ transporter, BicA, identified in marine cyanobacteria (Price, 2011). The HCO₃⁻/Na⁺ symporter SbtA is a multi-pass membrane protein that is induced at low dissolved CO₂. SbtA, identified in *Synechocystis* PCC 6803, has a high affinity for bicarbonate (K_{0.5} < 5 μM HCO₃⁻), and its function is dependent on the *smf* generated by the activity of the Na⁺/H⁺ antiporter (Shibata et al., 2002). SbtA is particularly interesting as it works at low bicarbonate and high sodium concentration. Genes related to the *sbtA* gene from *Synechocystis* PCC 6803 are present in the genome sequences of the natronophilic SOB *Thioalkalivibrio* K90mix, *Thioalkalivibrio thiocyanoxidans* ARh4, *Thioalkalimicrobium aerophilum* AL3, and *Thioalkalimicrobium cyclicum* ALM1, but not in *Thioalkalivibrio sulfidophilus* HL-EbGr7. In all natronophilic chemolithoautotrophic SOB, however, a gene for low-affinity high-flux HCO₃⁻ transporter, SulP, is present (Muyzer et al., 2011a,b; personal BLASTP search). A comparative analysis of gene products also revealed homologous sequences of *sbtA* in haloalkalitolerant cyanobacteria (*Synechococcus* spp., *Anabaena* spp., and *Cyanothece* spp.) (Muyzer et al., 2011b). Apparently, SbtA bicarbonate transporters are not restricted to autotrophic bacteria. From a survey of the protein sequence database, homologous genes for SbtA are identified in alkaliphilic *Bacillus* and *Bacillus*-related species (*B. pseudofirmus* OF4 – acc. nr. YP_003425900.1, *B. halodurans* C-125 – acc. nr. NP_244736.1, and *Caldalkalibacillus thermarum* – acc. nr. ZP_08531624.1). In a complementation experiment, a gene fragment that encodes a homolog to the SulP transporter family member, BicA, from the haloalkaliphilic, strictly heterotrophic *Alkalimonas amylolytica* N10 allowed an alkali-sensitive *E. coli* mutant to grow at high pH (Wei et al., 2007). Interestingly, the closest homolog of the BicA protein from *Alkalimonas amylolytica* (acc. nr. ABG37985.1) is an uncharacterized anion transporter from haloalkaliphilic methanotroph *Methylomicrobium alcaliphilum* (acc. nr. YP_004916439.1) with 61 % amino acid identity. It is tempting to speculate that the active uptake of HCO₃⁻ in heterotrophic haloalkaliphilic bacteria could be an indicative of inorganic C fixation by anaplerotic pathways that alternatively replenish the Krebs cycle during starvation periods (Ashworth and Kornberg, 1966).

3.6. ADAPTATION TO HIGH-LIGHT AND OXIDATIVE STRESS BY SPECIFIC MEMBRANE PIGMENTS

Apart from their specific roles in photosynthesis (i.e., bacteriochlorophylls and carotenoids) and light-driven transport processes (rhodopsins), pigments produced by several halophilic and haloalkaliphilic prokaryotes may intervene in high-light/UV protection and prevent oxidative-stress damages as antioxidants. Such biological functions may be attributed to carotenoids from archaeal membranes and halophilic bacteria (e.g., bacterioruberin from *Halobacterium salinarum*, salinixanthin of *Salinibacter ruber*) (Oren, 2002). Natronochrome and chloronatronochrome, two yellow pigments composed of a C15-polyene chain attached to a phenyl group, were chemically characterized from the membranes of non-photosynthetic natronophile *Thioalkalivibrio versutus* ALJ15 (Takaichi et al., 2004). Yellow-membrane-bound pigments have been extracted from the chemolithoautotrophic SOB *Thiohalospira* spp. (Sorokin et al., 2008a). No functional characterization has been done on these yellow polyene pigments, but their possible involvement in light- and oxidative-stress protection was suggested, as well as a function as an additional membrane barrier to H⁺ and Na⁺ leakage in a similar manner as squalene (Haines, 2001; Hauss et al., 2002). Xanthomonadin, a structurally related yellow pigment isolated from the outer membrane of the phytopathogenic gammaproteobacterium *Xanthomonas* spp., was demonstrated to improve the survival rate of cells under high light conditions and to protect lipids against peroxidation in vitro (Poplawsky et al., 2000; Rajagopal et al., 1997).

4. Conclusions

Most moderate and extreme haloalkaliphilic and natronophilic prokaryotes are mainly distributed within the groups of aerobic Gram-negative bacteria, anaerobic, low G+C, Gram-positive bacteria, and haloarchaea. Haloalkaliphiles and natronophiles take up and/or produce compatible solutes in a similar manner as halotolerant and halophilic prokaryotes. The unique feature, however, is met in natronophiles that demand high soda medium and must apparently face a lower osmotic pressure at the same sodium concentration than the true halophiles. This situation has presumably important consequences to the bioenergetics of natronophiles. One such consequence is the energy saved out of synthesizing and/or importing high amounts of organic compatible solutes, energy that could be further invested in a multitude of low-cost/high-benefit strategies to overcome the low *pmf*. H⁺-dependent ATP synthesis is universally found among aerobic alkaliphilic, haloalkaliphilic, and natronophilic bacteria. High production of low-potential cytochromes of the *c*-type, point mutations in the H⁺-translocating subunits of F-ATP synthase are complemented by a bifurcated electron transfer chain. Such adaptations build a highly efficient OXPHOS machinery under the conditions of an unfavorable electrochemical proton gradient. Last but not least,

a broad variety of Na⁺- and pH-regulated transporters are interconnected and differently expressed to maximize the exploitation of the favorable electrochemical sodium gradient (*smf*). *Smf* is the driving force for flagellar movement and, indirectly, for chemotaxis, as well as for acidification of cytoplasm, a critical process to resist the alkaline surrounding. Other adaptations that have not been discussed in this chapter are met in the structures and kinetics of intracellular and extracellular enzymes of haloalkaliphiles. Overall, the physiology and genetic evidences indicate a conserved, general pattern of salt and alkaline adaptation over a broad range of alkaline pH (9–11) and salinity (up to saturation) in haloalkaliphiles/natronophiles. Genomes of salt- and alkaline-tolerant strains of *Bacillus* spp. and *E. coli*, the most common experimental models, apparently encode the basic survival kits to respond the moderate osmotic and alkali shocks. However, those kits are not sufficient to cross to the regions where true haloalkaliphiles are flourishing. Further in-depth analyses of alkaliphilic and haloalkaliphilic genomes completed by integration with other “omics” and physiological evidences would definitively enlighten us on the bases as well as on the peculiarities of haloalkaline adaptation in prokaryotes.

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Biodata of **Ronald S. Oremland**, author of “*A Random Biogeochemical Walk into Three Soda Lakes of the Western USA: With an Introduction to a Few of Their Microbial Denizens.*”

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A RANDOM BIOGEOCHEMICAL WALK INTO THREE SODA LAKES OF THE WESTERN USA: WITH AN INTRODUCTION TO A FEW OF THEIR MICROBIAL DENIZENS

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1. Introduction

The Wadi Natrun in Egypt is probably the oldest and best-known soda lake environment for finding polyextremophilic microorganisms adapted to its high salinity, pH, and temperature. It clearly has been a resource and motivational factor for multiple generations of microbiologists interested in exploring the physical/chemical boundaries of microbial life, as is reviewed by Professor Oren in this volume (Oren, 2013). Yet curiosity-driven research is not the only factor egging on a young scientist's aspirations, as was my situation some 35 years ago. I began my professional career at the US Geological Survey (USGS) in 1977 with my dissertation and postdoc background in marine sciences and anaerobic processes. After a very shaky first few years upon coming aboard the USGS, I was told that my research direction should be devoted to inland waters, preferably freshwater streams. About this time three seminal scientific events occurred that got me thinking about extremophiles and their habitats: the publication of a series of papers on Solar Lake (Cohen et al., 1977; Jørgensen and Cohen, 1977), the discovery of chemoautotrophic-based marine life at hydrothermal vent sites (Lonsdale, 1977), and the 1980 eruption of Mount St. Helens followed by the microbial successions observed in nearby Spirit Lake (Baross et al., 1982). I began thinking that there surely must be some interesting environments within a 1-day drive of my laboratory in Menlo Park, California. It was during a chance encounter with my colleagues Yousif Kharaka and Steven Robinson that I was told of such a place: Big Soda Lake, in Fallon, Nevada. Not only was it saline and alkaline, it had been in a condition of meromixis for over 60 years which resulted in hypersaline and extremely reducing anoxic bottom waters. Thus, I could fulfill both my curiosity and obey the letter (but certainly not the spirit) of the "riot act" that was read to me. I would work on a system more saline and far more chemically reducing than what the oceans could provide. I had found my smelly little bit of (obscure) scientific heaven! From there it was only a short conceptual hop to Mono Lake and Searles Lake in California for the scientific purposes of comparisons, as my USGS career blossomed.

2. Big Soda Lake, Nevada

Big Soda Lake ($30^{\circ}31'N$; $118^{\circ}52'W$) occupies the remnants of a volcanic crater that last erupted in the Pleistocene (Rush, 1972). In the nineteenth century, the lake level was about 18 m lower than today, and the brine was “mined” by pumping it ashore followed by evaporation for the purpose of obtaining borax. The Newlands Reclamation Project (~1907) diverted water from the Truckee River to the region of Fallon, causing a rise in the local water table with a concomitant influx into the lake of freshwater which eventually resulted in a permanent, density-based stratification (meromixis) of the water column that has persisted since ~1925. A deep chemocline/pycnocline is located at 35 m depth, while the surface water undergoes winter mixing followed by summer stratification and an oxycline/thermocline located at ~20 m depth (Kimmel et al., 1978; Priscu et al., 1982; Cloern et al., 1983a; Oremland and DesMarais, 1983; Kharaka et al., 1984). The lake’s dimensions are approximately 1.5 km along its east–west axis by 1.2 km north–south (Fig. 1), with an area of ~1.8 km². The lake has a profundal depth of



Figure 1. A satellite image, courtesy of Google Earth, of Big Soda Lake and Little Soda Lake, Nevada. Note the *darker grey* area surrounding the lakes and the discernable line beyond surrounding the local farming community which outlines the volcanic blast zone consisting of extruded lacustrine materials and basaltic debris (Scale, E/W axis of Big Soda Lake boundary = 1.5 km).

Table 1. Some chemical constituents of Big Soda Lake mixolimnion and monimolimnion waters.

Constituent	TDS ^a	pH	Cl ^b	HCO ₃ ^{-b}	Na ^b	Ca ^b	NH ₃ ^{-b}	SO ₄ ^{2-b}	S ^{2-b}	Mg ^b	B ^b	CH ₄ ^{-c}
Surface	26	9.7	203	67	353	0.13	0	60	0	6.1	4	0.1
Bottom	88	9.7	789	395	1,174	0.02	2.7	73	13.1	0.2	15	55

Modified from Kharaka et al. (1984) and Oremland and DesMarais (1983).

^aTDS (salinity) total dissolved solids (g/L).

^bmM.

^cμM.

approximately 62 m. Some chemical characteristics of its surface water (mixolimnion) and its bottom waters (monimolimnion) are given in Table 1.

Annual primary productivity was estimated at ~500 gC m⁻², about 60 % attributable to a winter bloom of pennate diatoms. A significant contribution also came from the region of the summer oxycline, of which 30 % annual carbon fixation stems from bacterial chemoautotrophy with an additional 10 % contributed from anoxygenic photosynthesis associated with a red plate of *Ectothiorhodospira* (Cloern et al., 1983b). While the lake's surface water is only moderately saline, its high pH precludes the presence of fish. Nonetheless, the surface waters host abundant zooplankton (copepods and cladocerans), aquatic insects, and seasonal shoreline rooted macrophytes (*Ruppia* sp.) that become heavily epiphytized by diazotrophic *Anabaena* sp. communities during summer (Oremland, 1983). The water column bacterial communities were described based on microscopic examinations that were done concurrently with measures of heterotrophic activity made with radiotracers (Zehr et al., 1987). The water column was also assayed for sulfate reduction (Smith and Oremland, 1987), methane formation and anaerobic methane oxidation (Iversen et al., 1987), and fluxes of particulate matter across the chemocline (Cloern et al., 1987). The presence of dissolved methane in the monimolimnion, along with a clear ¹²C-enriched value of δ¹³C-methane, suggested a biological origin of this gas (Oremland and DesMarais, 1983). Incubated bottom sediment slurries had methanogenic activity, but only amendments with methanol, as opposed to acetate or formate, caused a pronounced stimulation of activity (Oremland et al., 1982a). The high concentrations of sulfate in this system as opposed to seawater (73 vs. 28 mM) gave rise to the concept that certain methylotrophic substrates (e.g., methanol, methylated amines, methylated sulfides) were not vied for with the thermodynamically more efficient sulfate-reducing bacteria and were termed "noncompetitive" methane precursors as opposed to competitive substrates like acetate and H₂ (Oremland and Polcin, 1982; Oremland et al., 1982b; King, 1984; Kiene et al., 1986). The bottom sediments, along with an isolated methylotrophic coccoid methanogen, showed a pronounced pH optimum at 9.7, indicating a clear adaptation to the lake's harsh bottom water conditions (Oremland et al., 1982a). Anoxic sediments from this lake and other sites including Mono Lake and San Francisco

Bay had the capacity to biologically form ethane from ethylated sulfur compounds like ethanethiol and diethyl sulfide (Oremland et al., 1988). Littoral sediments were also scrutinized for denitrification by measuring N_2O reductase activity (Miller et al., 1986). Big Soda Lake remains to be investigated from the perspective of employing culture-dependent and culture-independent methods to better define its resident microbial populations and their adaptations to its ambient conditions. Recent visits to the lake indicate that the hydrological conditions are changing, with a notable sinking of the depth of the deep chemo-pycnocline suggesting a slow breakdown of meromixis.

3. Mono Lake, California

Located in a closed basin formed by geologic subsidence nearly 0.75–1 million years ago (Christensen et al., 1969), Mono Lake (38°N; 119°W) abuts the eastern escarpment of the Sierra Nevada mountain range (Fig. 2). The lake is situated on the western edge of the Great Basin Desert, with its present dimensions running roughly 20.6 km along its east–west axis, and about 13.8 km along its north–south axis (area = ~150 km²). In the Pleistocene the lake covered five times the area than it does today, contained 18-fold more water, and may have been a branch of the much larger Lake Lahontan that covered a great deal of western Nevada. The change in climate from wet to arid resulted in evaporative contraction of Mono's size and the concentration of its major chemical constituents (Stine, 1990). The currently exposed former lakebeds are clearly visible from the satellite image displayed in Fig. 2. Unlike Big Soda Lake which occupies the remnants of an exploded volcanic caldera, Mono Lake occupies no such caldrion. However, volcanism and volcanic features are common in the basin and have contributed to the lake's current morphometry. Volcanism in the region commenced some 750,000 years ago with the mega-scaled eruption of the Long Valley caldera, located ~40 km south of the lake. Volcanic activity migrated northwards over time, and active heat flow persists into the basin (Hill et al., 1985). The Mono Craters (some 40 in all) are prominent features along the lake's south shore, while the its two major islands, Negit and Paoha, were formed as a consequence of volcanic activity, the latter island having hot springs located along its southeastern corner. Mark Twain's classic book "Roughing It" devotes an entire chapter to Mono Lake, mostly dealing with his misadventures on Paoha Island. Early scientific investigations concerning the lake's general limnological properties (Mason, 1967) and sediment organic geochemistry (Reed, 1977) stimulated further research interest. Broader societal interest centered on the consequences of the prolonged diversion of freshwater runoff into the lake to supply drinking water for the city of Los Angeles. These diversions lowered the lake level by some 15 m over four decades, roughly doubling its salinity (from 50 to 90 ppt) and raising concerns that the lake would eventually become too saline to support metazoan invertebrate life (which support large populations of migratory waterfowl) and that its shallow regions



Figure 2. A satellite composite image of Mono Lake, courtesy of Google Earth. Note the striations around the lake's former lakebed, indicative of much higher water stands in the Pleistocene. In contrast, the exposed white region near the lake's current edge is the amount of lake-water volume lost attributable to diversion of freshwater runoff since 1940 (Scale, E/W axis of the lake boundary = 20.6 km).

Table 2. Some chemical constituents of Mono Lake.

Constituent ^a	TDS	pH	Cl	HCO ₃ ⁻	Na	Ca	Mg	SO ₄ ²⁻	As	B	CH ₄ ^b
—	90	9.8	500	400	1,300	0.05	1.3	130	0.2	46	40

Compiled from Dana et al. (1977), Oremland et al. (1993), and Johannesson and Lyons (1994).

^aGiven as mM (or g/L for TDS) unless noted otherwise.

^bμM as dissolved in the bottom water.

would become exposed dry playas that would generate toxic dust storms. A political settlement was eventually reached with the goal of stabilizing the lake level at its 1941 level by allowing more freshwater runoff into the lake. Some of the major chemical constituents of Mono Lake are given in Table 2.

Our interest in Mono Lake was piqued in the early 1980s by the observation that the high precipitation and snow melt runoff of freshwater into the lake (1982–1984) caused by a strong El Niño event resulted in a dilution of surface water salinity by ~10 %. This set up a meromictic condition that was to persist for 8 years (Jellison and Melack, 1993; Jellison et al., 1993; Miller et al., 1993), a situation that was to recur during the El Niño winter of 1994–1995 and persist for another 5 years (Humayoun et al., 2003). We made two serendipitous observations during our early visits to Mono Lake that led to a couple of unexpected discoveries with regard to (1) multiple sources of methane outgassing from the lake (Oremland et al., 1987) and (2) a small-sized (1–3 μm) eukaryotic phytoplankton in the water column that was responsible for most of the lake's primary production (Roesler et al., 2002).

Despite the great abundance of sulfate ions in its waters, Mono Lake has considerable dissolved methane present within its bottom waters during stratification (Table 2) along with an abundance of sulfide (~2 mM). Methanogenic activity was detectable both in the lake's pelagic and littoral sediments (Kiene et al., 1986; Oremland and King, 1989; Oremland et al., 1993; Oremland and Miller, 1993) arising primarily from "noncompetitive" precursor substrates (e.g., methylated amines, methylated sulfides, and methanol), ultimately derived from breakdown of osmoregulatory compatible solutes like dimethylsulfoniopropionate and glycine betaine. A small amount of biogenic ethane could also be formed from precursors like ethanethiol and diethyl sulfide (Oremland et al., 1988). Sediment core profiles taken from the pelagic region indicated methane saturation was reached in the upper 60 cm, where there was still abundant sulfate present (25–70 mM) (Oremland et al., 1987). This pore water methane was isotopically "light" ($\delta^{13}\text{C}_{\text{CH}_4} = -75\text{‰}$) and contained detectable amounts of ethane and propane ($\text{C}_1/\text{C}_2 + \text{C}_3 = 600\text{--}750$), indicating biogenic origins. Sulfate reduction, as determined with ^{35}S -radiotracer, also was detectable in these cores, although rates measured in the top 3 cm ($250 \mu\text{mol L}^{-1} \text{day}^{-1}$) declined exponentially over the upper 10 cm and were about 100-fold higher than most elsewhere in the core (Oremland et al., 1993). Methane-oxidation activity, both aerobic and anaerobic, was measured in the stratified water column of the lake (Oremland et al., 1993; Joye et al., 1999), as was oxidation of methyl bromide (Connell et al., 1997). Culture-independent methods detected the presence of methanogens (Scholten et al., 2005), methanotrophs (Carini et al., 2005; Lin et al., 2005; Nercessian et al., 2006), and nitrifiers (Ward et al., 2000; Carini and Joye, 2008) in the water column.

The first surprise in this context was our observation of gas seeps around the lake. Intuition argued against them being composed of methane because of the lake's high sulfate content. But paradoxically, they turned out to be methane rich. Mono Lake therefore leaks methane to the atmosphere in the form of continuous bubble ebullition from thousands of seeps located around the lake (Oremland et al., 1987). What differentiates these seeps from the methane cycle described above is that they are radiocarbon dead (age $\gg 20,000$ years), while methane in the bottom water, which has its origin in the anoxic sediments, is not.

Hence, the seeps are associated with a natural gas deposit underlying the lakebed that escapes its confinement along geologic fracture lines. Ebullition rates for individual seeps ran from $\sim 50 \text{ mL CH}_4 \text{ min}^{-1}$ to as high as $4 \text{ L CH}_4 \text{ min}^{-1}$. Seeps located in the vicinity of the hot springs and active heat flow on the southeastern corner of Paoha Island had a strong thermogenic character (methane depleted in ^{12}C and contained abundant ethane, propane, and butanes), while seeps located elsewhere had a strong biogenic character (methane enriched in ^{12}C ; lacking ethane and propane).

The second surprise was our observation of an optically turbid layer located in the vicinity of the oxycline/pycnocline/redoxcline of the lake's water column. We assumed this to be caused by a dense "plate" of photosynthetic bacteria, like the *Ectothiorhodospira*-dense layer that occurs in Big Soda Lake. But rather than being pink, the water recovered from this depth ($\sim 16 \text{ m}$) was green-tinged. Microscopic examination revealed the abundant presence of unicellular eukaryotic algae in the $1\text{--}3\text{-}\mu\text{m}$ size range. A study was launched by C.W. Culbertson to investigate the ecophysiology of this microbe. It was dubbed "Mickey" because its spherical cytoplasmic main body had two superimposed smaller "ears" that contained its chlorophyll, giving it a resemblance to Mickey Mouse when viewed head on, thereby facilitating direct counting via epifluorescence microscopy because of its unique morphology. "Mickey" could be cultivated in the lab and was designated as *Picocystis* sp. strain ML (Roesler et al., 2002). Strain ML exhibited growth over a wide range of pH (5–11; optimum 6–8) and salinity (0–250 ‰; optimum = 50 ‰). Cells contained both dimethylsulfoniopropionate (DMSP) and glycine betaine (GBT) as internal osmolytes. Internal DMSP levels showed no trend when cells were grown over a salinity range of 19–123 ‰ (0.08–0.14 pg cell^{-1}), but GBT increased markedly at salinities above 40 ‰ and became the dominant osmoregulant at the highest salinity ($\sim 0.8 \text{ pg cell}^{-1}$ at 123 ‰). Strain ML only demonstrated light-dependent growth, but was able to grow well under initially anoxic (N_2 headspace) and reducing conditions when supplemented with 100 μM sulfide plus 100 μM ammonia, thereby mimicking the sub-oxic environment of the 16 m chemocline. Cells contained primarily chlorophyll *a*, but also substantial amounts ($\sim 30 \%$) of chlorophyll *b*, as well as several ancillary pigments (several xanthins and carotenes). When captured brine shrimp (*Artemia monica*) from the lake were placed in a suspension of "Mickey," their numbers decreased rapidly over a 15-h feeding incubation, thereby indicating the importance of strain ML in trophic energy transfer to the lake's dominant zooplankton. Highest cell densities of "Mickey" ($2 \times 10^5 \text{ cells mL}^{-1}$) occurred in the chemocline during water column stratification. Annual primary production during meromixis was estimated at 22.4–38.5 mol C m^{-2} , but runs roughly 50 % higher during the lake's "normal" monomictic (one turnover per year) cycle (Jellison and Melack, 1993). Estimates of the contribution that "Mickey" makes to the lake's annual photoautotrophic carbon fixation were determined by conducting seasonal water sample incubations made with ^{14}C -bicarbonate incorporation followed by size fractionation. Results indicated that this microorganism holds importance beyond its small

size and contributes between 25 and 50 % of annual primary productivity. Mono Lake surface water contains abundant phosphate (~600 μM ; J. T. Hollibaugh, 2003, personal communication) but is severely nitrogen-limited. A diazotrophic community was detected that inhabited the internal matrices of a small, tufted free-floating algal assemblage (*Ctenocladus circinnatus*) found in the lake (Oremland, 1990). Nitrogen fixation was attributed to both a light-driven, aerobic component, presumably cyanobacterial, and a dark/anaerobic component presumably bacterial. However, the estimated activity did not appear to make a quantitatively relevant contribution to sustaining water column primary productivity.

The issue of the contamination of the western portion of the San Joaquin Valley's soils with naturally occurring selenium salts prompted the next phase of this work. We were seeking extremophiles better suited to grow in saline/alkaline agricultural wastewaters so as to efficiently remove selenium from the aqueous phase via its precipitation as Se(0). Since we had first isolated a selenate-respiring bacterium, *Sulfurospirillum barnesii*, from a freshwater slough (Oremland et al., 1994; Laverman et al., 1995; Stolz et al., 1999), we turned to Mono Lake as a source of anaerobes better suited to more extreme conditions. Two new species of low G+C haloalkaliphilic anaerobes were isolated from the sediments of Mono Lake, *Bacillus arsenicoselenatis* strain E1H (renamed *B. arseniciselenatis*) and *B. selenitireducens* strain MLS10 (Switzer Blum et al., 1998). Both grew by oxidizing lactate to acetate+CO₂, with strain E1H growing via reduction of Se(VI) to Se(IV), and strain MLS10 via reduction of Se(IV) to Se(0). Both were adapted to high pH (optima between 8.5 and 10.0) and salinity, with strain E1H having growth between 20 and 120‰, while strain MLS10 had a much broader range (20–220‰). Both species also proved capable of growing via dissimilatory reduction of As(V) to As(III), and the respiratory arsenate reductase (*arrA*) of *B. selenitireducens* was shown to align with the family of Mo-containing arsenate reductases of other mesophilic arsenate respirers (Afkar et al., 2003). Both species also proved capable of growth via dissimilatory reduction of oxyanions of tellurium, but they were sensitive to the presence of concentrations >1 mM Te-oxyanions added to the medium (Baesman et al., 2007). Therefore, an isolation protocol was imposed by using 10 mM tellurite [Te(IV)] as the enrichment selection factor to find a more robust organism in this regard. The result was isolation of *B. beveridgei* strain MLTeJB to honor of the late Professor Terry Beveridge. This microorganism could use Te(IV), Te(VI), Se(IV), Se(VI), and As(V) (amongst others) as respiratory electron acceptors to sustain growth with the concomitant oxidation of lactate (Baesman et al., 2009).

The observation that our isolates from Mono Lake all had a clear affinity to employ arsenate as an electron acceptor coupled with the unusual abundance of this oxyanion in the lake water (Table 2) led us to conduct experiments designed to measure biological As(V) reduction in the lake's stratified water column by employing the radiotracer ⁷³As(V) (Oremland et al., 2000). Field incubations demonstrated that anoxic samples recovered from below the chemocline were able to reduce ⁷³As(V) to ⁷³As(III), yielding rate estimates ranging between

0.5 and 5.9 $\mu\text{mol L}^{-1} \text{day}^{-1}$ with the highest rates evident just below the chemocline. It was estimated that this vertically integrated activity could mineralize as much as ~14 % of annual primary productivity occurring during meromixis. In a subsequent study that used a consideration of seasonal mass balances of arsenic oxyanions, a similar estimate was arrived at (~10 %) (Hollibaugh et al., 2005). Both studies found cell densities of arsenate-respiring bacteria in the monimolimnion, as determined by MPN techniques, to be in the 10^2 – 10^3 cells mL^{-1} range. The next question was what were the types of bacteria and/or archaea that were responsible for the observed activity? A water column culture-independent microbial diversity study was made using 16S-based rRNA genes cloned from DNA collected at various depths (Humayoun et al., 2003). There was a fundamental difference observed between the populations in the surface waters as opposed to the more richly diverse population in the monimolimnion, but this approach did not tackle the question of assigning species-specific responsibility for arsenic metabolism. Aspects of the earlier work conducted on arsenic biogeochemistry of Mono Lake were reviewed by Oremland et al. (2004).

While conducting experiments with manipulated anoxic bottom water aimed at assigning the responsibility mentioned above two serendipitous discoveries were made: (1) the nitrate-linked biological oxidation of arsenite and (2) the sulfide-linked biological reduction of arsenate. The first case led to the isolation and characterization of *Alkalilimnicola ehrlichii* strain MLHE-1, a haloalkaliphilic gammaproteobacterium capable of chemoautotrophic growth via nitrate respiration using arsenite, hydrogen, or sulfide as electron donors, and of aerobic, heterotrophic growth upon acetate (Oremland et al., 2002; Hoefl et al., 2002, 2007). This microbe was to prove seminal in further discoveries because its genome lacked homologs of arsenite oxidase (*aoiB*) but had two evident homologs of respiratory arsenate reductase (*arrA*) even though the strain showed no capacity for As(V) reduction. The arsenate reductase was found to be operating in a reverse functionality in vivo (Richey et al., 2009), and knockout mutants of the strain demonstrated that one of the annotated arsenate reductases was essential for cell growth on arsenite plus nitrate (Zargar et al., 2010). A new clade of anaerobic arsenite oxidase genes were assigned and designated *arxA* because of their proximity to arsenate reductases in lieu of aerobic arsenite oxidases (*aoiB*). The second discovery led to the isolation of strain MLMS-1, an anaerobic deltaproteobacterium that was also an obligate chemoautotroph and the first example of an obligate arsenate respirer (Hoefl et al., 2004). Further work with culture-independent and culture-dependent molecular characterization approaches indicated a greater diversity amongst the deltaproteobacteria of Mono Lake that had this capacity to oxidize sulfide with arsenate (Hollibaugh et al., 2006).

In our early sojourns to the shores of Paoha Island, a mass of volcanically uplifted bottom sediments located in the middle of Mono Lake, we noted the presence of and smelled the sulfide emanating from a series of small hot springs located on the shoreline of the island's southeast corner. When examined more closely, these springs, most of which had temperatures of ~45 °C, were adorned

with red biofilms. We thought it likely that they were composed of anaerobic bacteria carrying out anoxygenic photosynthesis by using the aqueous sulfide present as their primary electron donor. Yet these anoxic waters also contained an abundance of other chemical reductants in addition to sulfide, which included dissolved methane and notably $\sim 100 \mu\text{M}$ arsenic, mostly in the form of As(III). We then initiated investigations using the live biofilm materials with the goal in mind of determining if As(III) could also serve an electron donor to support anoxygenic photosynthesis (Kulp et al., 2008). These early results were successful, in that we observed light-driven anaerobic As(III) oxidation, which did not occur in dark-incubated controls. We hypothesized that As(III) could also support growth, and Shelley Hoefft eventually isolated a pure culture of a red-pigmented gammaproteobacterium from the biofilm, preliminarily named *Ectothiorhodospira* strain PHS-1, which demonstrated As(III)-dependent growth, forming As(V) as a product in proportion to the amount of As(III) consumed. When PCR tested for the presence of arsenite oxidase using primers appropriate for aerobic As(III)-oxidizing bacteria (*aoiB*), no amplicons were obtained, yet strain PHS-1 successfully produced PCR amplicons when primers for respiratory arsenate reductase (*arrA*) were employed. The sequenced product aligned closely with that of the *arxA* of *A. ehrlichii*. A further characterization of the biofilm material revealed that it is mostly, but not entirely, composed of *Ectothiorhodospira*-like organisms and harbors functional genes of both the classic *arrA* type and the *arxA* type (Hoefft et al., 2010). The former gene is involved in employing As(V) as a respiratory oxidant formed by the latter's light-driven activities. These are carried out by different bacterial populations, with As(V) respiration being driven by chemoautotrophy using sulfide or H_2 as electron donors. The *arxA* was also upregulated in strain PHS-1, and the *arxA* gene itself was identified as a new clade of anaerobic Mo-containing arsenite oxidases phylogenetically more closely related to the *arrA* arsenate reductases than to the *aoiB* aerobic arsenite oxidases (Zargar et al., 2012).

One more finding concerning unusual microbes discovered in Mono Lake certainly bears mentioning, namely, the claim that a halomonad, strain GFAJ-1, could get by using arsenate instead of phosphate to sustain growth (Wolfe-Simon et al., 2011). This aerobic organism was isolated from shoreline mud by using arsenate in lieu of phosphate in the medium, and growth experiments indicated it could grow reasonably well with 40 mM As(V) substituting for the normally employed 1.5 mM phosphate. It certainly grew better and faster with this amount of phosphate than the As(V), but the implications of the growth experiments were profound, namely, that arsenate may successfully substitute for phosphate in nucleic acids. It implied that life may not be constrained to only the six major known elements (C, N, S, O, P, and H) that make up the bulk of living biomass. A variety of follow-up experiments and analyses in the original paper supported this claim, which included distribution of $^{73}\text{As(V)}$ radiolabel in the cells, EXAFS/XANES spectra, and nano-SIMS analysis of extracted DNA. The prepublication (on-line version) of the paper in the journal *Science* in December 2010 coincided

with a widely disseminated NASA-sponsored press conference and media follow-up. The mood of the scientific and lay community quickly changed in a matter of a couple of days from astonishment to disbelief and anger, especially in the on-line “blogosphere” but also from reputable scientific news sources and commentaries in professional journals (e.g., Rosen et al., 2011; Silver and Phung, 2011). I will not recount my perspective on these details in this chapter, other than to say it was an extremely harrowing experience. Nonetheless, such extraordinary claims must be tested experimentally, especially by disinterested parties, to either support or refute the original contentions. The genome annotation of strain GFAJ-1 was recently published (Phung et al., 2012) along with commentary on its possible significance, or lack thereof, regarding the question of arsenic in DNA (Kim and Rensing, 2012). I will say at this juncture that the key item to be concerned with is the background level of phosphorus contained in the impurities of the reagents employed for culturing this microorganism. Discounting all the personalities involved, the media circus and the many strong (and at times quite nasty) opinions expressed on-line, the scientific process will resolve the ultimate controversy. In this regard, it is incumbent on me to briefly discuss the most recent experimental publications that have appeared after the first draft of this chapter was submitted.

The results of Reaves et al. (2012) and Erb et al. (2012) clearly do not support the original observations made in my laboratory with the newly isolated strain GFAJ-1 which gave evidence for arsenate-dependent growth at low background levels of P (Wolfe-Simon et al., 2011). Because we originally observed no growth at these background phosphorus concentrations ($\sim 3 \mu\text{M}$), we concluded that added arsenate insinuated itself into the bioenergetic and genetic operation of this microorganism and was carrying out P-related functions under conditions of severe P-limitation. We provided the strain to two established culture collections (ATCC and DSMZ), as well as to a number of labs that requested it for further testing either in confirmation or refutation of the original hypothesis posed. In recent weeks my own lab has reexamined this question after eliminating the problem of background P, and we too have failed to see any evidence for arsenate-dependent growth in the absence of phosphorus, although we have observed a modest arsenate-growth enhancement at 1–3 μM phosphate. This evidence in itself is clearly not sufficient to support our original hypothesis, but it does bear closer scrutiny in the months to come.

So what can account for this key disparity between the original growth data published in Wolfe-Simon et al. (2011) and that of Reaves et al. (2012) and Erb et al. (2012) as well of those of my own lab’s recent efforts? One possibility is that this trait has been lost over time (~ 18 months) with the continued transfers of the cell line made into media either replete with arsenate or with phosphate. Another opinion, as suggested by Basturea et al. (2012), is that a scavenging of ribosomal phosphate occurs in strain GFAJ-1. These workers demonstrated with *E. coli* that added arsenate at low background P resulted in similar growth to that originally observed with strain GFAJ-1 held under similar conditions. Finally, Kang et al.

(2012) noted that the induction of arsenite oxidation genes in *Agrobacterium tumefaciens* occurs only at low P concentrations ($<5 \mu\text{M}$), a phenomenon which presumably somehow aids in the scavenging of phosphate from the surrounding environment and might be applicable to the case of GFAJ-1.

So where do things stand now? While the progress of science is usually thought to move in a straightforward, logical fashion, in reality it often progresses more like that of a pinball in an arcade game. While the results of Reaves et al. (2012) and Erb et al. (2012) may eventually firmly close the door on the validity of the original arsenic-life hypothesis, at this point I would say it is still just a tad ajar, with points worthy of further study concerning the metabolic effects of arsenate upon cells encountering severe P-limitation.

4. Searles Lake, California

Located in the Mojave Desert of southeastern California ($35^{\circ}45' \text{ N}$, $117^{\circ}22' \text{ W}$) by the small town of Trona, Searles Lake occupies the basin floor of Searles Valley (dimensions $\sim 16 \text{ km N/S}$ and $\sim 10 \text{ km E/W}$). The basin consists of broad alkaline playas, evaporation ponds, salt flats, and an ephemeral shallow lake that owes its variable surface water content to the extent of any runoff from local winter precipitation coupled with industrial discharge water stemming from Searles Valley Minerals, Inc. (<http://www.svminerals.com/default.aspx>). These operations extract a number of valuable chemical salts (e.g., borax, soda ash, sodium sulfate) from heated brine solutions. The brines are pumped up from the subsurface underlying the lakebed. Hence, the extent of the lake's area is seasonally variable and as pictured below (Fig. 3) runs roughly 3.5 km along the W/SE axis and 2.1 km N/S axis, with a depth of $\sim 1.0 \text{ m}$. In our first excursion to the lake in 2004 led by Larry Miller, the region sampled had no surface water visible and consisted only of a thick ($\sim 12 \text{ cm}$) salt crust beneath which lay a salt-saturated, dense brine perhaps 30 cm deep, underlain further by anoxic sediments. When we returned early the following spring after a rainy winter, there was approximately 50 cm of water overlying the salt crust which was, despite this water's presence, still quite firmly intact. Table 3 gives the major constituents of the Searles Lake brine.

The impetus for examining Searles Lake came out of our interest in arsenate-respiring prokaryotes. As part of our review paper on arsenic metabolism (Oremland and Stolz, 2003), John Stolz produced a 16S rRNA gene sequence phylogenetic tree which provided a map of "DARPs" or dissimilatory arsenate-respiring prokaryotes. While there were over 20 species of diverse DARPs in the Domain Bacteria, the Domain Archaea had but two closely related species of hyperthermophiles, *Pyrobaculum aerophilum* and *P. arsenaticum*. Reasoning that there must be other examples of DARPs from the Archaea out there, we began a search for other "extremophile" conditions, assuming (incorrectly) that anaerobic, halophilic Archaea would thrive in such locales. We settled on the arsenic-rich environment of Searles Lake. After contacting some of the folks at Searles Valley



Figure 3. A satellite image of Searles Lake and the surrounding Searles Valley courtesy of Google Maps. A *push pin* notes the lake's general location, which is surrounded by dry playa, salt flats, and evaporation ponds (Scale, W/SE axis of the lake's boundary = ~3.5 km).

Table 3. Major constituents of the Searles Lake brine underlying its salt crust.

Constituent ^a	TDS	pH	Cl ⁻	HCO ₃ ^{-b}	Na ⁺	Ca ²⁺	Mg ²⁺	SO ₄ ²⁻	As ^c	B	CH ₄ ^d
–	~350	9.8	5,250	640	7,430	ND	ND	730	4	460	<1

Compiled from Smith (1979), Felmy and Weare (1986), and Oremland et al. (2005).

ND not determined.

^aUnits are as milli-molar (millimoles per kilogram of solvent).

^bCarbonate plus bicarbonate.

^cMillimolar.

^dMicromolar.

Minerals, they agreed to ship us anoxic sediment from which Jodi Switzer Blum isolated strain SLAS-1, a haloalkaliphilic anaerobe capable of growth at salt saturation (~350 g/L) and respiring arsenate. But alas, the isolate was not a member of the Archaea but belonged to the *Haloanaerobiales* of the Domain Bacteria. Nonetheless, it was clearly a polyextremophile, demonstrating growth only at alkaline pH (optimum ~9.4) and only at high salinities (>200 g/L) including at salt

saturation (~350 g/L) (Oremland et al., 2005; Switzer Blum et al., 2009). Strain SLAS-1 had a mixed metabolism, able to grow either as a chemoorganotroph (e.g., lactate/arsenate) or as a chemoautotroph (sulfide/arsenate) using inorganic carbon as its cell C source, although cell extracts lacked RuBisCo activity.

Tom Kulp found that sediments from Searles Lake also demonstrated clear biological activity with regard to their ability to reduce As(V) to As(III) under anoxic conditions, as well as their capability to oxidize As(III) back to As(V) in aerobic incubations. Larry Miller and Tom Kulp, along with the rest of my project (Shelley Hoeft and Shaun Baesman), made a field excursion to Searles Lake in order to obtain sediment cores, which, when processed, gave clear evidence for a reduction of As(V) to As(III) with vertical transit from surface oxic conditions into the anoxia prevalent in the sediments. This was written up in our paper in *Science*, which for the purpose of logical “storytelling” described the reverse order of the actual chronology of events (i.e., sediment profiles sediment slurry experiments isolation and characterization of strain SLAS-1). It still remains to be determined if an arsenate-respiring extreme halophile from the Domain Archaea exists. Nonetheless, *Halarsenatibacter silvermanii* strain SLAS-1 is a most unusual organism, especially with regard to its sinuous motility which is more akin to that of an eel than the “twiddle and run” of a “typical” bacterium like *E. coli*.

The enrichment culture from which we isolated *H. silvermanii* also had the clear ability to form sulfide, and ³⁵S-sulfate radiotracer demonstrated it was produced via sulfate reduction. Subsequent work resulted in the isolation of strain SLSR-1 from the enrichment, which had the capacity to grow via sulfate reduction, as well as via arsenate respiration (Switzer Blum et al., 2012). The organism is also an extreme halophile, capable of growth (and sulfate reduction) at salt saturation, which has been a topic of scientific curiosity for those interested in hypersaline ecosystems (Oren, 1999, 2011).

The question of respiratory arsenate reduction and sulfate reduction in Searles Lake sediments was investigated further and compared with similar experiments conducted in Mono Lake (Kulp et al., 2006, 2007). Although both sulfate reduction and arsenate reduction were robust in Mono Lake, only arsenate reduction could be detected in Searles Lake. Despite a number of attempts and manipulations to enhance sulfate reduction in the latter environment (e.g., substrate additions, lowering background sulfate, lowering the salinity), no activity could be solicited. Addition of 200 mM borate ions to Mono Lake sediments resulted in ~80 % diminishment in sulfate reduction as compared with controls, while increasing the salinity from 25 to 325 g/L completely eliminated detectable sulfate reduction. By extrapolation to Searles Lake, these results indicated that the combined effects of high salinity and high borate concentrations in the brine conspired against expression of sulfate reduction in situ. Nonetheless, free sulfide was detected in Searles Lake sediments, albeit at levels between 10- and 100-fold lower than in comparable Mono Lake sediments (e.g., 0.1–0.3 vs. 5–10 mM). The origin of this sulfide is not clear, but it could stem from a partially constrained

sulfur cycle associated with the redox reaction of lower oxidation states of sulfur (e.g., thiosulfate, sulfite, elemental sulfur) that give rise to sulfide upon reduction (or disproportionation) and do not require expenditure of energy to activate, as does sulfate. These processes have been studied closely in the soda lakes of Russia and have been recently reviewed in detail (Sorokin et al., 2011).

One final point worth bearing in mind as a reason to study the microbial ecology and biogeochemistry of hypersaline lakes is that they can be considered potential terrestrial analogs of possible biomes for life elsewhere in our Solar System. Hence, Mars is currently a cold and exceptionally dry environment. Any liquid water encountered upon or well beneath its surface is likely to be highly saline. Similar arguments can be made for the dense brines existing beneath the ice sheets of Europa (satellite of Jupiter) or of Enceladus (satellite of Saturn). Detection of in situ life, if these brines could be remotely sampled, would pose a challenge akin to that encountered for the Viking Mission to Mars in the 1970s, namely, what would constitute strong proof (or disproof) for an active microbial population? To this end we tested the ability of isolated anaerobes (*B. selenitireducens* and *H. silvermanii*) and anoxic sediments from Mono and Searles Lakes to generate electricity by using an anode of a microbial fuel cell as an electron acceptor. Power generation occurred in live samples and was greater in the lower salinity samples and cultures from Mono Lake than from Searles Lake. Nonetheless, biological electricity generation was notably occurring in the latter, suggesting that this approach to life detection has merit (Miller and Oremland, 2008).

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HALOPHILIC, ACIDOPHILIC, AND HALOACIDOPHILIC PROKARYOTES

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1. Introduction

During Earth's evolution, accompanied by geophysical and climatic changes, a number of ecosystems have been formed which differ by the broad variety of physicochemical and biological factors composing our environment (Pikuta et al., 2007). Traditionally, extreme conditions can refer to physical extremes such as temperature, pressure, and radiation, but also to geochemical extremes such as desiccation, salinity, pH, and toxins (van den Burg, 2003). Many microorganisms survive under physically and geochemically extreme conditions, and these are termed as extremophiles, including thermophilic, psychrophilic, piezophilic, radioresistant, xerophilic, halophilic, acidophilic, alkaliphilic, metallotolerant, toxitolerant, and oligotrophic. This chapter deals with polyextremophile organisms that are both halophilic and acidophilic.

2. Halophilic Prokaryotes

Hypersaline environments with more than 10 % to saturated salts (NaCl, Na₂SO₄, MgCl₂, etc.) are widely distributed on Earth, and they are mainly represented by saline lakes, solar salterns, and other aquatic systems as well as saline soils (Oren, 2002b). Microorganisms that inhabit those habitats are designated as halophiles. Extreme halophiles can grow optimally in media with 2.5–5.2 M NaCl, and moderate halophiles grow optimally in media with 0.5–2.5 M NaCl (Kuhnsner and Kamekura, 1988). These can be found in the hypersaline environments, as well as in rock salt and solar salt. The metabolic diversity of halophiles is very wide: they include oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens (Oren, 2002a). With increasing salinity the diversity of metabolic types decreases dramatically. The maximal salinity limit, at which each dissimilatory process occurs, is correlated with the amount of energy generated and energetic cost of osmotic adaptation in the environment (Oren, 1999).

Halophiles are found in each of the three domains: *Archaea*, *Bacteria*, and *Eukarya*. Within *Bacteria* we know halophiles within the phyla *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, and *Thermotogae*. Within the *Archaea* the most salt-requiring microorganisms are found in all genera in the family *Halobacteriaceae* and in some genera of the family *Methanosarcinaceae*: *Methanohalobium*, *Methanohalophilus*, *Methanosalsum*, etc. (Boone et al., 2001).

Haloarchaea, extremely halophilic aerobic *Archaea*, are classified within the family *Halobacteriaceae* of the order *Halobacteriales*. They require high concentration of NaCl for growth, with optimum concentrations of 10–30 %. Currently, the family *Halobacteriaceae* contains 39 genera comprising 137 species. Most haloarchaea are red or orange pigmented due to the presence of C-50 carotenoids, but some strains are colorless, and those with gas vesicles form opaque, white, or pink colonies. Haloarchaea exhibit feature characteristic of the *Archaea*, including eukaryotic-like transcription and translation machineries, ether-linked lipids, and a cell wall S-layer composed of glycoprotein. Many strains grow at neutral to slightly alkaline pH, and some alkaliphilic strains grow only at alkaline pH. However, no strains within the family *Halobacteriaceae* had been reported until not long ago that grow only in acidic pH conditions.

3. Acidophilic Prokaryotes

Acidic environments (pH <4) are also distributed on Earth, in natural geothermal areas, solfataric fields, acidic vents, sulfuric pools, etc., as well as man-made environments, areas associated with human activities such as mining of coal and metal ores (Seckbach, 2000). Acidic environments are interesting because, in general, the low pH is the consequence of microbial metabolism and not a condition imposed by the system as is the case in many other extreme environments (temperature, high salinity, alkaline pH, etc.) (González-Toril et al., 2003).

Acidophiles are an ecologically and economically important group of microorganisms, distributed through *Archaea*, *Bacteria*, and *Eukarya* (fungi, algae, and protozoa). They are hyperthermophilic, moderately thermophilic, or mesophilic (Oren, 2010). The general definition of acidophiles is that they are organisms that display pH optima for growth significantly lower than 7. There is no common agreement on the pH boundary that delineates acidophily, but a useful guide is that extreme acidophiles have an optimum pH for growth of lower than 3.0 and moderate acidophiles grow optimally at pH 3–5. Many moderately acidophilic prokaryotes can grow at pH <3, including mesophiles (*Thiomonas* and the *Acidobacteriaceae* spp.), moderate thermophiles (some *Alicyclobacillus* spp.), and extreme thermophiles (some *Sulfolobus* spp.). While acid-tolerant organisms have pH growth optima of >5, they are metabolically active in acidic environments (Johnson, 2007, 2012).

Acidophiles possess networked cellular adaptations to regulate the pH inside the cell. Extreme acidophilic bacteria have chemolithotrophic, chemolithomixotrophic, or chemoorganoheterotrophic metabolisms. They are spore-forming and non-spore-forming, aerobic, microaerophilic, or obligately anaerobic (Pikuta et al., 2007). The cell membranes of these bacteria have a positive or negative reaction to the Gram stain, and sometimes the cell wall is reduced to a single membrane. They are able to receive energy from hydrogen, iron, sulfur, or organic molecules.

Most extreme acidophiles belong to *Archaea*, including *Acidianus*, *Desulfurolobus*, *Metallosphaera*, *Stygiolobus*, *Sulfolobus*, *Sulfurisphaera*, *Sulfurococcus*, *Thermoplasma*, and *Picrophilus* (Bertoldo et al., 2004). The most acidiphilic archaeal strains, *Picrophilus oshimae* and *P. torridus*, are able to exist at a minimum pH of -0.2 (Schleper et al., 1996). These species grow within a moderately thermal regime and were found near a hydrothermal spring with solfataric gases. Another extremely acidophilic archaeon *Ferroplasma acidarmanus* grows at pH 0 in acid mine drainage in Iron Mountain, California. This species does not have a cell wall, and the cell membrane is the only barrier between the cytoplasm and concentrated sulfuric acid with high concentrations of heavy metals, copper, arsenic, cadmium, and zinc in the surrounding medium. None of these acidophiles is halophilic.

4. Microbial Communities of the Acid-Saline Environment

Although hypersaline environments and acidic environments are distributed on Earth, natural acidic and saline lakes are relatively rare. Acidic hypersaline lakes have been recognized in Western Australia (Alpers et al., 1992), northwestern Victoria, Australia (Long et al., 1992; Macumber, 1992), and Chile (Risacher et al., 2002). There are hundreds of ephemeral, acid-saline lakes, and regional acid-saline groundwater, as well as nearby neutral-hypersaline lakes on the highly weathered Archean ($\sim 4\text{--}2.5$ billion years old) igneous and metamorphic rocks, including granite, gneiss, anorthosite, quartzite, and ironstone of the Yilgarn Craton area (~ 1.78 million km^2) in the southern part of Western Australia (Mann, 1983; McArthur et al., 1991) (Fig. 1).

The lakes vary in size between 4,000 m^2 and 800 km^2 ; are shallow (<0.5 m deep), saline ($\sim 60\text{--}280\%$ of TDS (total dissolved solids)), and Na-Mg-Cl-SO₄ rich; and contain Al, Fe, and Br and variable amounts of Ca and K. Hundreds of individual lakes of different pH values exist in this region, and they contain extremely acidic (pHs <4), moderately acidic (pH 4–6), neutral (pH 6–8), and moderately alkaline (pH >8) lake water and groundwaters. Even where lake waters are neutral or moderately alkaline, groundwaters tend to be extremely acidic. Weather, climate, and seasonal variations affect the size, shape, depth, and water geochemistry of these lake systems (Benison and Bowen, 2006).

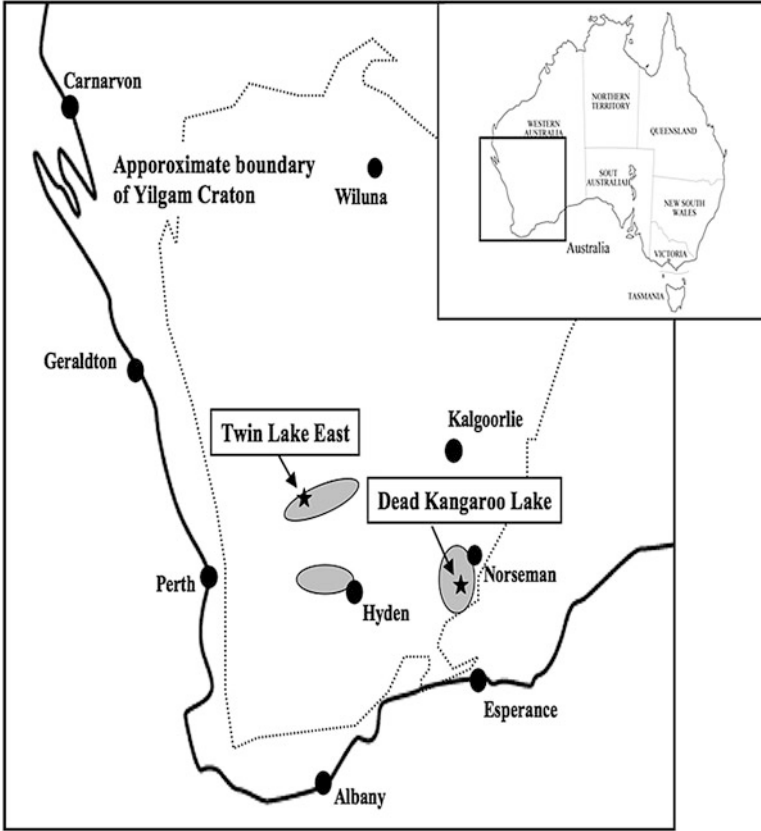


Figure 1. Approximate locations of acid-saline lakes in southern Western Australia. Acid lakes areas are shaded (Modified from Fig. 1 of Mormile et al., 2009).

The acid-saline lake and groundwater systems in Western Australia are strikingly similar to sedimentary deposits on Mars. They both have a similar, yet highly unusual, assemblage of minerals, including chloride salts, calcium sulfates, hematite, jarosite, phyllosilicates, and opaline silica, as well as a siliciclastic component (Clark et al., 2005; Benison and Bowen, 2006; Osterloo et al., 2008).

The presence of microorganisms in Western Australia acid-saline lakes that can withstand and even thrive in low-pH, high-salinity, metal-rich waters, together with fluctuating surface conditions and high UV radiation, indicates that specific life-forms can indeed live in conditions likely to have existed on early Earth (Sugisaki et al., 1995) and on Mars (Benison and Bowen, 2006).

Mormile et al. (2009) determined the microbial diversity of two acid-saline lakes of the Yilgarn Craton, Twin Lake East, and Dead Kangaroo Lake. Twin Lake East is a small round lake located 99 km south of Norseman in the southeastern part of the Yilgarn Craton. At the specific time and location of sampling,

the lake water had a pH value of 3.0, 160‰ TDS, a temperature of 14 °C, and was 22 cm deep. This lake represents a halite-dominated acid lake situated on Archean host rocks which has the lowest pH. One more acid-saline lake, Dead Kangaroo Lake, is a small round lake that is part of a large lake system called the Bandee Lakes located 25 km east of Kellerberrin in the central part of the Yilgarn Craton. The lake water had a pH value of 4.3, 40‰ TDS and a temperature of 17 °C and was 2 cm deep. This lake is situated on sediment composed mostly of quartz sand and reworked gypsum sand.

In June 2005, they collected lake water to characterize the microbiological population of the two lakes. They extracted DNAs from lake water samples retrieved from each of these lakes, amplified by PCR using a bacterial primer set for the clone libraries, and analyzed by denaturant gradient gel electrophoresis (DGGE). The clone libraries from Twin Lake East possessed 24 unique sequences representing three different phyla, *Proteobacteria* (18), *Bacteroidetes* (4), and *Actinobacteria* (2), and were closely related to 13 different genera (*Altererythrobacter*, *Caulobacter*, *Devosia*, *Dyella*, *Erythrobacter*, *Gillisia*, *Hymenobacter*, *Modestobacter*, *Novosphingobium*, *Porphyrobacter*, *Segetibacter*, *Sphingomonas*, and *Sporichthya*).

On the other hand, in Dead Kangaroo Lake, the 18 clones sequenced were closely related to those of five genera, *Marinobacter*, *Marinomonas*, *Methylophaga*, *Paracoccus*, and *Rhodobacter* of the phylum *Proteobacteria*. Unfortunately and sadly enough, Mormile et al. did not determine the diversity of haloarchaeal acidophiles. Could any acidophilic haloarchaea have been present in these lakes?

5. Acidophilic Haloarchaea

5.1. SEARCHING FOR ACIDOPHILIC HALOARCHAEA

Of the 137 species belonging to the family *Halobacteriaceae*, *Halococcus qingdaonensis* is the only one that has been reported to be able to grow at acidic pH 4.0 (Wang et al., 2007). Is this because of the scarcity of acidic hypersaline environments on Earth? Or, were we just idle in searching for acidophilic haloarchaea? Since *Hcc. qingdaonensis* was isolated from an enrichment of a crude sea-salt sample in a medium with pH 7.0, there is a possibility that acidophilic haloarchaea are thriving in saline environments of neutral pH. Fortunately, various salt samples are available on the market in Japan. We purchased many salt samples including natural sea salt, rock salt, and lake salt, domestic, imported, and gifts from abroad. A medium (MH1) of the following composition (per liter) was used for enrichment cultures: 4.0 g casamino acids (Difco), 2.0 g yeast extract (Difco), 2.0 g L-glutamic acid, 2.0 g trisodium citrate·2H₂O, 5.0 g K₂SO₄, 1.0 g MgCl₂·6H₂O, 1.0 g NH₄Cl, 1.0 g KH₂PO₄, 4 mg Fe₂SO₄·6H₂O, 200 g (3.4 M) NaCl, 2.0 ml trace metal solution, pH adjusted to 4.0 or 4.5 with 40 % KOH, and 20 g Bacto-agar (Difco) when necessary. The trace metal solution contained (per liter of distilled water): 2.0 g

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 1.0 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 g ZnCl_2 , 0.1 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.04 g AlCl_3 , 0.02 g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, and 0.02 g H_3BO_3 , pH adjusted to 4.0 with HCl. The two media were autoclaved for 20 min at 121 °C. The salt samples (1.0 g each) mentioned above were dissolved in 4 ml of MH1 liquid medium. After incubation at 37 °C for 1 week without shaking, 0.1 ml of each culture were spread on MH1 agar plates (pH 4.5).

After incubation at 37 °C for 2 weeks, no colonies were obtained from enrichment at pH 4.0. However, 39 out of 583 salt samples tested gave colonies on the enrichment at pH 4.5. Ten salt samples were imported solar salt (from China, Indonesia, Italy, and the Philippines), seven were rock salt (from Bolivia, Colombia, Italy, Nepal, and Pakistan), two were salt prepared in Japan from imported solar salts (from Australia and Mexico) (dissolved in seawater and boiled for several hours), four were Japanese solar salt, nine were salt produced in Japan from seawater by boiling, three were prepared by low-temperature drying or spray-drying methods in Japan, and the origin of four additional samples was not clear. Colonies were picked up and transferred to fresh agar plates of pH 4.5, and pure cultures were obtained by plating serial dilutions and repeated transfers on the agar plates. More than 70 strains were isolated, most of which were not pigmented, and only five strains were colored pale pink to orange. All strains grew within a pH range of 4.5–7.2, and none of them showed growth at pH higher than 7.5. The most acidophilic strain MH1-52-1 showed growth in a very narrow pH range, between pH 4.2 and 4.8.

These results were surprising since halophilic microorganisms, growing at 20 % NaCl, and acidophilic organisms, growing at pH 4.5–6.0, but not at pH 6.5, were so easily isolated from so many different salt samples of various origins, suggesting strongly that the haloacidophiles may be distributed in nature throughout the world. It was also surprising that alkaliphilic halophiles able to grow at pH 9.5–12.0 were isolated from the same six salt samples that yielded the acidophilic halophiles: one rock salt from Pakistan and five solar salt samples from China, Indonesia, Korea, Japan, and the Philippines. These salt samples harbored both acidophilic and alkaliphilic halophilic *Archaea* of the genera *Halorubrum* and *Halostagnicola*.

5.2. PHYLOGENY ANALYSIS OF ACIDOPHILIC HALOARCHAEA

Total DNAs of 74 strains isolated were extracted by the method of Cline et al. (1989), and the 16S rRNA genes were amplified by PCR with the following forward and reverse primers: 5'-ATTCCGGTTGATCCTGCCGG-3' and 5'-AGGAGGTGATCCAGCCGCAG-3'. DNA polymerase was Ex Taq polymerase (Takara Bio, Japan). The PCR was done by 25 cycles of denaturation (20 s, 96 °C), annealing (20 s, 58 °C), and extension (2 min, 72 °C). The 5'-terminal 500 bp of the amplified genes were sequenced using the BigDye Sequencing Kit Ver. 3.1 (Applied Biosystems) by the ABI 310 DNA sequencer (Minegishi et al., 2008).

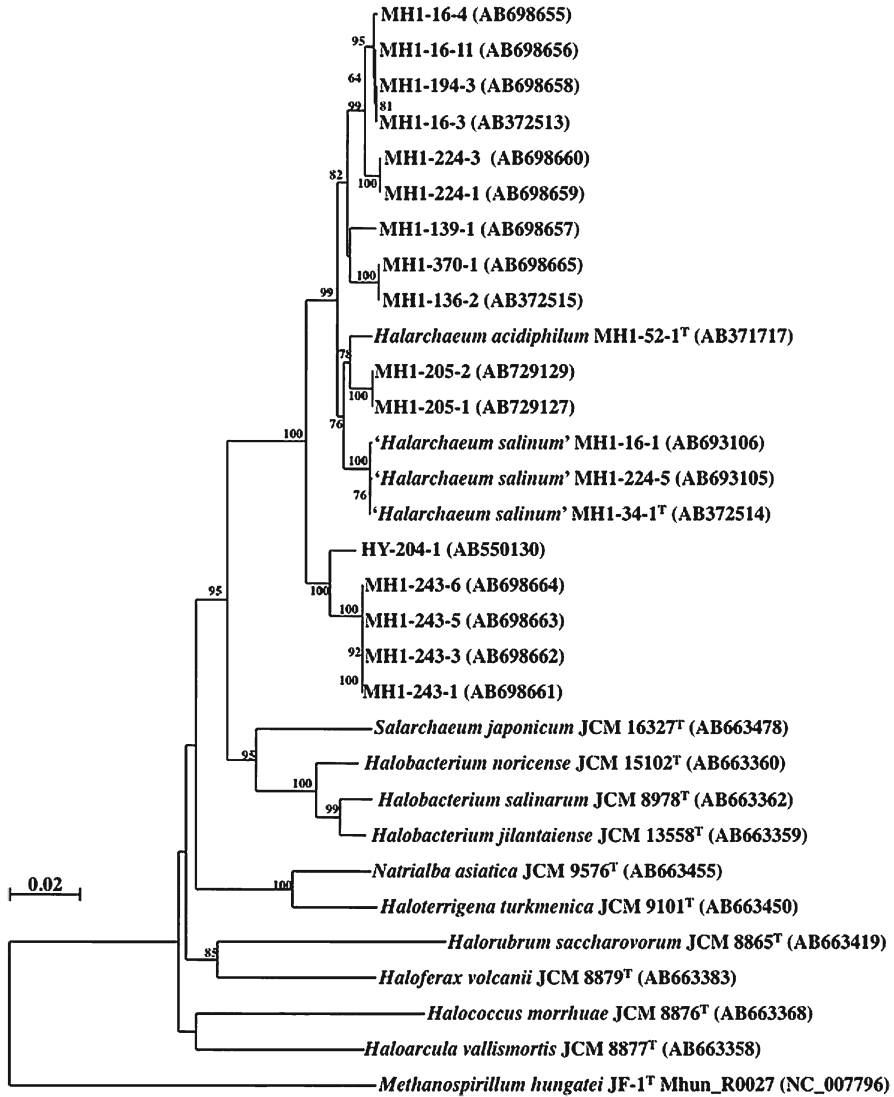


Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between 20 strains and type species of the genera within the family *Halobacteriaceae*. Three additional species of the genus *Halobacterium* were also added since these were most closely related with the four isolates. Bootstrap values (%) are based on 1,000 replicates and are shown for branches with more than 50 % bootstrap support. The sequence of *Methanospirillum hungatei* was used as the outgroup. Bar, 0.02 substitutions per site.

The 74 isolates can be divided into nine groups based on their sequence similarities. Twenty representative strains were selected and their full-length sequences were determined. A phylogenetic tree (Fig. 2) suggested that these 20 strains were not

scattered throughout the family *Halobacteriaceae* but rather formed one loose cluster that may constitute a novel genus within the family. For example, the sequence of the strain MH1-52-1 (AB371717), the most acidophilic strain, showed 91.7 % similarity to that of *Halobacterium noricense* A1^T (AJ548827). This strain has been proposed as *Halarchaeum acidiphilum* gen. nov., sp. nov. (Minegishi et al., 2010). DNA-DNA hybridization values among *Halarchaeum acidiphilum* strain MH1-52-1 and the rest of the 20 strains assessed by the fluorometric method of Ezaki et al. (1989) were well below the threshold value of 70 % generally accepted for the definition of a novel species (Wayne et al., 1987; Stackebrandt and Ebers, 2006), suggesting strongly that they may represent different species of the genus *Halarchaeum*. More recently, we proposed strain MH1-34-1^T, MH1-16-1, and MH1-224-5 as *Halarchaeum salinum* sp. nov. The 16S rRNA gene sequences of the strains were almost identical (99.8–99.9 % similarity), and the closest relative was *Halarchaeum acidiphilum* MH-1-52-1^T with 98.4 % similarity. The level of DNA-DNA relatedness among the three strains was 90–91 %, while relatedness between each of the three strains and *Hla. acidiphilum* MH1-52-1^T was 51–55 %. The type strain MH1-34-1^T was able to grow at 12–30 % (w/v) NaCl at pH 4.5–7.2 (optimum, pH 5.2–5.5) and at 15–45 °C (Yamauchi et al., 2013).

5.3. PHYSIOLOGY OF ACIDO-HALOARCHAEA

The most acidophilic strain *Hla. acidiphilum* MH1-52-1^T was isolated from “Salt of Coral,” a solar salt imported from Australia, to which powdered coral has been added. The pH of 25 % aqueous solution of this salt was 9.0. The three strains of *Hla. salinum* were isolated from commercial salt samples produced from seawater in Indonesia, the Philippines, and Japan. *Hla. acidiphilum* MH1-52-1^T could grow at pH from 4.2 to 4.8, with optimal growth at pH 4.4–4.6 in liquid MH1 medium (5 mM Mg²⁺) (Fig. 3). In media with higher Mg concentration, 25 mM, growth was observed at pH 4.0–6.0 with an optimum at pH 4.0.

Colonies of *Hla. acidiphilum* on agar plates were nonpigmented, but strains of *Hla. salinum* were orange or pink pigmented. The temperature range for growth was 15–45 °C, with optimum growth at 37 °C. Strain MH1-52-1^T grew in 18–30 % (w/v) NaCl, with optimum growth at 21–24 % (w/v) NaCl. Growth was observed from 1 mM up to 500 mM Mg²⁺ in media with 20 % (w/v) NaCl, with an optimum at 50 mM. Cells aggregated in media with more than 20 mM Mg²⁺ after incubation for 7 days. In media with 250 mM Mg²⁺, growth was observed at pH 4.0–6.0 with an optimum at pH 4.5 after incubation for 3 days.

It may be correct to point out here that an often-used medium, JCM medium No.168, adjusted to pH 4.5 does not support growth of *Hla. acidiphilum* MH1-52-1^T. This medium has the following composition: 5.0 g casamino acids, 5.0 g yeast extract, 1.0 g sodium glutamate, 3.0 g trisodium citrate·2H₂O, 20.0 g Mg₂SO₄·7H₂O, 2.0 g KCl, 36 mg FeCl₂·4H₂O, 0.36 mg MnCl₂·4H₂O, and 200 g NaCl, per liter. This medium adjusted to pH 7 supports growth of many

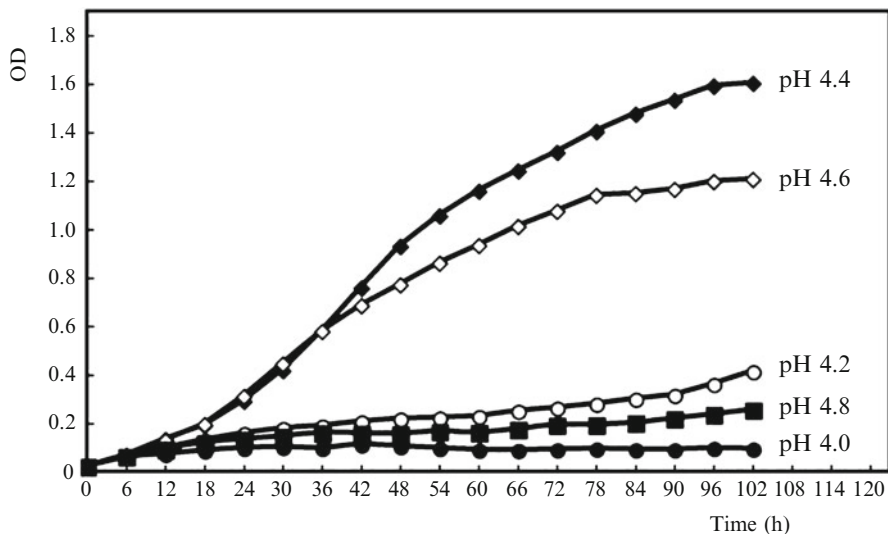


Figure 3. Growth curves of *Halarchaeum acidiphilum* strain MH1-52-1 at 37 °C in MH1 media adjusted to pH 4.0–4.8.

haloarchaeal strains, but medium adjusted to pH 4.5 failed to support growth of MH1-52-1^T. This fact suggests that some components of medium MH1 are crucial for growth at acidic pH. A modified MH1 medium, without KH_2PO_4 and metal solution, however, supported the growth of *Hla. acidiphilum*. There is a probability that more and more acidophilic haloarchaeal strains will be isolated by using further modified media, or other culture conditions.

Although acidophilic haloarchaea capable of growth at below pH 4 have not been isolated so far, our present data suggest that acidic saline environments such as acidic hypersaline lakes in southern Western Australia may inhabit interesting novel types of acido-haloarchaea.

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LIFE IN MAGNESIUM- AND CALCIUM-RICH HYPERSALINE ENVIRONMENTS: SALT STRESS BY CHAOTROPIC IONS

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1. Introduction

Most hypersaline environments on Earth are derived from seawater by evaporation. Seawater is dominated by sodium chloride as the main salt. Sodium constitutes 86 % of the cation sum (0.482 M in “standard” seawater of 35 ‰ salinity), with lower concentrations of Mg^{2+} (0.056 M), K^+ (0.011 M), and Ca^{2+} (0.011 M). When seawater evaporates to form hypersaline brines (so-called thalassohaline brines), the ionic composition of seawater is initially preserved. When the salinity increases, sequential precipitation of calcium carbonate (calcite, at total salt concentrations above 6–8 ‰) and calcium sulfate (as gypsum, $CaSO_4 \cdot 2H_2O$, that starts precipitating when the total dissolved salt concentration has increased to >120–150 g/l) causes minor changes in the ionic ratios. Only during the precipitation of NaCl as halite, when the total salt concentration exceeds 300–350 g/l, do we witness a great change in the ratio between monovalent and divalent cations. The bittern brines that remain after most of the sodium ions have been removed from the water are dominated by magnesium as the main cation.

Most halophilic and halotolerant microorganisms prefer to live in near-neutral brines in which Na^+ and Cl^- are the main ions. Some hypersaline environments are highly alkaline. At high pH, the solubility of the divalent cations Mg^{2+} and Ca^{2+} is greatly limited. The microbiology of such haloalkaline environments is discussed elsewhere in this book (Banciu and Sorokin, 2013; Oren, 2013). Microorganisms living in those environments have to cope with the additional stress by the high pH, which causes problems to the bioenergetics of the cells which have to maintain an internal pH below that of the medium, compensating the reverse pH gradient by a high inside-negative membrane potential. Environments at neutral or slightly acidic pH dominated by divalent cations can be even more stressful to microbial life because of the “chaotropic” – destabilizing – nature of calcium and magnesium ions when present at high concentrations. An excess of such chaotropic ions over “kosmotropic” – stabilizing – ions causes severe stress, this in addition to the osmotic stress inherent to solutions with high solute concentrations.

This chapter first discusses the specific effects of chaotropic and kosmotropic ions on microorganisms, as expressed in the “Hofmeister series” in which the different ions are arranged on the basis of their stabilizing or destabilizing effect. This is followed by an evaluation of the intracellular concentrations of chaotropic ions, a topic about which not much is known yet. Then a number of case studies of the microbiology in high divalent cation concentration environments are discussed: the bittern ponds in solar salterns in which brine is stored from which most of the Na^+ has been precipitated as halite, the Dead Sea, a natural salt lake with extremely high – and still increasing – magnesium and calcium concentrations and ever-decreasing concentrations of sodium so that the ratio between chaotropic and kosmotropic ions is rapidly increasing; the brines of Discovery Basin on the bottom of the Mediterranean Sea that contain 5 M MgCl_2 and little else; and the CaCl_2 brines of Don Juan Pond in Antarctica, an environment in which the boundaries of life are exceeded although claims of the presence of living microorganisms have been made in the past. Finally, a discussion will be devoted to the recently discovered “chaophilic” fungi, organisms that grow better in chaotropic media with extremely high magnesium ion concentrations than in NaCl -dominated media.

An understanding of the limits of the existence of halophilic microorganisms at high concentrations of chaotropic divalent ions is of great importance for the assessment of the possibility of life on Mars. The recent discovery of seasonal flows of liquid, most likely composed of chlorides of magnesium, sodium, and/or calcium, at different locations on Mars (McEwen et al., 2011), makes the issue of life at high divalent cation concentrations particularly relevant today.

2. Divalent Cation Concentrations and Halophily: The Hofmeister Series

A major reason why divalent cations such as calcium and magnesium at high concentrations are toxic even to the best salt-adapted microorganisms is their destabilizing action on biological structures (McGenity and Oren, 2012). The relative stabilizing/destabilizing action of different cations on proteins was first documented at the end of the nineteenth century when Hofmeister (1888) showed that different salts have different efficiencies at salting out egg-white protein and that some salts do not cause salting out at all. The phenomenon is not fully understood even today, but the conventional view is that competition between dissolved salt and dissolved protein for water of hydration results in a loss or gain in solubility. The so-called Hofmeister series or lyotropic series reflects the order of effectiveness of ions in precipitating or “salting out” hydrophilic sols. The lyotropic series for the common monovalent cations, from stabilizing to destabilizing, is $\text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+$. For inorganic monovalent anions, the order is $\text{Cl}^- > \text{NO}_3^- > \text{ClO}_3^- > \text{I}^- > \text{CNS}^-$ (Hofmeister, 1888; Brown, 1990). The stabilizing ions are named kosmotropic, the destabilizing ones chaotropic. Chaotropes weaken electrostatic interactions and

destabilize biological macromolecules. Kosmotropes such as compatible solutes strengthen electrostatic interactions. The series for the monovalent ions shows the order of increasing ionic radii and increasing free energy of hydration. Di- and multivalent ions can be fitted into the series but their radii and hydration energies do not usually conform to their place in the series. Calcium and to a lesser extent magnesium behave as chaotropic ions. High concentrations of chaotropic ions can be compensated to some extent by stabilizing kosmotropic ions (Hallsworth et al., 2003).

A recent reevaluation showed the Hofmeister series to be a sequence in water-ordering power of ions. Ions in solution may disturb the characteristic tetrahedral structure of water. To compare the effects of applied pressure and high salt concentrations on the hydrogen-bonded network of water, neutron diffraction was used, a technique that gives a microscopic measure of the Hofmeister effect. Ions induce a change in water structure equivalent to the application of high pressures, the size of the effect being ion-specific. These changes may be understood in terms of the partial molar volume of the ions relative to those of water molecules (Leberman and Soper, 1995; Parsegian, 1995). The equivalent induced pressure of a particular ion species is correlated with its efficacy in precipitating, or salting out, proteins from solution. But the factors that determine whether an ion stabilizes or destabilizes a molecular surface are still incompletely understood (Parsegian, 1995).

Early investigations on the interactions between chaotropic (destabilizing) and kosmotropic (stabilizing) ions determining growth of halophilic microorganisms were performed already in the 1930s when Lourens Baas Becking devoted extensive studies to the physiology of the unicellular flagellated green algae *Dunaliella viridis* and *D. salina* (Baas Becking, 1930, 1934; Oren, 2011). Much of the behavior of *Dunaliella* and other halophilic microorganisms in their natural habitats was explained by Baas Becking on the basis of antagonism between the different ions in the brines. Calcium proved particularly toxic, especially in slightly acidic media, but magnesium could relieve the toxicity of calcium to some extent. The higher the NaCl concentrations, the more magnesium was required to detoxify calcium to enable growth of *Dunaliella*. For example, in 1 M NaCl, the antagonistic relation Mg:Ca is 4:5, while in 4 M NaCl the proportion becomes many times as great (20:1). The red Archaea of the family *Halobacteriaceae* were found to require high salinity, pH, and a certain amount of magnesium, but they did not develop in cultures containing high calcium concentrations. Cyanobacteria were found to grow best in lower NaCl and low Mg:Ca ratios. The summary statement by Baas Becking (1931) was: "Thus, during the evaporation of sea water, we may predict the environment will become suitable, in succession for: *blue-green algae and fungus* → *Dunaliella viridis*, first swarmers and zygotes, then palmella → *D. salina* → *red bacteria*."

Another set of observations by Baas Becking related to the influence of chaotropic anions on the viability of *Dunaliella*. *Dunaliella* proved resistant to very high concentrations of toxic anions such as chromate, cyanide, and

thiocyanate: “Anions have little or no influence on *Dunaliella*. It is intriguing to watch this alga swimming for hours in concentrations of cyanide, thiocyanate, or chromate” (Baas-Becking, 1931). The true physiological basis for the survival of *Dunaliella* in molar concentrations of chromate, cyanide, and other unusual anions is yet to be elucidated, but Baas Becking’s explanation for the phenomenon is an interesting one. He attributed the phenomenon to the presence of a strongly negative charge of the cell membrane and the flagella, which can be neutralized by cations in a lyotropic series, rendering the membrane impermeable to anions and selectively permeable to cations.

A study of the ionic composition of the media supporting growth of different strains of halophilic Archaea, comparing *Halobacterium salinarum* which prefers monovalent cations with *Haloferax volcanii*, an isolate from the divalent-rich Dead Sea, was published by Edgerton and Brimblecombe (1981). *Hbt. salinarum* is a true extreme halophile that grows optimally at water activities of 0.78–0.79, values close to the water activity of NaCl-saturated brines ($a_w \sim 0.75$; Grant, 2004). *Hfx. volcanii* should be termed moderately halophilic, with best growth at $a_w = 0.925$. Calculations of the activities of the individual ions in such media, using the Pitzer equations (Pitzer, 1973; Pitzer and Kim, 1974), showed that in brines of increasing salinity, activity coefficients often first decrease, then increase with concentration. Sulfate has a low activity coefficient (<0.1), even at moderate ionic strengths (Edgerton and Brimblecombe, 1981).

There are, however, also specific stabilizing effects of divalent cations on the cell envelope of halophilic Archaea. Cells of many pleomorphic types such as *Haloferax* and *Haloarcula* species become spherical when suspended in media devoid of divalent cations. Differences in Mg^{2+} requirements of halophilic Archaea are likely to be reflected in peculiarities of protein chemistry of the cell envelope (Cohen et al., 1983).

3. Intracellular Concentrations of Divalent Cations in Halophilic Microorganisms

There are only very few data on the intracellular concentrations of divalent cations within microorganisms grown in media rich in magnesium or calcium. Such measurements are not straightforward as the calculations depend on very exact estimates of the volumes of intracellular and extracellular water in cell pellets. Presence of a periplasmic volume complicates the calculations. Moreover, it is necessary to discriminate between free ions and ions bound to cellular structures – DNA, RNA, and proteins. Only few attempts have therefore been made to estimate the intracellular Mg^{2+} and Ca^{2+} concentrations in halophilic microorganisms.

In a comparative study, intracellular concentrations of different ions were measured in the non-halophilic *Escherichia coli*, the moderately halophilic bacterium *Salinivibrio costicola*, the moderately halophilic, high divalent cation-requiring archaeon *Haloferax volcanii*, and the extremely halophilic archaeon *Halobacterium*

salinarum (*cutirubrum*). Intracellular magnesium concentrations in *E. coli*, *S. costicola*, and *Hbt. salinarum* were estimated at 29.7, 39.8, and 102.4 mmol/kg of cell water, respectively (de Médicis et al., 1986). How much of this magnesium is free in solution and how much is bound to cellular structures is unknown. Unfortunately, no data were presented on the magnesium concentrations measured inside *Hfx. volcanii*, possibly due to the analytical problems connected with the high magnesium concentrations in the growth medium.

Attempts to estimate the intracellular magnesium and calcium concentrations in *Halorubrum sodomense*, a halophilic archaeon with an unusually high magnesium requirement and magnesium tolerance, isolated from the Dead Sea, showed apparent intracellular Mg concentrations to be lower than those in the outside medium, but still the estimated values were surprisingly high (Table 1). It must be remembered that part of the “intracellular” magnesium and calcium may be bound to the cell wall, membranes, and macromolecules in the cytoplasm. Artifacts are possible as well due to the difficulty to estimate lower intracellular concentrations in the presence of high concentrations in the surrounding medium, the uncertainty in the determination of the exact contribution of intracellular volume within cell pellets, etc.

Little is known about the mechanism of regulation of intracellular Mg^{2+} concentrations in halophilic Archaea. In *Halobacterium salinarum*, an outward calcium transport mechanism was identified mediated by a Na^+/Ca^{2+} antiporter (Belliveau and Lanyi, 1978).

4. Case Studies

4.1. SOLAR SALTERN S

The brines in the evaporation ponds of solar saltern brines up to the stage of halite crystallization are typical thalassohaline solutions, dominated by NaCl. However, during the precipitation of halite in the crystallizer ponds, the relative concentrations of Mg^{2+} and Ca^{2+} increase sharply, so that organisms growing in the salt-saturated ponds need to be able to tolerate high divalent cation concentrations as well, in addition to the general salt tolerance required (Javor, 1989; Oren, 2002). Few systematic studies have been performed on the upper limit of divalent ion tolerance in halophilic Archaea and other organisms that make up the biota of saltern crystallizer ponds. Javor (1984) described a strain GNM-3 from the Guerrero Negro, Mexico, salterns, with an unusually high magnesium requirement and tolerance. This red-pink, motile strain containing gas vesicles and bacteriorhodopsin, probably belonging to the genus *Halobacterium*, grows optimally in the presence of 1.2 M $MgCl_2$ in the presence of 3 M NaCl.

A prominent member of the biota of saltern crystallizer ponds worldwide is the flat square archaeon *Haloquadratum walsbyi*. Strain C23, the type strain of *Hqr. walsbyi*, had no specific optimum for $MgCl_2$, but concentrations above 1 M

Table 1. Apparent intracellular cation concentrations of *Halorubrum sodomense* ATCC 33755^T, grown at different concentrations of magnesium and calcium.

	Na ⁺ _{out}	Na ⁺ _{in}	K ⁺ _{out}	K ⁺ _{in}	Mg ²⁺ _{out}	Mg ²⁺ _{in}	Ca ²⁺ _{out}	Ca ⁺ _{in}	Total _{in}	Total _{out}
High magnesium, low calcium medium	2.14	0.66	0.01	3.10	0.95	0.33	0.05	0.035	3.15	4.13
Intermediate magnesium, intermediate calcium medium	2.14	1.00	0.01	3.14	0.95	0.42	0.25	0.19	3.15	4.75
Low magnesium, high calcium medium	2.14	0.52	0.01	3.47	0.50	0.18	0.50	0.19	3.15	4.36

Modified from Oren (1986a).

Concentrations are given in molar units. The general outline of the experiments, estimation of intracellular and extracellular volumes in cell pellets, etc. were as given by Oren (1986b). Na⁺ and K⁺ were assessed by flame photometry. Intracellular Ca²⁺ concentrations were estimated by measuring the distribution of ⁴⁵Ca in cell pellets and culture supernatants of cells grown in medium supplemented with ⁴⁵CaCl₂. Magnesium was estimated colorimetrically using a modification of the procedure described by Taras (1948).

yielded higher cell densities than lower concentrations, while growth peaked at 0.4–0.6 M MgSO_4 . Strain HBSQ001 grew optimally at 0.2 M MgCl_2 but required 0.6 M MgSO_4 to reach the same density. In both strains, growth declined markedly at high MgCl_2 concentrations compared to MgSO_4 , suggesting that the high chloride ion concentration (>5 M) may have been inhibitory (Burns et al., 2007). In spite of the presence of energy-demanding cation efflux systems, the high external magnesium concentrations may lead to an increase in the internal magnesium concentration that is higher than in other organisms. Bolhuis et al. (2006), who analyzed the genome of *Hqr. walsbyi*, argued that magnesium ions have a stabilizing effect on the DNA duplex, the secondary structure of RNA, and DNA-RNA heteroduplexes. In case of an already stable high-GC genome, as present in most members of the *Halobacteriaceae*, the additional stabilizing effect of magnesium might therefore result in DNA rigidity that may interfere with essential processes like DNA replication and transcription. *Hqr. walsbyi* is a genome with an unusually low G+C content (47.9 mol %). It was proposed that the drift to an AT-rich genome might be induced as a long-term evolutionary adaptation to this over-stabilization by magnesium.

Divalent cations dominate in the bittern brines that remain after most of the common salt has precipitated. Such bittern ponds are often considered to be devoid of life. No bacteria or archaea grew in the enrichment cultures or on agar plates inoculated with bittern brines (Javor, 1983, 1984). Still the possibility exists that viable cells remain in the bitterns. In a culture-independent molecular (16S rRNA sequence-based) community analysis of magnesium-rich bittern brine from a Tunisian saltern at 380–400 g/l total salt concentration, viability tests based on the staining behavior with acridine orange suggested 46 % of the cells to be viable but not detectable by culturability tests (Baati et al., 2011). This brine contained only 2.7 g/l Na but had 96.7 g/l Mg and 256.9 g/l chloride, had a measured water activity of 0.68, and harbored 1.4×10^7 prokaryotes/ml (by microscopic count). It remains to be ascertained to what extent the staining procedure employed can really be used to reliable information on the presence of viable cells. Unfortunately, the authors did not employ the LIVE/DEAD BacLight strain adapted for use in hypersaline environments by Leuko et al. (2004). Sequences retrieved by Baati et al. (2011) include *Salinibacter*-related ones (*Bacteroidetes*), *Haloquadratum* (58 % of the archaeal sequences), and *Halorubrum* spp. The bitterns of the La Trinitat saltern in Spain yielded many isolates of yeasts, showing that there also are eukaryotic microorganisms adapted to low water activity and high divalent ion concentrations (Butinar et al., 2005; see also Sect. 5).

4.2. THE DEAD SEA

The Dead Sea is a unique hypersaline lake that is not only dominated by divalent cations but in which the concentrations of magnesium and calcium are constantly

Table 2. Dead Sea ionic composition, salt concentrations.

Year	1959–1960		1977	1996	2007
	Upper water mass	Lower water mass			
Na ⁺ (M)	1.57	1.84	1.73	1.59	1.54
K ⁺ (M)	0.17	0.19	0.18	0.20	0.21
Mg ²⁺ (M)	1.49	1.75	1.81	1.89	1.98
Ca ²⁺ (M)	0.41	0.43	0.43	0.44	0.47
Cl ⁻ (M)	5.56	6.18	6.34	6.34	6.48
Br ⁻ (M)	0.06	0.07	0.07	0.07	0.08
SO ₄ ²⁻ (M)	NR	NR	0.005	0.005	0.005
(Na ⁺ +K ⁺)/(Mg ²⁺ +Ca ²⁺) ^a	0.92	0.93	0.85	0.77	0.71
Total dissolved salts (g/l)	298	335	339	339	347

Data were derived from Beyth (1980) and unpublished data (I. Gavrieli, the Geological Survey of Israel, personal communication).

NR not reported.

^aMolar ratio.

increasing: since the early 1980s, the water column is saturated with respect to NaCl, and massive amounts of halite precipitate to the bottom, increasing the salt layer by about 10 cm per year. As a result, the ratio between monovalent and divalent cations has sharply decreased in the past decades (Table 2). Activity coefficients of the major ions, calculated by Krumgalz and Millero (1982) for the Dead Sea for 1979, as based on the equations of Pitzer (1973), were Na⁺, 0.961; K⁺, 0.548; Mg²⁺, 2.276; Ca²⁺, 1.034; and Cl⁻, 3.37; Edgerton and Bribblecombe (1981) gave estimated activity coefficients of 0.821, 0.527, 1.072, 0.579, and 2.298, respectively.

A variety of microorganisms have been isolated from the Dead Sea over the years; these include Archaea of the family *Halobacteriaceae*, bacteria, unicellular algae, and fungi, and there also is evidence for the presence of different types of viruses. With respect to their adaptation of life at high salt concentrations and to the specific conditions prevailing in the divalent cation-dominated brines, most research effort has been devoted to the halophilic Archaea. Species isolated from the lake and described include *Haloferax volcanii* (Mullakhanbhai and Larsen, 1975), *Haloarcula marismortui*, *Halorubrum sodomense* (Oren, 1983), and *Halobaculum gomorrense* (Oren et al., 1995). *Hfx. volcanii* is less salt demanding than most other representatives of the *Halobacteriaceae* but is markedly tolerant toward the presence of high magnesium concentrations, some growth still being possible at 2 M Na⁺ and 1.4 M Mg²⁺ (Mullakhanbhai and Larsen, 1975). The optimal medium for growth of *Hrr. sodomense* contains 0.8 M MgCl₂ + 12.5 % NaCl. In the presence of 2.1 M NaCl, 0.6–1.2 M Mg²⁺ is needed for optimal growth. When grown at lower magnesium concentrations, growth rates were reduced and cells became irregular spheres. Calcium can at least partially replace

magnesium, and good growth was found in medium containing 1 M CaCl_2 , 15 mM MgCl_2 , and 2.1 M NaCl . Relatively good growth was even observed with as little as 0.5 M NaCl when the MgCl_2 was raised to 1.5 or 2.0 M (Oren, 1983). *Hbc. gomorreense* is no less magnesium tolerant, with optimal growth at 0.6–1 M Mg^{2+} in the presence of 2.1 M NaCl . At least 1 M Na^+ was needed in the presence of 0.8 M MgCl_2 , and media with less than 0.2 M Mg^{2+} supported poor or no growth (Oren et al., 1995).

Based on this information, it is clear that the salt concentrations in the Dead Sea with its current >2 M Mg^{2+} and about 1.5 M Na^+ are far above the optimum even for the best salt-adapted and chaotropic ion-adapted Archaea. Also species of the unicellular green algal genus *Dunaliella*, the sole primary producer in the Dead Sea, cannot grow under these conditions. Therefore, the development of massive blooms of microorganisms in the lake is currently possible only following exceptionally rainy winters when the upper meters of the water column become diluted by fresh water and the salinity drops significantly. Such blooming events were witnessed in 1980 and in 1992, when up to 2×10^7 and 3.5×10^7 red halophilic Archaea were found per ml in the surface layers (5–10 m, the maximum depth being about 300 m). In other periods when no density stratification existed, some viable Archaea remained still present, but their metabolic activity was minimal. Thus, the uptake rates of glycerol and amino acids in 1991 (following a number of dry years) were in the order of 0.02–0.05 nmol/l h, as compared to 10–40 nmol/l h during the bloom in the summer of 1980 (Oren, 1992). A metagenomic study of the Dead Sea microbial community in 2007, more than 10 years after the termination of the 1992–1995 bloom, showed the presence of a small but highly diverse community of Archaea in the surface waters, phylogenetically affiliated with genera such as *Halorhabdus*, *Halosimplex*, *Halomicrobium*, *Halogeometricum*, *Haloplanus*, *Natronomonas*, and *Halalkalicoccus* with no closely related cultured representatives. This in contrast to the metagenome of the 1992 bloom material which was essentially composed of a single lineage remotely affiliated with the genus *Halobacterium* on the basis of 16S rRNA sequences, but showing an entirely different polar lipid pattern (Bodaker et al., 2009, 2010).

The metagenome of the 1992 community showed enrichment in COGs (Clusters of Orthologous Groups) of Mg^{2+} and Co^{2+} channels. Enrichment in putative archaeal CorA magnesium channels in the 1992 library (up to 11-fold for COG 0598) points to a potential, yet unknown, resistance mechanism used by the Dead Sea halophiles. Although CorA is usually associated with Mg^{2+} influx activity, when exposed to high extracellular Mg^{2+} concentrations, CorA can mediate Mg^{2+} efflux (Gibson et al., 1991; Papp-Wallace and Maguire, 2008).

The magnesium concentration in the Dead Sea now rapidly approaches the value of 2.3 M, considered as the upper limit for life when there is no significant concentration of a kosmotropic ion to offset the inhibition by chaotropic ions (the limit determined by Hallsworth et al., 2007; see Sect. 4.3). Thus, when the current trend of decreasing water levels and halite precipitation will continue,

conditions may soon become too extreme for life of even those microorganisms best adapted to the adverse conditions presented by the Dead Sea brine.

4.3. THE LIMITS OF LIFE AT HIGH MAGNESIUM CHLORIDE: STUDIES ON DISCOVERY BRINE

A natural environment with magnesium chloride concentrations far higher than those presently encountered in the Dead Sea is Discovery Basin, located at a depth of 3.58 km below the surface of the Mediterranean Sea, 200 km off the western coast of Crete. This deep-sea brine pool originated about 2,000 years ago by dissolution of bischofite ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) that had formed during the desiccation of the Mediterranean Sea around 5.5 million years ago. The existence of a large-scale bischofite formation deposited during the Messinian salinity crisis demonstrates that the eastern Mediterranean had become evaporated to near dryness during that time. The undersea brine pool was discovered in December 1993–January 1994. It has a surface area of about 7.5 km², a temperature of 35–38 °C, and a total dissolved salts concentration of about 470 g/l, and it contains almost pure magnesium chloride (concentrations in mol/kg H₂O: Mg²⁺, 5.15; Cl⁻, 10.15; Na⁺, 0.084; K⁺, 0.090; Ca²⁺, 0.001; SO₄²⁻, 0.110; Br⁻, 0.110) (Wallmann et al., 1997, 2002).

The existence of this magnesium chloride brine and the salinity gradient formed at the interface between the Mediterranean Sea water and the concentrated brine provided a unique opportunity to explore the limits of life at increasing concentrations of a chaotropic salt, not compensated by significant concentrations of stabilizing kosmotropic cations. The first microbiological exploration of Discovery brine suggested the presence of a significant microbial community down to the bottom of the brine pool. At the interface, the microscopic counts (DAPI stain) were 6.1×10^4 cells/ml, decreasing to 1.9×10^4 /ml at the bottom. Analysis of clone libraries and enzymatic assays provided evidence for the occurrence of dissimilatory sulfate reduction, methanogenesis, as well as heterotrophic activity (glutamate uptake) (van der Wielen et al., 2005). 16S rRNA gene clone libraries prepared from different depths in the brine along the salt gradient showed a high abundance of sequences affiliated with the genus *Halorhabdus* (*Halobacteriaceae*), a genus that contains at least one anaerobic representative, *Hrd. tiamatea*, which was isolated from a deep-sea brine in the Red Sea (Antunes et al., 2008, 2011). *Halorhabdus* clones constituted 11 % of the interface clone libraries and 33 % of the clone libraries from the brine itself (van der Wielen et al., 2005). Further analyses of gene libraries prepared from DNA isolated from the brines uncovered *cbbL* and *cbbM* genes coding for the ribulose-1,5-bisphosphate carboxylase/oxygenase both in the brine and in the brine/seawater interface but not in the overlying seawater. Diversity of these genes was low. The *cbbL* sequences were remotely affiliated with a *Thiobacillus* or with one of the RuBisCo genes of

Hydrogenovibrio marinus. The *cbbM* genes clustered with thiobacilli and formed a new group. This was presented as evidence for the existence of a potential for autotrophic CO₂ fixation in the 5 M MgCl₂ brines. However, attempts to amplify genes for ammonia monooxygenase, methane monooxygenase, and diheme cytochrome *c* (involved in bacterial thiosulfate oxidation) remained unsuccessful (van der Wielen, 2006).

Still, viable microorganisms could not be recovered from the brines below the interface, with the possible exception of *Bacillus* and relatives that may have survived in situ as endospores. These cannot be subcultured on media containing molar concentrations of MgCl₂ (Sass et al., 2008; Antunes et al., 2011). Cells of different species of non-halophiles and slight halophiles (*Alteromonas marina*, *Bacillus firmus*) survive in Discovery Basin brine for no longer than a few hours (Borin et al., 2008).

It is now clear that no active cells are present in the 5 M MgCl₂ brines. It was calculated that 5 M MgCl₂ would have a chaotropic effect of 212 kJ/g, more than twice that of a saturated phenol solution. And the Discovery brine has an a_w of <0.4, far below the value of ~0.6 considered as the lower limit to support life. The highest permissible MgCl₂ concentration in culture media with growth scored after 18 months of cultivation was 1.26 M, equivalent to a water activity (a_w) of 0.916. The fact that 16S rRNA genes and genes for different metabolic functions (*dsrAB* of sulfate reducers, *mcrA* of methanogens) could be amplified from the most saline layers is due to the fact that DNA is preserved well in the concentrated magnesium chloride solutions. When, however, a search was made for specific mRNA molecules, which are much more labile than DNA, it became clear that active cells were present only up to about 2.3 M MgCl₂. Messenger RNA for *dsrAB* was found up to 1.88 M, *mcrA* mRNA up to 2.23 M, concentrations equivalent to a_w 0.801 and 0.845, respectively. mRNA of *dsrAB* of *Desulfohalobiaceae* (a family of halophilic sulfate reducers) was restricted to the zone of 1.60–2.23 M MgCl₂. The upper limit of MgCl₂ in the absence of compensating stabilizing ions appears to be about 2.3 M (Hallsworth et al., 2007). The rapid degradation of mRNA and the stability of bacterial DNA in the high-magnesium brines were confirmed in a simulation experiment in which cells of a *Marinobacter* isolated from the seawater/Discovery brine interphase were incubated in the 5 M MgCl₂ brine. DNA was only moderately degraded after 45 days and both 16S rRNA and *gyrB* gene sequences could be amplified. *gyrB* mRNA could be recovered only in the first hour of exposure of the cells to the brine. In the absence of compensating kosmotropic ions, a concentration of 2.3 M MgCl₂ appears to be the upper limit for life (Hallsworth et al., 2007). DNA preserved in such strong brines could constitute a genetic reservoir of traits acquirable by horizontal gene transfer (Borin et al., 2008). Despite the presence of energy-yielding redox couplings in the MgCl₂-rich chemocline (sulfate reduction, methanogenesis), the macromolecule-destabilizing effects of MgCl₂ above a concentration of 2.3 M must be considered to be incompatible with life (McGenity and Oren, 2012).

4.4. THE MAGNESIUM SULFATE BRINES OF HOT LAKE, WASHINGTON

An intriguing but poorly investigated magnesium sulfate-dominated environment is Hot Lake, Washington. This is a stratified terminal lake with a salinity gradient from about 100 g/l salts in the surface waters to 400 g/l salts at the bottom. Bottom brine contained (g/l) Mg^{2+} , 53.6; SO_4^{2-} , 243; Na^+ , 16.8; Cl^- , 1.9; K^+ , 1.5; Ca^{2+} , 0.7; and $\text{HCO}_3^-/\text{CO}_3^{2-}$, 3.1 (Brock, 1979; Trüper and Galinski, 1986). The pH ranged from 8.7 at the surface to 7.0 at the bottom. Heliothermal heating caused the waters at 1.5–2.2 m depth to heat up to 40 °C. Oxygen was depleted at depths below 1.5 m.

Organisms detected in the lake include the green alga *Chara* and different types of cyanobacteria found at depths below 1 m (*Plectonema*, *Oscillatoria*, *Anacystis*, *Gomphosphaeria*), and a dense population of *Chlorobium*-like green sulfur bacteria was recorded in the upper part of the monimolimnion, slightly below 2 m depth at >200 g/l total salts. Higher organisms were found as well: the brine shrimp *Artemia salina* and the rotifer *Brachionus angularis* (Anderson, 1958). This intriguing ecosystem deserves a more in-depth study, also with respect to the adaptation of the organisms present to life at high magnesium concentrations.

4.5. THE CONCENTRATED CALCIUM CHLORIDE BRINES OF DON JUAN POND, ANTARCTICA: THE SUPREME CHALLENGE TO LIFE

Since it was discovered in 1961, Don Juan Pond, located in the Wright Valley, Victoria Land, Antarctica, has intrigued biologists searching for the most extreme environments on Earth supporting life. Don Juan Pond is an unfrozen shallow (average depth 11 cm) lake about 200 × 700 m in size. During the summer, two small streams drain the moraine to the west. The temperature of the water reaches –24 °C to –3 °C in October–December. The water is a concentrated calcium chloride brine with up to 474 g/l total dissolved salts. The freezing point of the brine was determined at about –48 °C, and the pH was around 5.4 (Meyer et al., 1962). The ionic concentrations vary during the seasons: in December 1968, the water density was 1.386 g/ml, and it contained 137.1 g/kg Ca^{2+} and 251 g/kg Cl^- ; in July 1974, a density of 1.208 g/ml was measured, with 74.1 g/kg Ca^{2+} and 148 g/kg Cl^- . Siegel et al. (1979) reported even higher Ca^{2+} and Cl^- concentrations. Concentrations of Na^+ , K^+ , and Mg^{2+} are very low (Matsubaya et al., 1979).

Early studies suggested that living microorganisms may be present in Don Juan Pond. Meyer et al. (1962) reported bacterial rods and cocci growing in colonies. Different bacteria (*Bacillus megaterium*, *Micrococcus* sp., *Corynebacterium*

sp.) and a yeast (*Sporobolomyces* sp.) were isolated from the brine. During a field study in the Austral summer of 1978–1979, a 3–5 mm thick microbial mat was observed, extending 500–600 m² over much of the western part of Don Juan Pond. It contained *Oscillatoria*-like filaments, *Chlorella*-like cells, *Dunaliella*-like flagellates, as well as nonmotile colorless and red bacteria. The mat contained 327,000 ppm dissolved solid material, compared to 485,000 ppm in the brine of pond. Chlorophyll *a* and other photosynthetic pigments were also detected, and photosynthetic activity (oxygen evolution) could be measured upon incubation of mat material at +4 °C (Siegel et al., 1979).

In spite of these old observations, there is no conclusive evidence of microbes to grow in CaCl₂-dominated Don Juan Pond, presumably because concentrated CaCl₂ brines, in addition to the extremely low a_w , destabilize biological macromolecules just as MgCl₂. A CaCl₂ brine of >470 g/l has a water activity below 0.45 (Grant, 2004; Horowitz et al., 1972; Oren, 1993; Siegel et al., 1979). The recently reported N₂O evolution from the pond is due to abiotic processes and not, e.g., by bacterial dissimilatory reduction of oxidized nitrogen compounds (Samarkin et al., 2010). Biological activity is possible on the shore of the lake, where salinities are reduced and active algal mats containing *Oscillatoria*-like filaments, unicellular cyanobacteria, diatoms, fungi, and other organisms can develop (Siegel et al., 1979, 1983).

5. Do “Chaophilic” Microorganisms Exist?

The organisms able to grow at the lowest water activities are not prokaryotes but fungi. Some fungi such as *Xeromyces bisporus* can grow at a_w 0.61 (Grant, 2004; Hocking and Pitt, 1999). Surprisingly, some fungi grow optimally under chaotropic conditions. Glycerol at high concentrations is chaotropic, and it disrupts and permeabilizes biological membranes. However, a strain of *Xeromyces bisporus* was recently described that grew fastest in a highly chaotropic condition containing 6.84 M glycerol ($a_w = 0.714$) and could even grow in 7.60 M glycerol ($a_w = 0.653$) in the absence of any compensating kosmotropic compounds. It apparently has a preference for conditions that disorder macromolecular and membrane structures. Thus, we may have here a representative of a previously uncharacterized class of extremophiles: “chaotolerant” or even “chaophilic” microbes (Williams and Hallsworth, 2009).

More such “chaotolerant” fungi were recovered from the MgCl₂-rich bittern ponds of the coastal salterns of Sečovelje, on the border between Slovenia and Croatia. Isolates of *Cladosporium*, other filamentous fungi, yeasts, and black yeasts could grow on media with up to 1.5 M MgCl₂ and higher without a kosmotropic salt such as NaCl to compensate the chaotropic effect of the medium (Sonjak et al., 2010).

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SURVIVAL STRATEGIES OF HALOPHILIC OLIGOTROPHIC AND DESICCATION RESISTANT PROKARYOTES

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1. Introduction

Viable halophilic and halotolerant Archaea and Bacteria have been found in ancient salt deposits around the world. The first cultivations of halophilic microorganisms from Permian salt sediments (about 250 million years old) were reported in the 1960s (Reiser and Tasch, 1960; Dombrowski, 1963) and met with considerable skepticism. Some 30 years later, detailed taxonomic descriptions of halophilic Bacteria and Archaea (haloarchaea) obtained from ancient evaporites began to be published (Norton et al., 1993; Denner et al., 1994; Stan-Lotter et al., 2002; Mormile et al., 2003; Gruber et al., 2004; Vreeland et al., 2007). Sequences of small ribosomal RNA (16S rRNA) genes and other molecules allowed more meaningful comparisons of isolates with known strains than was possible before. In many cases, no exact matches of sequences from subsurface isolates with those of known strains from surface waters were found (McGenity et al., 2000). This does not necessarily mean that they do not exist in surface environments, merely that they have not been isolated yet from there. In one other case, three strains of *Halococcus salifodinae* with identical 16S rRNA sequences were found in three geographically separated subsurface regions, all of Permo-Triassic age: strain B1p from Permian Zechstein rock salt, mined at Bad Ischl, Austria; strain Br3 from solution-mined Triassic Northwich halite; and strain BG2/2 from a core of Permian Zechstein salt, Berchtesgaden, Germany (Stan-Lotter et al., 1999; McGenity et al., 2000). A detailed characterization of the three independently isolated halococci revealed that they were very similar and should be considered as strains of the same species (Stan-Lotter et al., 1999). During the Permian period, the large hypersaline Zechstein Sea covered an area of about 250,000 km² over much of northern Europe. This sea would have provided a connection between the areas from which the three strains of *Hcc. salifodinae* were isolated (Stan-Lotter et al., 1999; McGenity et al., 2000; Radax et al., 2001). It is conceivable that *Hcc. salifodinae* was present in the Zechstein Sea and became trapped in the evaporating salts.

Besides microorganisms, the retrieval of DNA from ancient sediments was also reported, yielding haloarchaeal and bacterial 16S rDNA amplicons from salt samples estimated to be up to 425 million years old (Radax et al., 2001; Fish et al., 2002). Recently, successful cultivations from several well-dated bore cores (Schubert et al., 2010; Gramain et al., 2011) added more support to the notion of viable haloarchaea in evaporites of great geological age. Survival of cells over millennia of years in dry sedimentary rocks on Earth would have important implications for the search for life on other planets, where sediment-like structures exist (Squyres and Knoll, 2005).

The halophilic microorganisms in ancient evaporites have experienced hypersaline environments, which were characterized by extremely high ionic strength, elevated temperatures, and probably high levels of UV radiation. Periodic evaporation of water had taken place, which caused concentration of salts and exposure of cells to desiccation. In addition, nutrients were depleted, and over time, the environment became oligotrophic, which denotes conditions of low carbon, phosphate, and/or nitrogen availability. Plausible mechanisms for the long-term survival of extremely halophilic Bacteria and Archaea in salt sediments have yet to be clarified. Although the long-term survival of spores from halophilic bacilli (Bacteria) in Permian salt crystals had been proposed, these data were considered rather controversial (see Schubert et al., 2010, for a discussion). With respect to haloarchaea, genome sequences suggested that these microorganisms do not produce spores (Onyenwoke et al., 2004). The occurrence of other types of haloarchaeal resting states or dormant forms, such as cysts, has been discussed (Grant et al., 1998), but could not yet be demonstrated unequivocally.

This chapter presents a description of mechanisms or strategies of halophilic microorganisms to cope with the multiple stresses of high salt (or low water activity), oligotrophic conditions, and long-term desiccation. Some mechanisms are known from other members in the prokaryotic world; some are novel and appear – at present – unique to extremely halophilic Archaea. If those strategies can be inferred to have been in use for millennia is debatable, but some reasons for this assumption will be discussed. Future directions, where research will likely be headed and new results can be expected, will be presented briefly.

2. General Strategies

2.1. RESPONSE TO HIGH CONCENTRATIONS OF SALT: OSMOADAPTATION

The general strategies for adaptation of microorganisms to hypersaline environments have been described extensively. Therefore, only a brief synopsis will be given here and the reader is advised to consult the excellent reviews and books on these topics (e.g., Grant, 2004; Oren, 2002; Galinski, 1995).

Microorganisms which are exposed to a low water activity (a_w) environment have to apply strategies to avoid water loss by osmosis. The term water activity is widely used to define the availability of water in a particular environment. When water interacts with solutes and surfaces, it is not available for other hydration interactions. Water activity (a_w) denotes the amount of water available for hydration of materials; a value of 1.0 indicates pure water, whereas a value of 0 indicates the total absence of free water molecules; addition of solutes is lowering a_w to values below 1.0. The a_w of saturated salt lakes, for example, is 0.75, which is the typical environment of numerous halophiles.

There are two basic strategies for microorganisms: (1) several halophiles use counterbalancing levels of inorganic ions (usually KCl) to achieve osmotic stability and (2) the majority of halophiles produces or accumulates low-molecular-mass organic compounds, the so-called compatible solutes (also termed osmolytes) which are compatible with the cell machinery. They protect against inactivation, inhibition, and denaturation of enzymes and macromolecular structures under conditions of low a_w . Compatible solutes belong to several classes of compounds, like amino acids and derivatives, alcohols, and sugars and derivatives (Grant, 2004).

2.2. OLIGOTROPHY AND STARVATION

Oligotrophs are organisms that can live in nutrient-poor environments. They are characterized by slow growth and low rates of metabolism, which lead generally to low population density. Oligotrophs occur in deep oceanic sediments, caves, glacial and polar ice, deep subsurface environments, ocean waters, and leached soils. The concentration of total organic carbon in these environments is typically in the range of one to a few milligrams per liter (Egli, 2010). Such environments were long considered to be “deserts” for life, and microbial forms, which were sometimes seen in the microscope, were assumed to be dead, dormant, or at least severely starved bacterial cells (Egli, 2010). Only during the last years was it recognized that most of these microbes are perfectly alive, metabolizing, and ready to grow when given the chance (Egli, 2010). One mode of adaptation to nutrient limitation consists of increasing the surface to volume ratio which increases the capacity for nutrient uptake relative to cell volume. Most oligotrophs achieve a high surface to volume ratio by reducing their cell diameter and effectively forming miniaturized cells. In this way, the organism’s capacity to scavenge available energy-yielding substrates will be increased (Morita, 1982). A high surface to volume ratio is thus typical for many oligotrophic bacteria. From a 0.2 μm filtrate of Mediterranean Sea water, a halophilic oligotrophic bacterium, *Vibrio calviensis*, was isolated (Denner et al., 2002). The haloarchaeon *Haloquadratum walsbyi* manages the increase of surface area in a somewhat different way, namely, by extremely flattening itself (Bolhuis, 2005; see below).

Reducing the cell diameter is sometimes called “dwarfing” (Kjelleberg et al., 1983; Nyström, 2004). So far, several bacterial dwarfs have been described, but

recently also an archaeal dwarf was identified in arid soil (Rutz and Kieft, 2004). There is a limit to miniaturization, which is due to the required minimum contents of DNA, ribosomes, RNA, and proteins. The theoretical prediction of the minimum volume is 0.005–0.01 μm^3 , which was nearly reached by starved cells of the marine bacterium *Candidatus Pelagibacter ubique*, a member of the SAR11 clade (Steindler et al., 2011).

Data from dormant or starved bacteria and spores indicated reduced amounts of ATP, for example, the ATP content of dormant spores of several *Bacillus* species was about two orders of magnitude lower than that of actively growing *Bacillus* cells (Setlow and Kornberg, 1970); marine *Vibrio* species, which are deemed dormant when in the oceans, lost ATP during starvation experiments (Oliver and Stringer, 1984); and several strains of *Staphylococcus* showed a decline of ATP when adhering to polymer surfaces and entering a dormant, miniaturized state (Stollenwerk et al., 1998). Prokaryotic dormancy is not easy to define, as discussed extensively by Kell and Young (2000), one reason being that “we have to wait for a cell to divide and can only state that it was alive, but not that it is alive” (an observation going back to the microbiologist J.R. Postgate). In any case, a low amount of cellular ATP has generally been associated with a dormant state of various bacteria and spores.

2.3. DESICCATION AND LOW WATER ACTIVITY

Desiccation is the removal of water from a biological system. This can be accomplished by exposure to dry heat, to reduced pressure or vacuum, or to low water activity (a_w). Most biological systems are adversely affected by the loss of water. Microorganisms are no exception, save for those that have evolved defensive measures to escape the loss of viability which is typically associated with water loss. A quantitative definition of complete desiccation has been stated as drying to <0.1 g of water per g of cell mass (Alpert, 2005). Sensitive prokaryotes do not survive drying to 30 % water content (Billi and Potts, 2002), but desiccation tolerant cells use structural, physiological, and molecular mechanisms to survive water contents of 10 % or less (Alpert, 2005).

The synthesis of protective polymers is a response of several microbial taxa to desiccation. Involvement of extracellular (or exo-) polysaccharides (EPS) in resistance to desiccation has been shown early for several bacterial mucoid strains (Ophir and Gutnick, 1994). Subaerial biofilms on rocks, including salt-stressed surfaces, take advantage of EPS for retaining water for long periods and facilitating access to water vapor in the atmosphere (Gorbushina, 2007). Rock-inhabiting cyanobacteria (*Chroococcidiopsis* sp.) produce polysaccharide-rich envelopes, which increase desiccation tolerance by providing a repository for water as well as a matrix which stabilizes desiccation related enzymes and molecules (Billi, 2012). Unlike bacterial EPS, which usually contain less than four different monomers,

cyanobacterial EPS are complex heteropolysaccharides composed of more than six different monosaccharides (Billi, 2012).

Disaccharides can act as protective agents during drying. Trehalose and sucrose have been reported as the best stabilizers of membranes and proteins of several microorganisms by replacing water molecules that are lost from biological systems following desiccation (Garcia, 2011, and references therein). Cells of *Nostoc commune* were shown to increase their trehalose content during desiccation (Sakamoto et al., 2009) and treatment with 0.2 M NaCl also induced trehalose accumulation to a similar level as observed by desiccation.

A combination of tolerance to desiccation and resistance to ionizing irradiation was found for *Halobacterium* sp. strain NRC-1 (Kottemann et al., 2005) and several other prokaryotes (see below). Efficient DNA repair systems were assumed to be responsible, similar as for the highly radiation-resistant *Deinococcus* strains, since *Halobacterium* species do possess sets of such enzymes. However, recent research showed that the basis of resistance is provided by the ability to cope with protein oxidation, which is caused by radiation, desiccation, and possibly other stressors. In particular, antioxidants consisting of Mn-containing compounds are active (Fredrickson et al., 2008; for an extensive treatment of this subject, see Webb and DiRuggiero, 2013).

Morphological changes of cells such as shrinkage and alterations in shape and surface properties in response to desiccation were listed by Potts et al. (2005). The behavior of *Bacillus subtilis* with respect to decreasing water activity was examined in detail and changes from rods to filaments to coils or coiled superstructures to roundish cells were reported, concomitant with a decrease of a_w from 0.95 to 0.936 (de Goffau et al., 2011). Close to the a_w growth limit, only globular cell shapes occurred.

3. Specific Strategies of Halophilic Prokaryotes

3.1. A UNIQUE PROTEIN OF *HQT. WALSBYI* PROTECTS AGAINST DESICCATION

Haloquadratum walsbyi is truly unique among haloarchaea and even prokaryotes, since its cells are square and flat, forming often extended sheets (Parkes and Walsby, 1981); its GC content is much lower (48 %) than that of other haloarchaea (60–70 %); its gene density is quite low (76 %); and it contains halomucin, the largest archaeal protein known so far (Bolhuis et al., 2006; Dyall-Smith et al., 2011). Halomucin of *Hqt. walsbyi* strain HBSQ001 consists of 9,159 amino acids and its sequence and domain organization are similar to those of animal mucins, which are known to protect various tissues against desiccation (Bolhuis et al., 2006). Halomucin is secreted and apparently surrounds the cells as a cloud of protein, as was deduced from a specific antibody stain (Dyall-Smith et al., 2011). Due to the fact that it is highly glycosylated

and sulfated, it is thought to form a water-rich capsule around the cells and protect against conditions of desiccation or extremely low water activity (Bolhuis et al., 2006).

3.2. SURVIVING OLIGOTROPHIC CONDITIONS AND STARVATION

Increasing the surface to volume ratio is a mode of adaptation to nutrient limitation by many oligotrophic microorganisms. *Hqt. walsbyi* uses a different strategy for this purpose – it flattens itself to a thickness of 0.1–0.5 μm and achieves thus what is probably the highest surface to volume ratio in the microbial world (Bolhuis, 2005; Bolhuis et al., 2006). Whereas spherical-shaped microorganisms have to remain small, the squares can become unlimitedly large since the surface to volume ratio solely depends on their thickness (Bolhuis et al., 2006). The ability of *Hqt. walsbyi* for efficient phototrophic growth by spreading out the flat cells on the water surface, much like a molecular solar panel, is also unique among prokaryotes (Bolhuis et al., 2006) and represents an adaptation to the oligotrophic environment of hypersaline salterns with high contents of Mg^{++} ions.

A novel finding of a morphological change (miniaturization) of some haloarchaea in fluid inclusions as a response to low water activity (Fendrihan et al., 2012) is described in more detail below.

3.3. SPHERE FORMATION IN FLUID INCLUSIONS

Fluid inclusions are present in natural halite (Roedder, 1984) and were considered early as possible habitats for microorganisms (Norton and Grant, 1988). A strain of *Halobacterium salinarum* was indeed isolated from a fluid inclusion in a 97,000-year-old halite crystal from Death Valley (Mormile et al., 2003). Cells within fluid inclusions can be visualized in laboratory-grown halite, as was demonstrated for the non-halophilic *Pseudomonas aeruginosa* (Adamski et al., 2006). When cells of *Hbt. salinarum* NRC-1 were pre-stained with the LIVE/DEAD kit and then embedded in laboratory-produced salt crystals by evaporation of the liquid, the bright green fluorescence of stained cells outlined the morphology of the characteristic rectangular fluid inclusions of halite (Fig. 1, left panel). At higher magnifications, individual cells became visible (Fig. 1, right panel). Most of them appeared of roundish morphology. The data suggested that haloarchaeal cells accumulated preferentially in the fluid inclusions of artificial halite, which formed during desiccation, and that rod-shaped cells were transformed into spheres (Fendrihan and Stan-Lotter, 2004; Fendrihan et al., 2012). Most haloarchaea possess an S-layer (surface layer), which forms the outer envelope of the cell and consists of glycoproteins, held together by non-covalent interactions (Sára and Sleytr, 2000). They do not contain the covalently linked rigid peptidoglycan,

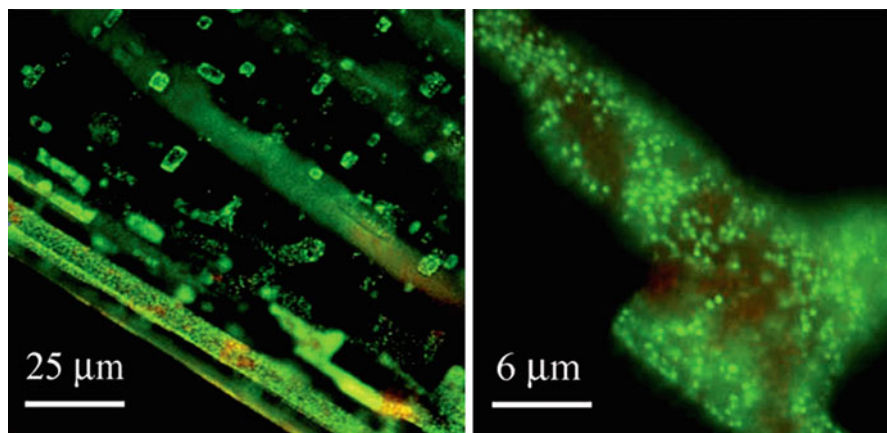


Figure 1. Localization of pre-stained cells of *Halobacterium salinarum* NRC-1 in fluid inclusions of laboratory-produced halite. Low magnification (*left*) and higher magnification of an individual fluid inclusion (*right*). Cells were stained with the LIVE/DEAD® *BacLight*™ bacterial viability kit prior to embedding in halite; epifluorescence microscopy was performed after entrapment of cells for 3 days.

which constitutes the cell wall of many bacteria. The haloarchaeal S-layer can be removed, for example, by chelating agents, leaving large fragile spheroplasts (Cline and Doolittle, 1992).

Recently small roundish particles with less than 1 μm diameter were described in fluid inclusions of 22,000–34,000-year-old halite from Death Valley, California, and, following surface sterilization and dissolution of crystals, successfully cultured and identified as three different genera of haloarchaea (Schubert et al., 2010). The small particles were ascribed to a form of miniaturization. We examined the spherical particles in artificial fluid inclusions (Fig. 1) in detail and could show that sphere formation is apparently a response to low external water activity (a_w) of several haloarchaeal species (Fendrihan et al., 2012). Our results suggested that rod-shaped cells of *Halobacterium* species gradually converted to small spheres (see Fig. 2) upon a decrease of the external a_w to less than 0.75. From one rod, three to four spheres were formed. The diameter of the spheres was $0.40 \pm 0.02 \mu\text{m}$ (Fendrihan et al., 2012). The water activity of liquids in fluid inclusions is difficult to measure; still, values of a_w of <0.75 in halite were reported (Yang et al., 1995). An explanation for the spherical particles, which were observed in fluid inclusions of ancient and well-dated halite (Schubert et al., 2010), could thus be the transformation from former rod-shaped haloarchaea to roundish forms as a response to lowered external a_w . We could confirm this notion by the rapid production of spheres in the laboratory upon exposing haloarchaeal cells to buffered 4 M LiCl solution, which exhibits an a_w of about 0.73 (Fig. 3; Fendrihan et al., 2012). Exposure of haloarchaeal rod-shaped cells turned those cells immediately into spheres. The haloarchaeal spheres stayed viable for years,

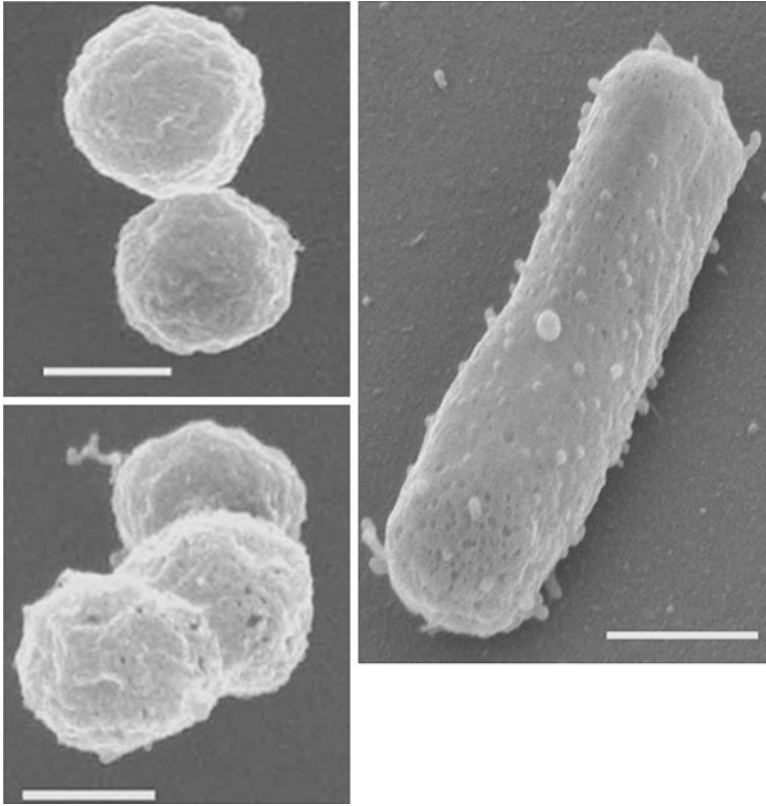


Figure 2. Scanning electron micrographs of a rod (right panel) and spheres (left panels) of *Halobacterium salinarum* NRC-1. Spheres had formed within fluid inclusions of laboratory-grown halite and were obtained after dissolution of salt crystals. Bars, 270 nm (Photographs taken by C. Frethem).

when kept in buffered 4 M NaCl, and, following addition of nutrients, proliferated to normal rods. An initial biochemical characterization showed that spheres contained an about 50-fold lower content of ATP, compared to rods (Fendrihan et al., 2012).

The intriguing findings by Schubert et al. (2009, 2010) of spherical particles of about the same size as those described here in fluid inclusions of ancient halite, which could be propagated to haloarchaeal cultures, can be interpreted as evidence for the occurrence of viable long-term survivors in geologically old sediments. Schubert et al. (2009, 2010) called the spherical particles “miniaturized cocci,” due to their appearance in the phase contrast microscope. However, the haloarchaea which were grown from the halite samples which contained these particles were not halophilic cocci. Instead, they were members of the genera *Halorubrum*, *Natronomonas*, and *Haloterrigena*, which are known to form

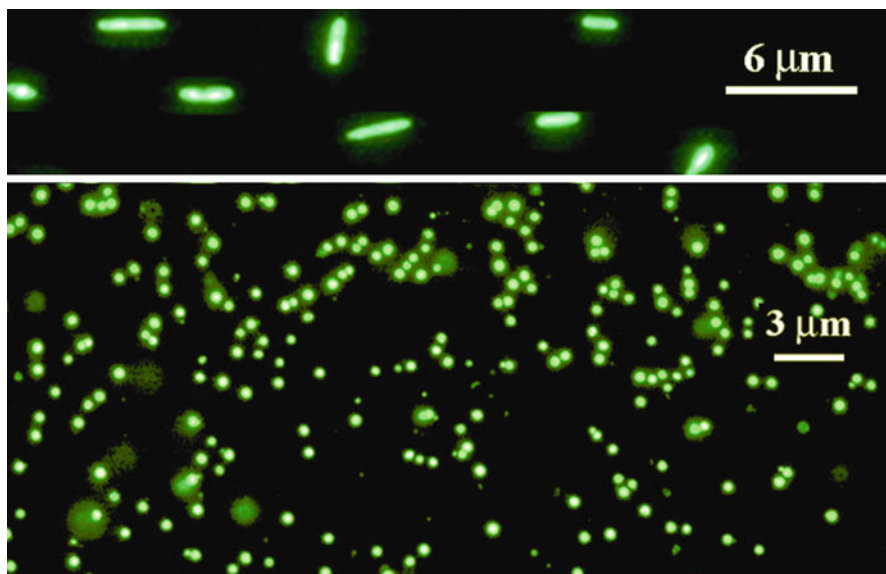


Figure 3. Rods (upper panel) and spheres (lower panel) of *Halobacterium salinarum* NRC-1, following staining with the LIVE/DEAD kit. These spheres were produced by exposure of rods to Tris-buffered 4 M LiCl.

rod-shaped or oval cells (<http://www.the-icsp.org/taxa/halobacterlist.htm>). This suggested that the spherical particles in natural fluid inclusions might be in a dormant resting state, which is reversed to growth as rod-shaped or pleomorphic cells, once the conditions for proliferation are met.

Miniaturization of cells as consequence of starvation and morphological changes as consequence of desiccation have been reported for several bacterial species (see above). The spheres from haloarchaea appear to represent a form of miniaturization in response to lowered external water activity. They could possibly be viewed as dormant cells, since they seem to be very similar to the particles detected in native fluid inclusions of old sediments (Schubert et al., 2010), which apparently survived in that state for ages and remained culturable.

3.4. FURTHER ASPECTS AND FUTURE RESEARCH

Fredrickson et al. (2008) suggested that protein oxidation is a key factor in desiccation resistance of phylogenetically diverse bacteria. The connection between resistance to desiccation and radiation had been established previously (Daly et al., 2007) and survival of such conditions may have a universal basis, being dependent on the presence of mechanisms which protect against or limit protein

oxidation. This has already opened up studies on the potentially wide diversity of protective molecules and systems (see Webb and DiRuggiero, 2013).

Attempts are now being carried out to identify novel genes in haloarchaea that might be involved in survival of extreme conditions. Capes et al. (2012) were screening nine complete haloarchaeal genomes and showed that nearly 800 protein clusters were present in all haloarchaea, with a subset of 55, which were considered critical or essential for success in extreme environments. One protein, Ral (tucHOG0456) from *Halobacterium salinarum* NRC-1, was suggested to function in double-stranded DNA break repair and desiccation and radiation tolerance.

Proteome studies of survival in the dry state might focus on the so-called desiccome, a term suggested by Potts et al. (2005), which comprises the proteins involved in desiccation stress. Expanding on this proposal, Leprince and Buitink (2010) discussed models which might allow a quantitative approach to desiccation tolerance, using functional genomics tools, and called the emerging field “desiccomics.” Some progress on the level of genes was reported by identification of Sip18 hydrophilin in yeast, a protein with antioxidant properties acting as a desiccation stress modifier (Rodriguez-Porrata et al., 2012).

The formation of spheres described above rapidly yields simple roundish mini-cells from several haloarchaea, but the process appears quite complicated. Many questions still need to be resolved, for instance, the mechanism of apparent multiple fission, which is used by the haloarchaeal cells. A few prokaryotes are known which do not or not only use binary fission, for example, some produce multiple endospores (*Metabacterium polyspora*; *Anaerobacter polyendosporus*), or multiple live offspring (*Epulopiscium* species), or apply multiple fission to enlarged cells (pleurocapsalean cyanobacteria), as reviewed by Angert (2005). The distribution of multiple genomes, which are known to occur in high copy numbers in haloarchaea (Breuert et al., 2006), among the spheres has to be elucidated. The processes involved in the greatly increased lag phases, a possible influence of reduced a_w on surface protein conformation as well as the role of proteins and other molecules, which are released during the transit from rods to spheres (Fendrihan et al., 2012), need to be clarified.

4. Extraterrestrial Halite

If halophilic prokaryotes on Earth can remain viable for very long periods of time, then it is reasonable to consider the possibility that viable microorganisms may exist in similar subterranean salt deposits on other planets or moons. This notion becomes all the more plausible in view of the detection of halite in extraterrestrial materials. Halite has been identified in Martian meteorites (Treiman et al., 2000), in the Murchison and other carbonaceous meteorites (Barber, 1981), and in the Monahans meteorite, together with sylvite (KCl) and water inclusions (Zolensky et al., 1999). Recently, Postberg et al. (2009) focused on ice grains from the plumes of Enceladus, using the Cosmic Dust Analyzer instrument aboard

Cassini and found that, although all the grains were dominated by water ice, about 6 % of them were quite salty, containing roughly 1.5 % of a mixture of sodium chloride, sodium carbonate, and sodium bicarbonate. All these discoveries make a consideration of potential habitats for halophilic life in space intriguing.

5. Conclusions and Considerations for Astrobiology

Extremely halophilic Archaea are of astrobiological interest since viable strains have been isolated from million-year-old deposits of halite (McGenity et al., 2000; Stan-Lotter et al., 1999, 2002; Fendrihan et al., 2006; Vreeland et al., 2007; Gramain et al., 2011), suggesting the possibility of long-term survival under desiccation. Potential extraterrestrial microbial life has come in focus particularly since the discovery of bacteria-like microfossils in the Martian meteorite ALH84001 (McKay et al., 1996). The apparent longevity of haloarchaeal strains in dry salty environments is relevant for astrobiological studies in general and, in particular, for the search for life on Mars. A detailed investigation of the mode of sphere formation and their properties could lead to insights on how haloarchaea inside natural fluid inclusions, where low a_w is prevalent, may survive. Fluid inclusions were also found in billion-year-old meteorites and constitute apparently a very old type of structure in the universe (Zolensky et al., 1999). Thus, such studies may also be useful for the design of experiments aimed at the detection of potential extraterrestrial forms of microbial life in sediments of great age.

6. Summary

Halophilic microorganisms can survive extreme desiccation and starvation, sometimes apparently for geological time periods, that is, millions of years. The mechanisms which appear to be involved include dwarfing of cells, formation of spores, reduction of ATP (and other molecules), formation of protective capsules, incorporation of trehalose, and probably production of dormant stages such as small spherical particles.

Recent data suggested the presence of halite on Mars, as well as on several moons in the solar system. To increase the chances for finding extraterrestrial life, halite-containing regions should be considered and the search should include the focus on very small particles, which might perhaps be living fossils.

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RADIATION RESISTANCE IN EXTREMOPHILES: FENDING OFF MULTIPLE ATTACKS

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1. Extremophiles and Radiation Resistance

Over the last few decades, studies of extremophiles have revealed an astonishing array of adaptations to the harsh environmental conditions to which those organisms are exposed (Cavicchioli et al., 2011). However, an extreme environment, from our point of view, is not extreme for the organisms that are specifically adapted to this environment. Hyperthermophilic organisms not only thrive at temperatures near the boiling point of water, but they also require those high temperatures for their cellular machinery to function. As an example, the glutamate dehydrogenase from *Pyrococcus furiosus*, a hyperthermophilic archaeon, does not function below 45°C (Klump et al., 1992). Similarly, cells of the halophilic archaeon, *Halobacterium salinarum*, will lyse if the osmotic pressure of their aqueous environment decreases below 3 M salt (DasSarma and DasSarma, 2012).

An interesting case is that of radiation-resistant bacteria. They have garnered a great deal of attention from scientists seeking to expose the mechanisms underlying their incredible survival abilities. These microorganisms were most likely not exposed to extremes of ionizing radiation (IR) over geological times (Mattimore and Battista, 1996), raising the question of their adaptation to such high doses of radiation. Early work on the desiccation resistance of the extremely radiation-resistant bacterium, *Deinococcus radiodurans*, and more recent environmental studies have revealed that it is the adaptation to extremely dry environments and high tolerance to desiccation that impart IR resistance to these organisms (Mattimore and Battista, 1996; Fredrickson et al., 2008). In other words, the IR resistance in bacteria is an incidental mechanism evolved to resist the cellular damage induced by desiccation (Fredrickson et al., 2008). In that regard, IR-resistant organisms are true polyextremophiles.

The distribution of radiation-resistant organisms in the phylogenetic tree of life is not limited to bacteria (Fig. 1). Recent work has revealed the high level of IR resistance of several eukaryotes: the basidiomycete fungus *Ustilago maydis* (Holliday, 2004), the freshwater invertebrate animal *Philodina roseola* (Gladyshev and Meselson, 2008), the water bear *Milnesium tardigradum* (Horikawa et al., 2006), and the roundworm *Caenorhabditis elegans* (Johnson and Hartman, 1988). Among the Archaea, the halophilic archaeon *H. salinarum*, in addition to being

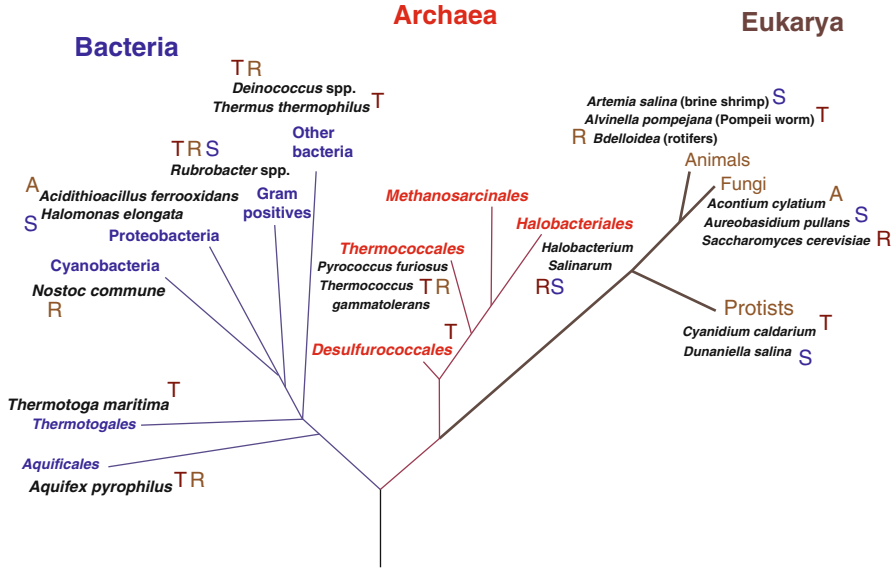


Figure 1. Phylogenetic tree of life with the distribution of extremophiles and radiation-resistant organisms. T thermophiles, S halotolerants and halophiles, A acidophiles, R radiation and desiccation resistant.

adapted to high salt, also shows a high level of resistance to desiccation, high pressure, UV radiation, and IR (Kish et al., 2012; Robinson et al., 2011; Kottemann et al., 2005). This is also true for a number of thermophilic archaea, including the sulfate-reducing *Archaeoglobus fulgidus*, methanogens such as *Methanocaldococcus jannaschii*, and the hyperthermophiles *P. furiosus*, *Thermococcus radiotolerans*, and *Thermococcus gammatolerans* (Beblo et al., 2011; DiRuggiero et al., 1997; Jolivet et al., 2003, 2004). Despite the seeming prevalence of radiation-resistant thermophiles, it would be unjustified to assume this is true of all thermophiles; several have been shown to be radiation sensitive, such as the archaeon *Sulfolobus solfataricus* (Rolsmeier et al., 2011). Table 1 lists radiation-resistant organisms with their D_{10} value – the dose of radiation in gray (Gy) that reduces the survival of a population by 90 %. Radiation resistance is strongly linked to genome size and the number of DNA double-strand breaks (DSBs) resulting from exposure to IR, which is approximately 0.004–0.01 DSB/Gy/Mbp. Because of the large difference in genome size, at a dose of IR of 1 kGy, the roundworm *C. elegans* experiences 400 DNA DSBs, whereas the bacterium *D. radiodurans* faces only 158 DSBs at 12 kGy (Daly, 2012). As a result, the D_{10} of eukaryotes is much lower than that of bacteria, but those organisms are considered to be highly resistant to IR.

While IR-resistant organisms are distributed across the three domains of life (Fig. 1), this distribution can vary dramatically between organisms of the same family and even between species. For example, *Thermus thermophilus* is as radiation

sensitive as *Escherichia coli* (D_{10} 0.7 kGy) but belongs to the same clade as one of the most IR-resistant bacterium known to date, *D. radiodurans* (D_{10} 12 kGy) (Omelchenko et al., 2005; Table 1). This raises an important question regarding the evolution of radiation resistance and whether or not the mechanisms underlying IR resistance are shared between the three domains of life.

In this chapter, we first discuss the cellular effects of IR and the parallels with desiccation, we follow by the current concepts regarding radioprotection and damage repair and the role of Mn antioxidants in radiation resistance, and finally we discuss the mechanisms underlying the radiation resistance of polyextremophilic archaea.

2. Cellular Effects of Ionizing Radiation

Ionizing radiation damages cellular components by direct and indirect effects (Riley, 1994). While direct ionization within the cell results in molecular damage, the vast majority of cellular insults under aqueous conditions are caused by indirect effects, through the actions of reactive oxygen species (ROS) formed by the radiolysis of water (Fig. 2) (Riley, 1994). Water radiolysis generates hydroxyl radicals (HO \cdot), protons, and free electrons (Eq. 1).



Hydroxyl radicals react indiscriminately with all macromolecules in the cell and with each other to form hydrogen peroxide (H_2O_2) (Eq. 2), and free electrons react with dissolved oxygen to form superoxide ($\text{O}_2^{\cdot-}$) (Eq. 3). DNA-associated water molecules that undergo radiolysis become an immediate threat for nucleic acids, generating oxidized DNA bases and sugar moieties, abasic sites, strand breaks, and cross-links to proteins. These damages often produce complex clustered lesions resulting in DNA DSBs from attempted repair (Dianov et al., 2001; Regulus et al., 2007; Kish and DiRuggiero, 2008). DNA is further damaged through its association with free iron in the cells (Dianov et al., 2001; Ward, 1994). Proteins are attacked by hydroxyl radicals introducing carbonyl residues, amino acid radical chain reactions, cross-linking, and ultimately resulting in protein inactivation and denaturation (Daly, 2009). By analogy to chemical oxidative stress, it is hypothesized that the low reactivity and high specificity of superoxide and H_2O_2 for iron-sulfur and heme groups produce consequential damage to [4Fe-4S] clusters of labile dehydratases (Imlay, 2006). This results in the release of free Fe^{2+} in the cytoplasm and enzyme inactivation, failure of metabolic pathways, and the synthesis of aromatic and sulfur amino acids (Imlay, 2006, 2008).

Table 1. Examples of IR-resistant polyextremophiles.

Extremophile	D_{10} (kGy) ^a	Haploid genome size (Mbp) ^b	Characteristics	References
Eukarya				
<i>Adineta vaga</i>	1	180	Desiccation resistant	Gladyshev and Meselson (2008)
<i>Philodina roseola</i>	1	180	Desiccation resistant	Gladyshev and Meselson (2008)
<i>Caenorhabditis elegans</i>	1.2	100	Thermotolerant	Johnson and Hartman (1988)
<i>Ustilago maydis</i>	6	20	Thermotolerant, halotolerant	Holliday (2004)
<i>Milnesium tardigradum</i>	1	75–800 ^c	Thermotolerant, desiccation resistant	Horikawa et al. (2006)
Archaea				
<i>Methanocaldococcus jannaschii</i>	1	1.7	Thermophile	Beblo et al. (2011)
<i>Archaeoglobus fulgidus</i>	1	2.2	Thermophile	Beblo et al. (2011)
<i>Haloferax volcanii</i>	1.5	4.0	Halophile	Delmas et al. (2009)
<i>Pyrococcus furiosus</i>	3	1.9	Thermophile, halotolerant	DiRuggiero et al. (1997)
<i>Halobacterium salinarum</i>	5	2.7	Halophile, desiccation and pressure resistant	Kottmann et al. (2005)
<i>Thermococcus gammatolerans</i>	6	2.1	Thermophile	Jolivet et al. (2003)
Bacteria				
<i>Aquifex pyrophilus</i>	2.8	1.6 ^d	Thermophile	Beblo et al. (2011)
<i>Rubrobacter xylanophilus</i>	6	3.2	Thermophile, halotolerant, desiccation resistant	Carreto et al. (1996)
<i>Rubrobacter radiotolerans</i>	10	3.4	Thermophile, desiccation resistant	Suzuki et al. (1988)
<i>Deinococcus geothermalis</i>	10	3.3	Thermophile, desiccation resistant	Daly (2009)
<i>Deinococcus radiodurans</i>	12	3.3	Desiccation resistant	Daly (2009)

^a D_{10} value – the dose of radiation in gray (Gy) that reduces the survival of a population by 90 %.

^bGenome sizes at <http://www.ncbi.nlm.nih.gov/genome>

^cGenome size unknown, range given from <http://www.genomesize.com/>

^dEstimated from the closely related *Aquifex aeolicus*.

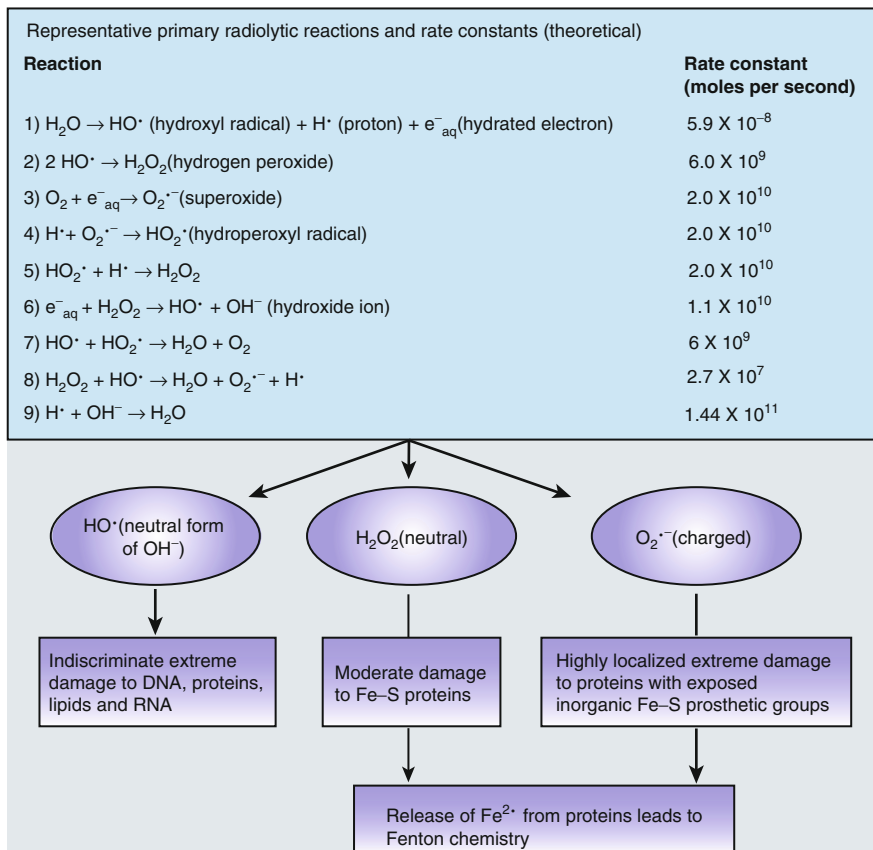
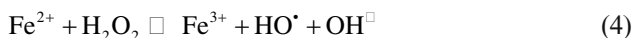


Figure 2. Theoretical cellular reactions generating ROS following IR. *Top:* expected reactions resulting from the radiolysis of water and their rate constants. *Bottom:* cellular targets of ROS (From Daly 2009, reproduced with permission from Macmillan Publishers Ltd.).

The released Fe^{2+} participates in the Fenton reaction (Eq. 4), which is the electron transfer from ferrous ion to H_2O_2 and the formation of superoxide (Imlay, 2008). The resulting $\text{HO}\cdot$ inflicts a barrage of oxidative damage upon all cellular components.



The mechanistic link between desiccation and IR resistance can be found in the formation of ROS resulting from both stresses (Nauser et al., 2005). During desiccation, loss of control of the electron transport chain, decrease membrane integrity compromising gas diffusion, impaired antioxidant systems, and macromolecule distortions as the result of volume changes contribute to the accumulation of ROS. This high level of ROS, in particular hydroxyl and peroxy radicals,

causes a major oxidative stress to the cell (Kranner, 2002; Kranner and Birtic, 2005). Thus, both desiccation and IR inflict severe oxidative damage to macromolecules in the cell that must either be prevented or repaired in order for the cell to survive.

3. Radioprotection and Damage Repair

In the 1960s, DNA was considered to be the principal target of radiation, and DNA damage was responsible for its lethal effects (Hutchinson, 1966). Scientists believed that IR-resistant microorganisms survived high-level radiation because they possessed unique and highly efficient DNA repair mechanisms. Several pathways for the repair of DNA DSBs after ionizing radiation have been proposed for bacteria, including homologous recombination; single-strand annealing; extended synthesis-dependent strand annealing, where cells need to contain another intact copy of the damaged DNA region; and nonhomologous end joining, which does not require a second homologous copy of DNA to join two contiguous fragments (Blasius et al., 2008; Confalonieri and Sommer, 2011; Slade and Radman, 2011). Recent work showed that the steps of DNA repair from IR damage were surprisingly ordinary in contrast to the extreme nature of the chromosome fragmentation and that the DNA repair proteins involved were not unique to IR-resistant organisms (Confalonieri and Sommer, 2011; Daly, 2012; Gutman et al., 1994).

In contrast to bacteria, DSB repair in the Archaea is less well characterized. In *P. furiosus*, DNA end processing is carried out by Rad50/Mre11 complexes that attach to the DNA ends and recruit nuclease and helicase proteins (NurA and HerA, respectively) to form 3' overhangs (Hopfner et al., 2001). This in turn recruits RadA, a RecA homolog (Constantinesco et al., 2004; Hopfner et al., 2001). In *H. salinarum*, *nurA* and *herA* homologs are missing, and while Rad50/Mre11 proteins are present, Rad50 is not required for homologous recombination (Kish and DiRuggiero, 2008). Additionally, mutants of *H. salinarum* deficient in both Rad50 and Mre11 ($\Delta rad50\text{-}\Delta mre11$) are just as IR resistant as the wild type, though the repair of DNA DSBs occurs less efficiently (Kish and DiRuggiero, 2008). These facts together with the demonstration that IR-sensitive and IR-resistant organisms suffer the same number of DNA DSBs for an equivalent dose of IR (~0.01 DSB/Gy/Mbp) depart from the dogma that DNA damage and in particular DNA DSBs are the most cytotoxic lesions resulting from exposure to IR. From early studies in 1940 (Dale, 1940), work from Gebicki's group (Du and Gebicki, 2004; Nauser et al., 2005), and recent reports from Daly's group (Daly, 2009; Daly et al., 2007), it is now established that proteins are the major targets for oxidation following exposure to IR. Thus, the idea that protein protection might govern radiation resistance has severely challenged the conventionally held view that DNA damage is paramount in radiation toxicity. The current model is that by protecting protein function from the damages inflicted by IR, DNA can be repaired by competent proteins, and the cell can survive (Fig. 3).

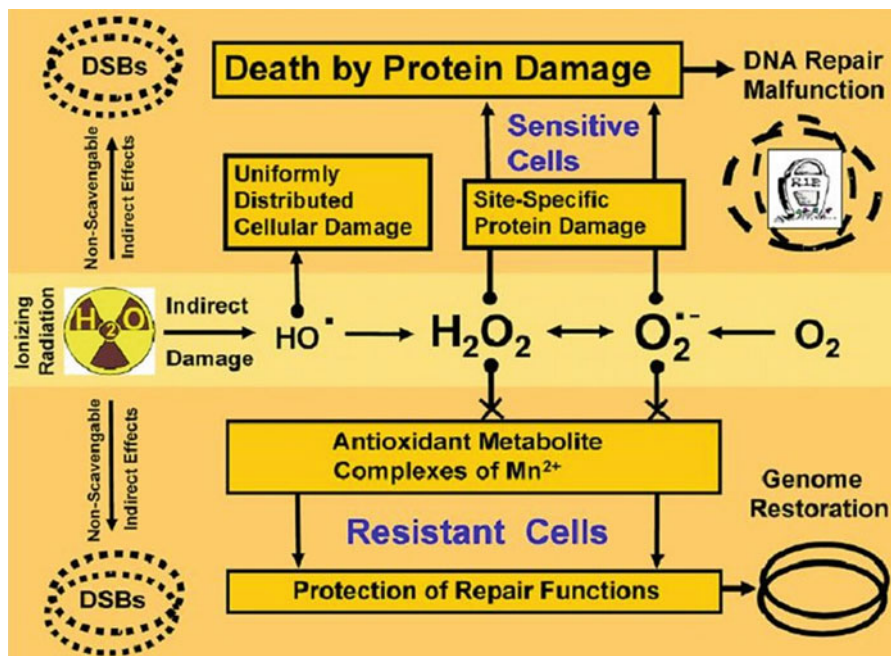


Figure 3. Model for death by protein damage in IR-sensitive cells and damage avoidance in IR-resistant cells (From Daly 2012, reproduced with permission from Elsevier).

4. Enzymatic Defense and IR

The major defenses of the cell against oxidative stress from exposure to H_2O_2 and redox cycling drugs – producing superoxide – are enzymatic (Imlay, 2008). Superoxide dismutases (SODs), catalases, and peroxidases are highly induced upon oxidative stress, and mutants of those proteins are greatly sensitive to chemical oxidants (Imlay, 2008). Surprisingly, SOD and catalase mutants of *H. salinarum* showed the same level of survival to IR as strains with active SODs and catalases (Fig. 4, Robinson et al., 2011), suggesting that those enzymes were not required for the survival of *H. salinarum* to IR; similar results were obtained with SOD and catalase mutants of *D. radiodurans* and *E. coli* (Markillie et al., 1999; Scott et al., 1989). This is quite paradoxical since the major stress from IR is oxidative stress (Daly, 2009) and both SOD and catalase are major ROS detoxification enzymes (Imlay, 2008). Furthermore, in bacterial systems and in *H. salinarum*, SODs and catalases were induced by several orders of magnitude in response to redox cycling drugs and H_2O_2 (Imlay, 2008; Kaur et al., 2010), but no increase in mRNA or protein levels for SODs, catalases, or peroxidases was detected in *H. salinarum* after IR (Whitehead et al., 2006). The question then is as follows: What protects the macromolecules of IR-resistant organisms since the enzymatic defenses of the cell against oxidative stress are not engaged?

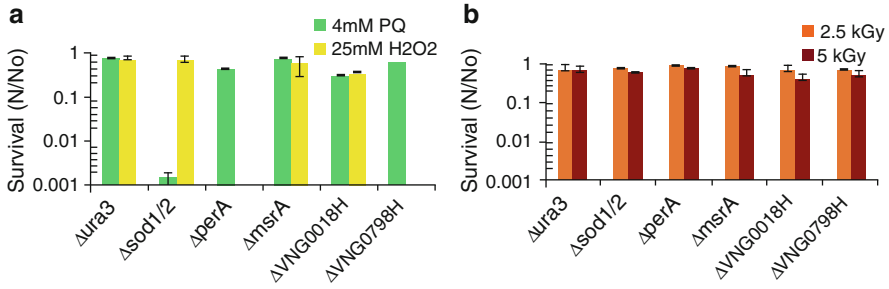


Figure 4. Survival of *H. salinarum* $\Delta ura3$ and mutant strains exposed to H_2O_2 , paraquat, and IR. Survival was calculated as the average ratio (N/No) of surviving colony-forming units from treated (N) compared to untreated (No) cultures. **(a)** Doses of chemical oxidants: paraquat (PQ) and H_2O_2 ; **(b)** doses of ionizing radiation (From Robinson et al. 2011, reproduced with permission).

5. Manganese (Mn) Antioxidants

“Mn antioxidants” were first discovered in *Lactobacillus plantarum* where accumulation of millimolar concentrations of Mn suppressed oxidative stress and substituted for the lack of superoxide dismutase (SOD) (Archibald and Fridovich, 1981, 1982b). High levels of Mn also rescued *E. coli* and *Saccharomyces cerevisiae* SOD-deficient mutants (Al-Maghrebi et al., 2002; Chang and Kosman, 1989), and small molecule complexes of Mn have been shown to exhibit superoxide-scavenging activity in vitro (Archibald and Fridovich, 1982a; Barnese et al., 2008). Recently, the high Mn/Fe ratio found in IR-resistant bacteria and archaea revealed a direct link between Mn and protection of proteins from oxidative damage by ROS (Fredrickson et al., 2008; Daly, 2009; Kish et al., 2009). Work with *D. radiodurans* elegantly established the key role played by Mn-peptide complexes in the extreme radiation resistance of this organism (Daly et al., 2010), and in yeast, in vivo studies showed the important function of Mn-orthophosphate complexes in oxidative stress (McNaughton et al., 2010) (Fig. 5). In *H. salinarum* enzyme-free cell extracts rich in Mn, phosphate, amino acids, and peptides provided a great level of enzyme protection against the deleterious effect of IR (Robinson et al., 2011), and recent studies with *Rubrobacter* species showed that the association of Mn and trehalose was essential for the extreme radiation resistance observed in these organisms (Webb and DiRuggiero, 2012). High levels of intracellular concentration of trehalose were also reported in the IR-resistant cyanobacterium *Chroococciopsis* (Billi et al., 2000). Investigating the mechanisms of catalytic removal of superoxide by Mn compounds, Barnese et al. (2012) found that Mn phosphate and Mn carbonate, but not Mn pyrophosphate and citrate, can catalyze superoxide disproportionation in vitro at rates sufficient to mimic enzymatic SOD. They also noted that carboxylate and phosphate motifs, found in amino acids and nucleotides, are the most commonly available ligands for Mn in vivo (Fig. 5). In addition to its antioxidant

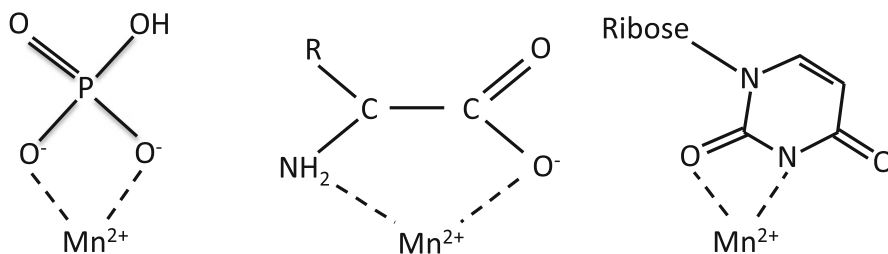


Figure 5. Model for manganese antioxidants. Mn²⁺ in complex with orthophosphate (*left*), with free amino acids or peptides (*center*) and with nucleosides (*right*) catalytically scavenges superoxide radicals (O₂^{•-}). Mn²⁺ in complex with free amino acids or peptide and orthophosphate catalytically decompose hydrogen peroxide (H₂O₂). Nucleosides, free amino acids, peptides, and other small organic metabolites scavenge hydroxyl radicals (HO[•]) (Daly et al., 2010).

activity, Mn may also act by functionally substituting for Fe in the Fe-S cluster of enzymes and thereby mitigating the deleterious effects of Fenton chemistry during oxidative stress (Sobota and Imalay, 2011).

In *Bacillus*, spores resistant to wet and dry heat benefited from the accumulation of Mn coordinated with small molecules including dipicolinic acid (DPA), and possibly α -/ β -type small, acid-soluble proteins (Ghosh et al., 2011). DPA also formed antioxidant complexes with Ca²⁺ and phosphate, indicating that other divalent metal ions may contribute to protection from IR (Granger et al., 2011). Mn-mycosporine complexes were also attributed to facilitating radiation and desiccation resistance in cyanobacteria (Oren and Gunde-Cimerman, 2007; Rastogi et al., 2010). Cellular accumulation of Mn together with a variety of organic and inorganic ligands may be a widespread mechanism to surviving oxidative stress, and there is evidence that this may extend also to simple animals such as rotifers (Krisiko et al., 2012).

6. What Is the Basis for the Radiation Resistance of the Polyextremophile *H. salinarum*?

While *H. salinarum* is highly polyploidic, with 15–25 copies of its chromosome per cell (Breuert et al., 2006), no connection has been established between chromosome copy numbers and radiation resistance (Daly et al., 2004; Gladyshv and Meselson, 2008), nor does the presence of eukaryotic-like proteins involved in the repair of DNA DSBs account for the high level of survival of this organism to IR (Kish and DiRuggiero, 2008). However, recent work revealed the critical role played by nonenzymatic antioxidant processes in the resistance of *H. salinarum* to IR (Robinson et al., 2011; Kish et al., 2009). Scavenging of ROS by intracellular halides in *H. salinarum* resulted in increased protection against nucleotide modification and carbonylation of protein residues (Kish et al., 2009). Measurements

of *H. salinarum* cell interior revealed a high Mn/Fe ratio similar to that of *D. radiodurans* and other radiation-resistant microorganisms, underlying the role of Mn in radiation resistance (Kish et al., 2009; Daly, 2009). Protein-free cell extracts from *H. salinarum* provided a high level of protection for protein activity against IR in vitro. Compared with cell extracts of radiation-sensitive bacteria, *H. salinarum* extracts were enriched in manganese-antioxidant complexes, supporting an essential role in ROS scavenging for those small molecules in vivo (Robinson et al., 2011).

To further elucidate the metabolic routes instrumental to this enhanced radiation resistance, IR-“super”-resistant mutants (IR⁺) of *H. salinarum* were evolved from the wild-type strain over multiple cycles of exposure to high doses of IR (Webb et al., 2013; DeVeaux et al., 2007). Proteomic analysis of IR⁺ mutants revealed overexpression of enzymes from central carbon metabolism, channeling a substantial flux of carbon into pyruvate and therefore the generation of energy and reducing equivalents (Webb et al., 2013). The corresponding IR⁺ mutants also had increased intracellular Mn concentration, compared to the wild type, supporting the case of an important role for Mn in central carbon metabolism, via strictly Mn-dependent enzymes or enzymes highly stimulated by Mn (Kehres and Maguire, 2003; Liedert et al., 2012; Ogunniyi et al., 2010). Maintenance of redox homeostasis was also activated by the overexpression of coenzyme biosynthesis pathways involved in redox reactions. These findings support the idea that increased IR tolerance is most likely achieved by a “metabolic route” and underscore the physiological importance in aerobic fitness of Mn antioxidants.

Recent studies regarding single-strand DNA-binding protein (SSB) in halophiles suggested a key role for these proteins in radiation resistance. Single-strand DNA-binding proteins (SSBs), also called replication protein A (RPAs), bind to ssDNA with high affinity and provide protection against nuclease and chemical attacks. These proteins are essential for DNA metabolism including DNA replication, recombination, and repair in all domains of life (Wold, 1997). The basic architecture of RPAs is based on the oligonucleotide-/oligosaccharide-binding (OB) fold, a five-stranded β -sheet coiled into a closed barrel, but the number of OB-folds present varies from species to species (Bochkarev et al., 1999). Unlike in bacteria and eukaryotes, there is a wide diversity in the architecture of RPAs present in archaea. Two operons, RPA1 (with the genes *rfa2* and *rfa7* in *H. salinarum*) and RPA3 (with the genes *rfa3* and *rfa8* in *H. salinarum*), and a single gene, *rpa2* (*rfa1* in *H. salinarum*), have been found to encode RPA proteins in the halophilic archaea (Fig. 6). *H. salinarum* IR⁺ mutants all showed overexpression of the RPA3 operon (Webb et al., 2013; DeVeaux et al., 2007). The same operon was upregulated in previous studies following irradiation of *H. salinarum* (Whitehead et al., 2006), and more recently two independent studies (Skowyra and Macneill, 2011; Stroud et al., 2012) reported hypersensitivity to DNA damaging agents of *rpa3* mutants in *Haloferax volcanii*. Furthermore, *H. volcanii* constructs overexpressing the Rpa2 protein exhibited increased resistance to DNA damage (UV, MMS, and phleomycin) (Skowyra and MacNeill, 2011). These data clearly implicate RPA proteins in enhanced IR tolerance.

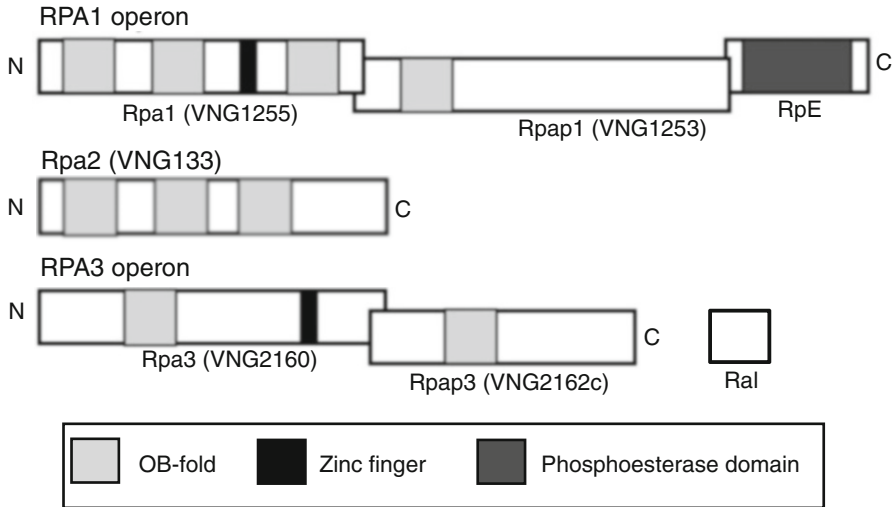


Figure 6. Operon organization and domain structures of *H. volcanii* and *H. salinarum* (gene names in parenthesis) of single-strand DNA-binding proteins (Adapted from Stroud et al. 2012, reproduced with permission).

7. What About Thermophiles?

While halophilic archaea and bacteria are adapted to desiccating conditions, imparting resistance to IR, it is not the case for many thermophiles and hyperthermophiles found to be radiation resistant. In fact, no direct correlation was found between desiccation tolerance and radiation resistance among (hyper)-thermophilic archaea (Beblo et al., 2009, 2011).

Archaea are of particular interest because they synthesize unusual, low-molecular-weight organic compounds such as β -amino acids, N^c-acetyl- β -lysine, mannosylglycerate (MG), and di-*myo*-inositol phosphate (DIP) known as compatible solutes (Fig. 7) (Martins et al., 1997; Santos and da Costa, 2002). These compounds are typically negatively charged in contrast to compatible solutes from mesophiles. Compounds such as DIP (found in *Pyrococcus/Thermococcus*, *Archaeoglobus*, and *Aquifex* species), di-glycerol-phosphate (in *Archaeoglobus* species), and MG (in *Pyrococcus/Thermococcus* and *Archaeoglobus* species) accumulate in the cell in response to supraoptimal growth temperature and osmotic shock, which are stress conditions likely to generate ROS (Müller et al., 2005). In addition, compounds such as DIP and MG have been shown to play a role in protein thermostabilization by protecting model enzymes against heat-induced denaturation, aggregation, and inactivation (Faria et al., 2004; Lamosa et al., 2003; Müller et al., 2005; Ramos et al., 1997; Scholz et al., 1992). The ability of

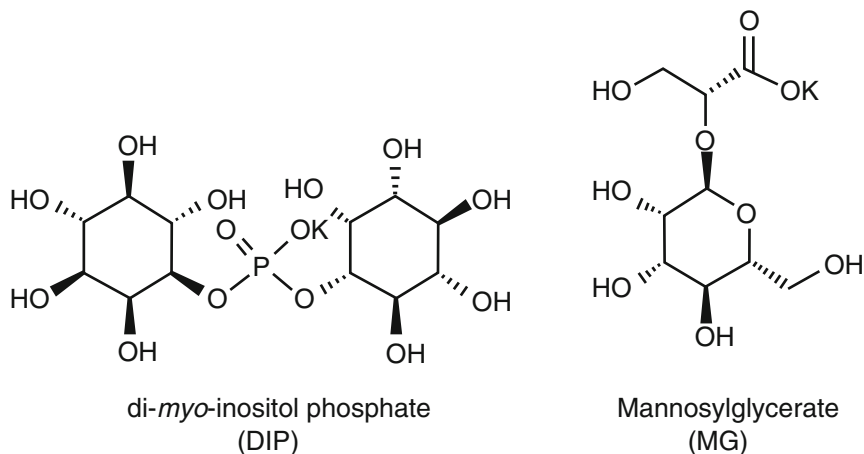


Figure 7. Compatible solutes in hyperthermophiles. Mannosylglycerate (*MG*) and di-myoinositol phosphate (*DIP*) are anions and depicted as potassium salts, K^+ being the main counterion in the organisms from which they originate.

such compounds to provide IR resistance is currently under investigation using *P. furiosus* and *Thermococcus gammatolerans* as model systems (Webb and DiRuggiero, unpublished), two hyperthermophiles highly resistant to IR (DiRuggiero et al., 1997; Jolivet et al., 2003; Table 1).

8. Relevance to Astrobiology

The understanding that adaptation to extreme environments might provide protection against high radiation levels is particularly relevant to the field of astrobiology. Lacking an atmosphere and magnetic shield to reduce the surface solar irradiance, microorganisms on the surface of Mars are exposed to far greater levels of UV-C (Cockell et al., 2000) and high-energy radiation than are microorganisms on Earth. Furthermore, there is evidence of evaporitic deposits containing high concentrations of chloride and bromide at both Meridiani Planum (Rieder et al., 2004) and Gusev crater (Haskin et al., 2005) gathered by the Mars Exploration Rovers and evidence of other evaporite deposits, possibly containing chloride, in the southern highlands of Mars reported by the Mars Odyssey Orbiter (Osterloo et al., 2008). The findings that the salt environment itself may be a protective factor for potential microbial life on the surface of Mars (Davila et al., 2008) indicate that chloride and bromide evaporite deposits showing water modification are excellent areas for surface investigations looking for evidence of life on Mars.

9. Conclusion

The study of extremophiles and how they meet the physical and chemical challenges found in the environmental extremes they inhabit lead to new insights on the mechanisms of stress response. Many extremophiles are found to be resistant to IR, suggesting that radiation resistance is a fortuitous consequence of a high tolerance to other environmental stressors (e.g., desiccation). Given the diversity of IR-resistant extremophiles and their natural environments, we do not know yet if there are universal features of IR resistance, such as high intracellular concentration of Mn (Daly et al., 2010; Robinson et al., 2011). The IR resistance found in *H. salinarum* is attributed to high intracellular concentrations of salts and Mn-antioxidant complexes that protect proteins from oxidative damage (Robinson et al., 2011; Kish et al., 2009). However, little is known regarding their physiology in the context of cellular adaptation to stress. The variety of Mn complexes found so far (Daly et al., 2010; Ghosh et al., 2011; Granger et al., 2011; Robinson et al., 2011) and the potential for compatible solutes from thermophiles to provide ROS-scavenging activity in the cell suggest that the adaptations of extremophiles to their environments provide a tremendous reservoir for novel radioprotective molecules and antioxidants against the deleterious effect of IR.

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**PART III:
THERMOPHILES**

**Kumar
Satyanarayana
Kawarabayasi**

**Bizzoco Weiss
Kelley**

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THERMOALKALIPHILIC MICROBES

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1. Introduction

The interest in studying extremophilic microorganisms has increased immensely over the last two decades. Extremophiles are organisms that are adapted to grow at or near to the extreme ranges of environmental variables. Most extremophiles are microorganisms that thrive optimally at environmental and physicochemical parameters unsuitable for the typical and widely studied mesophilic microorganisms such as *Escherichia coli*, *Bacillus subtilis*, and *Neurospora crassa* that cluster around temperature 37 °C, pH 7.4, 0.9–3 % salinity, and 1 atm pressure, the conditions that are congenial for human beings. R. D. MacElroy was the first who coined the term “extremophile” in a 1974 paper entitled “Some Comments on the Evolution of Extremophiles.” The definitions of extremophile and extreme are of course anthropocentric, from the point of view of the organism per se and the environment to which it is adapted to. A highly diverse group of organisms is known that can tolerate extreme conditions and grow, but not necessarily optimally in extreme habitats. These organisms have been called extremotrophs (Mueller et al., 2005). The distinction between extremophily and extremotrophy is not merely a semantic one but also highlights a number of fundamental issues relating to the experimental studies like:

- Methods which have been used to isolate putative extremophiles may be inappropriate.
- Putative extremophily may get compromised by subsequent serial cultivation under laboratory conditions.
- Claims of extremophily that may not have been tested rigorously.
- No further investigation whether organisms are adaptable to only small differences in environmental variables or really diverted from original conditions.

It also may be noted that many species can survive extreme conditions in a dormant state but are not capable of growing or reproducing indefinitely under those conditions. Particularly over the last century, exploration of other environments

has shown that a large number of organisms live under, or actually require, more “extreme” conditions (conditions hostile to humans and most of their microbial commensals) (Grant et al., 1990; Aguilar, 1996; Aguilar et al., 1998; Antranikian et al., 2005). Isolation of many more extremophilic microorganisms has become possible due to improved or more avid culture techniques, which has made possible to look into environments formerly considered uninhabitable. There is no environment which is not inhabited by living organisms; one just has to know how to recognize their presence. One of the most useful example of this fact is the Dead Sea, thought to be lifeless but actually containing large variety of exciting prokaryotic (Arahal et al., 1999) and even eukaryotic (Buchalo et al., 1998) life forms. The growth profiles could be the best criteria for characterizing extremophiles using marginal data, under certain culture or environmental conditions such as pH (pH_{opt} , pH_{min} , pH_{max}) and temperature (T_{opt} , T_{min} , T_{max}) ranges.

In general extremophiles are defined by one extreme of life, but in many cases natural environments pose two or more extremes, such as alkaline hot springs, alkaline hypersaline lakes, acidic hot springs, and dry sandy deserts. These environments harbor thermoalkaliphiles, halophilic alkaliphiles, thermoacidophiles, and UV radiation-resistant oligotrophs. Extremophiles adapted to more than two extremes, called polyextremophiles, are much less common than organisms with single extreme. While dealing with multiple extremophiles, it is problematic to give descriptions of their optimal and marginal growth data because the optimal growth parameters are interdependent as the value of one of the extreme growth conditions could be affected by the other.

For example, pH measurement of medium depends on temperature because of changing pKa values of different medium components at different temperatures (Wiegel, 1998). Thus, the pH of the medium when measured at room temperature will be different from its pH when it is measured at the elevated growth temperature using temperature-calibrated electrodes and pH meters. The difference in pH is small for neutral pH (less than 0.3 pH units), which becomes larger (more than 1 pH unit) at acidic or alkaline pH. To maintain authenticity and comparability among published data, it is important to know the conditions under which the pH was determined. Wiegel (1998) has proposed that authors indicate the temperature at which the pH is measured and the pH meter was calibrated with a superscript (e.g., $\text{pH}^{37^\circ\text{C}}$).

The wide distribution of thermoacidophiles is due to frequent occurrence of hot springs with acidic pH values (≤ 3.0). Acidic hot springs arise because of the presence of sulfuric acid formed by microbial and chemical oxidation of sulfur compounds. The scarcity of thermoalkaliphiles (with $\text{pH}_{\text{opt}} \geq 9.5$, $\text{temperature}_{\text{opt}} \geq 65^\circ\text{C}$) has been attributed to physiological causes. Growth under both of these extreme conditions requires specific adaptations of the cell wall and membrane composition to minimize permeability to protons and cations. Thermoalkaliphiles are also faced with the burden of acidifying cytoplasmic pH while growing in a scarcity of protons and various other bioenergetic problems, such as suboptimal proton-motive force and phosphorylation potential. However, the existence of

such isolates cannot be ruled out, as isolates like the obligately aerobic archaeal *Picrophilus* species have been reported at such temperatures on the acidic side of the pH scale from solfataric areas in Japan (Futterer et al., 2004). The genome of *Picrophilus torridus* contained genes that allow them to cope with cytoplasmic acidification and degradation of organic acids. Interestingly, all these adaptations have been acquired by horizontal gene transfer (Futterer et al., 2004). The acquisition of the genes required for surviving extremely acidic conditions by horizontal gene transfer is intriguing as this implies that hyperalkalithermophiles could have evolved by obtaining the necessary genes from extreme alkaliphiles and extreme thermophiles.

The study of extremophiles also stems from their possible utility in industrial processes and will provide possible clues as to how and where to look for extraterrestrial life (Stetter, 1996; Shock, 1997; Litchfield, 1998; Wiegel and Adams, 1998; Javaux, 2006; Lentzen and Schwarz, 2006; Villar and Edwards, 2006).

2. Life in Hot and Alkaline Conditions: General Considerations

Study of alkalithermophiles has been vindicated earlier by Wiegel and Adams (1998) as ancestor of life and model organism for the possible terrestrial life. The authors believe that life probably originated on mineral surfaces in moderate thermobiotic (e.g., 60–85 °C range), relatively shallow pools at the edges of the early Earth's oceans instead of hyperthermobioc environments. The necessary dynamic conditions for frequent association and dissociation of prebiotic and biotic structures, which lead to superior surviving combinations, were supplied by drastic changes in physicochemical parameters over space and time in such a thermoalkaline environment (Shock et al., 1998; Baross, 1998; Miller and Lazcano, 1998). These assumed selection conditions propose to a “bush-like origin” of life as suggested by Kandler (1998) and thus is different from the frequently assumed quasi monophylogenetic progenote. As such, some form of thermoalkaliphile can be considered as a logical descendant of hypothetical early life forms. In some cases, it is assumed that extremophiles are phylogenetically older ones (e.g., thermophilic *Clostridia*), whereas in other instances, the extremophiles are assumed to be secondary adaptations (Wiegel and Adams, 1998).

3. Definitions and Taxonomical Importance

3.1. ALKALIPHILES

Organisms that are able to grow at a high pH (pH ≥ 9) have two categories: The first group is categorized as alkali-tolerant species; those can grow at pH 9, but their optimum growth pH is around 7. In addition, they cannot grow at a pH higher than 10, for example, several *Virgibacillus* spp. (e.g., *Virgibacillus chiguensis*

(Wang et al., 2008) and *Anoxybacillus* spp. (e.g., *Anoxybacillus flavithermus* (Pikuta et al., 2000)). They are able to grow at pH 9 but not at pH 10, and their optimum growth pH is around 7. The second group of bacteria is categorized as alkaliphilic organisms. Alkaliphilic species can be defined as the organisms that can grow at above or at pH 10 and/or grow equally well or better in terms of growth intensity or velocity above or at pH 9 compared with those grown at a pH lower than 9. Alkaliphilic species can be further divided into obligate alkaliphiles, which cannot grow well below or at pH 8, and facultative alkaliphiles, which can grow well below or at pH 8. Some genera of bacteria include all three neutrophilic, alkali tolerant, and alkaliphilic (e.g., *Bacillus*). There are so many cases reported where in same species of bacteria, some of them are alkaliphilic and some are alkali-tolerant bacteria (e.g., *Bacillus horikoshii* (Nielsen et al., 1995)). Even among the same species of bacteria, there are possibilities that some are obligate and others facultative alkaliphiles like *Bacillus pseudofirmus* (Nielsen et al., 1995). It is considered that the above-described differences in categorized bacteria are due to the differences in the physiological function for adaptation at high pH and/or neutral pH.

3.2. THERMOPHILES

Thermophilic prokaryotes have been known as those with optimum temperature for growth between 45 and 80 °C (Cavicchioli and Thomas, 2000). Due to the development of better isolation techniques, Stetter (1996) was able to isolate *Pyrolobus fumarii* from black “smoker” chimneys in deep-ocean thermal vent environments which can grow up to 113 °C and above under hydrostatic pressure. This investigation led to change in the definition, and Stetter categorized thermophiles as hyperthermophiles (T_{opt} 80 °C or above), extreme thermophiles (T_{opt} 65–80 °C), and moderate thermophiles (T_{opt} 45–65 °C); these are the generally adapted definitions (Mesbah and Wiegel, 2008; Wagner and Wiegel, 2008).

The discovery of deep-sea hydrothermal vents in 1977 led to the first study of an ecosystem based on primary production of chemosynthetic extreme and hyperthermophilic bacteria (Priour et al., 1995). Mostly the hyperthermophilic organisms are archaea, and most of them perform common metabolic processes such as methanogenesis; anaerobic respiration via sulfate reduction, sulfur reduction, nitrate reduction, iron reduction, etc.; aerobic respiration; or even fermentation. For long time *P. fumarii* was considered as most thermophilic organism known with the record for highest T_{max} (113 °C), a T_{opt} of 106 °C, being unable to grow below 90 °C. However, the new record is held by *Methanopyrus kandleri* strain having a T_{max} of 122 °C under high atmospheric pressure isolated from the deep ocean near Japan (Takai et al., 2008). Representative genera include *Archaeoglobus*, *Thermoplasma*, *Thermoproteus*, *Acidianus*, *Pyrococcus*, *Thermococcus*, *Desulfurococcus*, and *Sulfolobus*, which can oxidize H₂S or elemental sulfur; the methanogens *Methanothermobacter*, *Methanococcus*, and *Methanopyrus*; and the nitrate reducers *Pyrobaculum* and *Pyrolobus*. These organisms are mostly

chemosynthetic or have metabolic process that no doubt plays a major role in the local environment and biogeochemical cycles. Hyperthermophilic bacteria are also included in the genera *Thermotoga* and *Aquifex*.

Most of the extremely thermophilic bacteria are anaerobic Firmicutes, which include cellulolytic *Caldicellulosiruptor saccharolyticus* (Rainey et al., 1994), the ethanol-producing *Thermoanaerobacterium* (Wiegel, 1992; Wiegel and Ljungdahl, 1996), as well as the acetogenic facultative chemolithoautotrophic *Thermoanaerobacterium kivui* (Leigh and Wolfe, 1983) and denitrifying *Ammonifex degensii* (Huber et al., 1996).

Some important and interesting for both basic and applied research are aerobic extreme thermophiles that include the well-known *Bacillus stearothermophilus* (Firmicutes) and some species within the Gram-negative genus *Thermus*, inhabitants of hot water boilers. Recently described novel thermophilic species include the citrate-fermenting *Sporolituus thermophilus* (Ogg and Patel, 2009); the novel microaerophilic, nitrate- and nitrite-reducing thermophilic bacterium *Microaerobacter geothermalis* (Khelifi et al., 2010); the deep-sea bacterium *Nautilia abyssi* (Alain et al., 2009); the thermal mud-inhabiting *Anoxybacillus thermarum* (Poli et al., 2009); and finally the novel bacterial phylum *Caldiseria* (Mori et al., 2009).

Some important moderate thermophiles and thermotolerant organisms are the cellulolytic *Clostridium thermocellum*, the acetogenic *Moorella thermoacetica*, and *Thermoanaerobacterium* (former *Clostridium*) *thermosaccharolyticum*, the “can-swelling” organism capable of growing in vacuum-packed foods (Kristjansson, 1992; Wiegel and Canganella, 2000; Prevost et al., 2010). The obligate mixotrophic *Thiomonas bhubaneswarensis*, the marine *Lutaonella thermophila*, the cellulolytic bacteria *Clostridium clariflavum* and *Clostridium caenicola*, the facultative microaerophilic *Caldinitratiruptor microaerophilus*, and a novel hydrogen-producing bacterium from buffalo dung have been described (Arun et al., 2009; Panda et al., 2009; Shiratori et al., 2009; Fardeau et al., 2010; Romano et al., 2010). Some important aerobic ones isolated in our laboratory are neutral amylase producer *Geobacillus thermoleovorans* NP54 (Malhotra et al., 2000), xylanolytic *Geobacillus thermoleovorans* (Sharma et al., 2007), and novel polyextremophilic endoxylanase-producing *Bacillus halodurans* TSEV1 (Kumar and Satyanarayana, 2011).

3.3. THERMOALKALIPHILIC MICROBES

Prokaryotes having ability to proliferate in environmental conditions having high pH extreme under elevated temperature are known as alkalithermophiles. Since they are able to sustain more than one extreme condition, these are considered polyextremophiles. Many definitions have been proposed that classify microorganisms based on their requirement or tolerance for salt, alkaline pH, and temperature (Kevbrin et al., 2004; Bowers et al., 2009). Since the minimum, optimum,

Table 1. Marginal data for defining alkaliphiles and thermophiles.

Alkali tolerant ^a	pH _{min} ≥ 6.0	pH _{opt} < 8.5	pH _{max} > 9.5
Alkaliphiles ^a	pH _{opt} ≥ 7.5	pH _{opt} > 8.5	pH _{max} > 9.5
Thermotolerant	T _{min} –	T _{opt} < 50 °C	T _{max} < 60 °C
Thermophiles	T _{min} –	T _{opt} > 50 °C	T _{max} > 60 °C
Extreme thermophiles	T _{min} usually ≥ 60 °C	T _{opt} > 75 °C	T _{max} > 85 °C
Hyperthermophiles	T _{min} usually > 65 °C	T _{opt} > 80 °C	T _{max} ≤ 122 °C

^aThe temperature at which pH values were measured with a calibrated pH meter (Wiegel, 1998).

and maximum salt concentration, pH, and temperature for growth are dependent upon each other and can vary with changes in the growth medium composition, it is very difficult to draw sharp boundaries for what a thermoalkaliphile is. The definition described by Kevbrin et al. (2004) is accepted: microorganisms that grow optimally at or above pH 8.5 and temperatures greater than or equal to 50 °C are deemed as thermoalkaliphiles. More simplified definition is given in Table 1.

Alkalithermophiles are found within both *Bacteria* and *Archaea*. Of the thermoalkaliphilic bacteria, the anaerobic thermoalkaliphiles fall into the class Clostridia, phylum Firmicutes. *Clostridium paradoxum* is considered as the most thermoalkaliphilic anaerobe, having a pH_{opt} for growth of 10.3 and a maximum of 11.3, with T_{opt} around 56 °C, whereas *Thermococcus alkaliphilus* and *Thermococcus acidaminovorans* are the most thermophilic ones (Keller et al., 1995; Dirmeier et al., 1998), growing optimally around 85 °C, with pH_{opt} of only 9.0. Among the aerobes, *Bacillus alcalophilus* B-M20 represents the most alkaliphilic thermoalkaliphile, with a pH_{opt} around 10.5 and T_{opt} of 60–65 °C. It has been observed that with increasing T_{opt}, the pH_{opt} becomes less alkaline and vice versa: at a T_{opt} of 72 °C, the pH_{opt} is around 9.2 (*Bacillus* sp. TA2.A1), and at a T_{opt} of 80 °C, the pH_{opt} is only around pH 8.0 (*Bacillus caldotenax* YTG).

Two moderately thermophilic novel species of *Anoxybacillus*, *Anoxybacillus tengchongensis* and *Anoxybacillus eryuanensis* (T_{opt} 50 °C and T_{opt} 55 °C), have been isolated from hot springs in Tengchong and Eryuan counties in Yunnan Province (Zhang et al., 2011). A novel strict aerobic genus and species of Firmicutes, *Caldalkalibacillus thermarum*, had been reported, which is a moderate thermophile (T_{opt} 60 °C) and alkaliphile (pH_{opt} 8.5) (Xue et al., 2006).

Recently three moderately thermophilic alkaliphilic novel strains of *Bacillus halodurans* have been isolated from different environmental samples in our laboratory; all three are having T_{opt} 45–52 °C and pH_{opt} 9–10.5 (unpublished work). All of them are good producers of thermoalkalitable hydrolases. One of them is a good source of thermoalkalitable endoxylanase having potential applicability in various biotechnological processes (Kumar and Satyanarayana, 2011).

Prokaryotes can adapt to a wide range of environments compared with other organisms due to their tremendous genetic diversities. Particularly bacteria have gone through generations of changes over a long period on earth. In addition,

bacteria are very well-known global material managers. Therefore, it can be said that the maintenance of environmental conditions on earth depends on the bacterial activity in ecological niches. On the basis of the above facts, it is important to know the environmental and taxonomic distributions of bacteria to understand their functions and communities in the environment. It is also beneficial to understanding the relationship between taxonomic diversity, geographical distribution, and the variation in adaptation strategy in an environment, along with the concomitant evolutionary process of bacteria. Knowledge of all these parameters will contribute to the understanding of the contribution of microorganisms to environmental sustainability on earth. However, it is not easy to understand the genetic diversity of bacteria in a short term, even with limited environment and limitations of bacterial categorization. Furthermore, it is not easy to consider the ecological function of such species of bacteria. In case we consider only small groups of polyextremophiles like alkaliphilic and thermophilic bacteria, it would be much easier to think about the above problems because bacteria can be isolated selectively from an environmental sample. In addition, alkaliphilic and thermophilic bacteria can be isolated from a wide range of environmental samples, and therefore, we will be able to consider a wide range of environments for a limited category of bacteria in comparison with other polyextremophilic bacteria. This approach will reduce the difficult problem in microbial ecology and will bring us a simplified model of bacterial taxonomic diversity and environmental distribution as compared to an exhaustive approach.

Based on the size and natural or artificial environments, Yumoto et al. (2011) have adopted four environmental bacterial habitats for alkaliphiles: large natural and artificial environments and small natural and artificial environments. If we apply these categories to the habitats of alkaliphiles, we can categorize the bacterial habitats as follows: large natural environments such as warm alkaline soda lakes, large artificial environments such as alkaline wastewater treatment systems (paper and pulp industries, textile industries, indigo dye processing units, and others), small natural environments such as the gut of termites, and small artificial environments such as laboratory enrichment culture for alkaliphilic thermophiles. To a greater or lesser extent, these four environmental categories can classify most of the microorganisms. The global material circulation and the human society are very much dependent upon the actual status and dynamics of bacterial flora between natural and artificial environments, and their symbiotic associations. However, it is not very easy to understand these differences by exhaustively considering all bacteria. If we consider a more solid proposal of a study of the above-mentioned problem on all four categories of bacterial habitats, the differences in physiological function between the same species of bacterial strains isolated from different habitats may also clarify the relationship between ecological niches and their physiological function. It is also an interesting question why distribution of alkaliphilic and thermophilic bacteria is not limited to only thermoalkaline environments but also can be isolated from conventional garden soil. To resolve such a problem, it is important to accumulate data on

the characteristics of corresponding species from different habitats. Targeting polyextremophiles will be useful from the physiological as well as microbial ecological points of view.

4. Habitats: Environmental Conditions Where They Thrive Well

Extreme habitats like hot springs, deep-sea hydrothermal vents, cold and hot deserts, and polar ice continue to provide a rich resource of novel microorganisms, appearing uniquely adapted for these extreme niches (Junge et al., 2002; Nagy et al., 2005; McCliment et al., 2006). Extreme environments that have received little attention are high-temperature soil systems such as those found on geothermally heated volcanic grounds.

It is generally assumed that the major environments from which thermoalkaliphiles can be isolated would be alkaline and thermobiotic such as naturally hot environments on earth ranging from terrestrial volcanic sites (including solfataras fields) to alkaline hot springs and the new alkaline hydrothermal vents of the “Lost City” or alkaline lakes like Lake Bogoria (Africa) and Rehai Geothermal Field, located in Tengchong County (China). There are also anthropogenic hot environments such as compost piles (usually around 60–70 °C but as high as 100 °C), slag heaps, industrial processes, and water heaters (Oshima and Moriya, 2008). As described in Sect. 1, the probability of isolation of alkaliphilic thermophiles is not restricted to environments having extreme conditions only; many thermoalkaliphiles have also been isolated from mesobiotic, slightly acidic to neutral habitats (Wiegel, 1998).

Thermoalkaliphiles have been generally categorized on the basis of their niches from which they have been isolated, their geographical distribution, and the geochemistry. The following cases can be distinguished based on spatial relationships: (1) narrow biogeography but with a relaxed biogeochemistry (organisms found are only in one very restricted location but various environmental niches like the nonspore-forming *Anaerobranca horikoshii* isolated from a specific location in Yellowstone National Park containing both acidic and alkaline springs next to each other (Engle et al., 1995)), (2) narrow and restricted biogeochemistry but relaxed biogeography (organisms can be isolated only from one type of environment but from different continents such as sporulating *C. paradoxum* found only in sewage sludge but in all tested sewage samples from various continents (Li and Poole, 1999)), and (3) relaxed biogeography and biogeochemistry (those thermoalkaliphiles which are ubiquitously distributed such as nonsporulating *Thermobrachium celere* in thermobiotic, mesobiotic, alkaline, and slightly acidic sediments from various continents (Engle et al., 1996)). An example of an aerobic thermoalkaliphile from a non-alkaline sample is *Bacillus* sp. Strain JB-99 isolated from slightly acidic sugarcane molasses (Johnvesly and Naik, 2001). Another example is *Thermalkalibacillus uzonensis* which was isolated from neutral green

mat samples from hot springs of Kamchatka (Zhao et al., 2006).

In short, alkalithermophiles have been isolated from mesobiotic as well as thermobiotic environments of natural (sediments, soil, manure piles) and anthropogenic origin. Furthermore, most of the aerobes and anaerobes are sporeformers, which make this group of the thermoalkaliphiles ubiquitous with respect to their geographical distribution.

5. Physiological Properties: Adaptation or Natural Selection?

The thermoalkaliphiles isolated so far are neither the most alkaliphilic nor the most thermophilic of the extremophiles. From the presently characterized thermoalkaliphiles, it appears that the larger the temperature optimum, the lower the pH optimum, and vice versa. This is not only true for species among different genera but also for different strains of the same species (Kevbrin et al., 2004).

Microorganisms utilize a number of adaptive mechanisms in order to enable them to proliferate in extreme environments, and this is true to an even greater extent with polyextremophiles. Life at alkaline pH values and high temperatures undoubtedly requires special adaptive physiological mechanisms. Each extreme growth condition, whether it is high salt concentration, alkaline pH, or high temperature, poses a number of physiological and bioenergetics problems. The physiological changes adopted by polyextremophiles may be a combination of changes adopted by microbes when they are exposed to individual extreme of life. Even within a given group, a very wide range of environmental limits may be tolerated. Some well-recognized adaptive mechanisms are discussed below.

5.1. ADAPTIVE MECHANISMS FOR ALKALINE CONDITIONS

The bioactive proteins (enzymes, hormones, and others) play a crucial role in organisms' survival and have distinct ranges of pH within which they can function, which propel living cells to maintain pH homeostasis. In addition, cellular bioenergetics is intricately dependent on the proton concentration. The central energy currency of bacterial cell is proton-motive force (PMF), and the pH gradient (ΔpH) across the bacterial cell membrane is one of the two PMF components (transmembrane pH gradient (ΔpH) and a transmembrane electrical potential ($\Delta\psi$)). Higher eukaryotes (including humans) follow strict pH homeostasis (internal pH 7.3) with external pH 7.4 (Casey et al., 2010). Neutrophiles can grow between pH 5.5 and 9.0, but they maintain their internal pH $\approx 7.5\sim 7.7$ (Padan et al., 2005; Slonczewski et al., 2009). In extremophiles, the strategies remain almost same as observed in neutrophiles, with some adaptations for responding to more extreme challenges. A large number of adaptive mechanisms are involved in maintaining pH homeostasis in alkaline environments, and some important

ones include:

- Changes in cell surface properties
- Increased expression and activity of Na^+ (K^+)/ H^+ antiporters
- Increased ATP synthase activity that couples H^+ entry to ATP generation
- Increased metabolic acid production through amino acid deaminases and sugar fermentation

The most important among the above are Na^+ (K^+)/ H^+ antiporters because there is a well-established correlation between Na^+ dependence and alkaliphily for mesophilic alkaliphiles and thermoalkaliphiles. There is a major difference between the aerobic and anaerobic alkaliphiles. Aerobic alkaliphiles are well known for pH homeostasis and are even able to raise or lower the external media pH to obtain optimal growth conditions (Horikoshi, 1991), whereas the anaerobic alkaliphiles change their intracellular pH with the extracellular pH and thus do not maintain a pH homeostasis; some possible reasons are discussed below.

The requirement of cytoplasmic Na^+ to support high levels of alkaliphile antiport activity is fulfilled by numerous Na^+ /solute symporters and two Na^+ channels: a voltage-gated Na^+ channel (Na_vBP) and another flagella-associated (MotPS) channel (Krulwich et al., 1985; Ito et al., 2004a, b; Fujinami et al., 2007; Krulwich et al., 2009).

Less is known about the antiporters which have major roles in anaerobic alkaliphiles or Gram-negative alkaliphiles, and these are present in very small numbers than aerobes (Mesbah and Wiegel, 2008). Polyextremophilic anaerobe *Natranaerobius thermophilus*, a halophilic and thermophilic alkaliphile, has been shown to possess a large complement of both Na^+ / H^+ and K^+ / H^+ antiporters (Mesbah et al., 2009; Krulwich et al., 2009). From this it can be concluded that cytoplasmic pH regulation and homeostasis are not controlled only by different antiporters. The properties of an antiporter that control its impact on cytoplasmic pH acidification include the stoichiometry and kinetics of the exchange.

In aerobic alkaliphilic *Bacillus* spp., proton uptake that accompanies the ATP synthesis mediated by F_1F_0 -ATP synthase contributes to pH homeostasis. While anaerobic and thermophilic alkaliphiles such as *N. thermophilus* and *Clostridium paradoxum* use their F_1F_0 -ATPases in the hydrolytic direction to generate a $\Delta\psi$, they have to avoid proton loss, and hence, they do so by using Na^+ -coupled F_1F_0 -ATPases instead of H^+ -coupled F_1F_0 -ATPases (Ferguson et al., 2006; Mesbah et al., 2009).

In aerobic alkaliphilic *Bacillus* spp., ATP synthases work in the synthetic direction. Their proton-translocating subunits *a* and *c* have specific sequence motifs which support function at high pH and protect cytoplasmic proton loss during ATP synthesis (Ivey and Krulwich, 1992; Wang et al., 2004; Liu et al., 2009; Fujisawa et al., 2010). Wang and his colleagues (2004) reported that mutations in these motifs of non-alkaliphile consensus sequence lead to the reduced ATP synthase activity, usually with shift in pH from 7.5 to pH 10.5. It has been very well established that magnitude of the defect in ATP synthase activity

correlates with a loss of the capacity for pH homeostasis during a sudden alkaline shift in external pH (Fujisawa et al., 2010; Wang et al., 2009). These mutations in motifs of ATP synthase also lead to proton leakiness (Liu et al., 2009; Fujisawa et al., 2010; Wang et al., 2009). Thus, it is possible that it was ATP synthase that adapted to promote both functions in pH homeostasis and ATP synthesis at high pH and elevated temperatures. Recently Preiss et al. (2010) had revealed atomic structure of the rotor of ATP synthase from the *B. pseudofirmus* OF4, a homo-oligomeric ring composed of 13 hairpin-like *c*-subunits, by three-dimensional X-ray crystallography. It was also evident that two major alkaliphile-specific motifs – AxAxAxA in the amino-terminal helix and PxxExxP in the carboxy-terminal helix – seem to influence the properties of the ion-binding site, including the presence of a water molecule, and are functionally important for high pH bioenergetics (Liu et al., 2009; Wang et al., 2009). Together these features of this *c*-ring have been proposed to support the high affinity of the binding sites for protons (Preiss et al., 2010), and some of these features decrease the growth capacity of *B. pseudofirmus* OF4 at a near-neutral external pH (Hicks et al., 2010). Similar types of adaptations are expected with other aerobic alkaliphiles growing at high temperatures.

Apart from Na⁺-based mechanisms, other strategies are also in play for survival in alkaline and thermally heated environment. Organisms growing at high temperatures are faced with the challenge of controlling cytoplasmic membrane permeability to protons, due to increased intramolecular motion of lipids at elevated temperatures. Because of this motion, water molecules are trapped in the lipid core, allowing protons to hop from one molecule to the other. Diffusion is a temperature-dependent process; thus, membrane permeability to ions will increase as well. Other ions, unlike protons, can diffuse through the membrane (Konings et al., 2002). The presence of specific secondary cell wall polymers associated with peptidoglycan has been reported that plays a crucial role for survival at pH 10.5 and high temperature, but these are not required at 7.5 in mesophilic conditions (Padan et al., 2005). Large proportions of cardiolipin and squalene are also reported in alkaliphilic *Bacillus* and is hypothesized that both these membrane components are involved in trapping protons at the membrane surface. The phospholipid fatty acid (PLFA) profile of *N. thermophilus*, when grown at its optimal growth conditions of pH^{55 °C} 9.5, 1.5 M NaCl, and 53 °C, revealed a unique pattern of branched chain dimethyl acetals, which are expected to play a critical role in cell permeation mechanism (Mesbah et al., 2007). It was also found that the presence of acidic amino acids in the cell wall of *Bacillus halodurans* supports growth at alkaline pH (Horikoshi, 2011). The peptidoglycan hydrolysate of polyextremophilic *Nv. wadinatrunensis* was of the type α -4- β and does not contain diaminopimelic acid isomers. It primarily consists of amino acids like aspartic acid, glutamic acid, ornithine, alanine, and glycine (Mesbah and Wiegel, 2012). The PLFA profiles of the three halophilic thermoalkaliphiles did not show any fatty acids longer than 18 carbons, the hallmarks of thermophilic bacteria, and there are only small amounts of some branched and unsaturated fatty acids

(Mesbah and Wiegel, 2012). These kinds of structural changes are being considered as reasons of less T_{opt} for polyextremophiles.

Another challenge for organisms growing at high temperatures is that they must have mechanisms to preserve protein structure at elevated temperatures. In general the preferred adaptations for extreme conditions are change of amino acid sequence of a protein by mutations, optimization of weak interactions within the protein at the protein and solvent boundary, and the influence of extrinsic factors such as metabolites and cofactors. Thermal adaptations are consequence of a number of subtle interactions, often characteristic for each protein species. Comparison of protein sequences of thermophilic and mesophilic bacterial sp. revealed amino acid substitutions involved in adapting proteins to higher temperatures. The main changes were an increase in hydrophobic amino acid residues (Haney et al., 1999). This adaptation minimizes surface energy and the hydration of nonpolar surface groups while burying hydrophobic residues and maximizing packing of the core (Yip et al., 1995). Another group of compounds, low-molecular-weight aliphatic polycations, polyamines are involved in numerous processes and are reported to play critical roles in the stabilization of nucleic acids and proteins during exposure to extremes of temperatures, either hot or cold (Tabor and Tabor, 1985). Polyamines stabilize DNA and RNA in cells of thermophiles; the presence of the polyamine tetrakis(3-aminopropyl)ammonium and spermidine is critical for protein biosynthesis in *Thermus thermophilus* near the optimal growth temperature of 65 °C (Uzawa et al., 1993; Terui et al., 2005).

6. Use of Thermoalkaliphiles in Biotechnology

The thermoalkaliphiles and halothermoalkaliphiles are promising in terms of production of biomolecules suited for a variety of applications. Enzymes from these microorganisms have found major commercial applications such as in laundry detergents, for efficient food processing, in finishing of fabrics, and in pulp and paper industries. Among various extremophiles, the immense potential of alkaliphiles has been realized since the 1960s, primarily due to the pioneering work of Horikoshi (1999). Products of industrial importance from alkaliphiles have been commercialized, the most successful of which are alkaline proteases and amylases for detergent and food industries. In a recent industrial survey, it has been shown that the enzyme industry worldwide is valued \$5.1 billion and is predicted to show an annual increase in demand by 6.3 %. Enzymes with process-specific characteristics and those used for animal feed processing, food processing, detergent, and ethanol production are envisaged to have increased demand (<http://www.freedoniagroup.com/World-Enzymes.html>). It is very important to notice that the production of extremozymes having requisite properties for respective industrial application is very low and uneconomical. Since most of the industrial process uses high temperature and alkaline pH, thermoalkaliphiles are well known to produce biomolecules for such applications. Some important

Table 2. Industrially important enzymes produced by thermoalkaliphilic bacteria.

Enzyme	Organism	Optimum production conditions		References
		T_{opt} (°C)	pH _{opt}	
Proteases	<i>Bacillus</i> sp. GUS1	70	8.0–12.0	Seifzadeh et al. (2008)
	<i>Nocardiopsis prasina</i> HA-4	50	7.0 and 10.0	Ningthoujam et al. (2009)
	<i>Bacillus circulans</i>	70–80	Alkaline pH	Rao et al. (2009)
	<i>Paenibacillus tezpurensis</i> AS-S24-II	45–50	9.5	Rai et al. (2010)
Amylase	<i>Bacillus halodurans</i> LBK 34	60	10.5–11.5	Hashim et al. (2005)
	<i>Bacillus halodurans</i> 38C-2-1	50–60	10.0–11.0	Murakami et al. (2007)
	<i>Bacillus</i> sp. PN5	90	10.0	Saxena et al. (2007)
	<i>Bacillus</i> sp. A3-15	70	11.0	Arikan (2008)
	<i>Bacillus flexus</i> XJU-3	40	10.0	Zhao et al. (2008)
	<i>Halobacterium salinarum</i> MMD047	40	8.0	Shanmughapriya et al. (2009)
Xylanase	<i>Streptomyces gulbargensis</i>	45	8.5–11.0	Dastager et al. (2009)
	<i>Rhodothermus marinus</i> ITI376	61	7.5	Dahlberg et al. (1993)
	<i>Dictyoglomus</i> sp. B1	70	8.0	Adamsen et al. (1995)
	<i>Bacillus</i> sp. SPS-0	60	8.2	Bataillon et al. (1998)
	<i>Clostridium absonum</i> CFR-702	75	8.5	Rani and Nand (2000)
	<i>Bacillus pumilus</i>	45	10	Duarte et al. (2000)
	<i>Bacillus</i> sp. NCIM59	50	10	Nath and Rao (2000)
	<i>Bacillus circulans</i> b AB16	55	8.0	Dhillon et al. (2000)
	<i>Bacillus</i> sp. JB-99	50	10	Virupakshi et al. (2005)
	<i>Paecilomyces thermophila</i> J18	50	7.0	Yang et al. (2006)
	<i>Geobacillus thermoleovorans</i> AP07	70	7.5	Sharma et al. (2007)
	<i>Streptomyces</i> sp. 7b	50	8.0	Bajaj and Singh (2010)
	<i>Bacillus halodurans</i> TSEV1	45	9.0	Kumar and Satyanarayana (2011)
	<i>Bacillus halodurans</i> TSPV1	50	10.0	(our unpublished work)

extremozymes obtained from moderate thermoalkaliphilic bacteria are presented in Table 2.

6.1. PROTEASES

Commercially, proteases have major uses as components of detergent formulations and contact lens solutions, in cheese production, in processing of meat products, and for the recovery of silver from photographic films (earlier it was recovered by burning of films) (Ito et al., 1998; Gupta et al., 2002). To be components of detergent

formulations, it is advantageous for proteases to have broad substrate specificity as well as function effectively at alkaline pH and high temperature. Proteases including thermoalkaline proteases are already reviewed earlier by many authors (Anwar and Saleemuddin, 1998; Gupta et al., 2002). Some recently reported thermoalkaline proteases produced mostly by aerobic thermoalkaliphilic bacteria are mentioned in Table 2. In the leather industry, routine hide-processing techniques entail the usage of harmful chemicals like sodium sulfide. Application of alkaline thermophilic keratinolytic proteases for the de-hairing step can result in improvement of leather quality as also less production of toxic waste (Gupta et al., 2002). There are several reports on the production methods and their functional utility in diverse fields such as agriculture and production of pharmaceuticals, cosmetics, and in protease preparations that cause cleavage of prion proteins (PrPSc) (Brandelli, 2008; Brandelli et al., 2010; Hirata et al., 2010).

6.2. AMYLASES

Thermostable α -amylases have been found to be beneficial for starch-processing and brewing industries, since these industrial processes utilize elevated temperatures to facilitate quicker and enhanced activity of reactants and to reduce process viscosity (Leveque et al., 2000; Pandey et al., 2000; van der Maarel et al., 2002). The enzyme preparations that contain alkaline debranching enzymes (pullulanases, amylopullulanase, neopullulanase, or isoamylase) in combination with amylases at high pH and temperature can remove stains more effectively (Ito et al., 1998). Ballschmiter and coworkers (2005) had reported, for the first time, a thermostable amylase having broad pH range from a thermoalkaliphilic *Anaerobranca gottschalkii* to exhibit transglycosylation on maltooligosaccharides as well as β -cyclodextrin glycosyltransferase (CGTase). CDs are cyclic molecules; a hydrophobic cavity is formed by them which facilitates inclusion of compounds of suitable size and polarity, which, in turn, changes the properties of these compounds and increases their industrial applications (Schmid, 1989). After the establishment of efficient industrial production of CDs using crude cyclomaltodextrin glucanotransferases (CGTase, EC 2.4.1.19), there are many reports on the production of thermostable and alkali-stable CGTases of *Bacillus* spp. and other bacterial strains (Matsuzawa et al., 1975; Martins and Hatti-Kaul, 2002; Thiemann et al., 2004; Yim et al., 1997; Atanasova et al., 2008). In spite of the apparent widespread occurrence of amylase-producing bacteria and efficient screening methods, a wide scope still exists for applications of amylases in other processes such as degradation of raw starch. Starch processing is widely used in the food industry for the production of maltodextrin and glucose syrups. It is performed in a two-step process consisting of liquefaction followed by saccharification. Thermostable α -amylases (EC 3.1.1.1) belonging mostly to

family GH13 are added before the heat treatment, usually performed at 105–110 °C, to catalyze the dextrinization of starch. Commercial preparations of α -amylases from hyperthermophilic archaea, namely, *Pyrococcus furiosus*, *Pyrococcus woesei*, and *Thermococcus litoralis*, having the ability to resist for several hours at the operational conditions (75–100 °C), have been exploited (Turner et al., 2007). However, ideally thermostable enzymes active and stable at low pH (about 4.5) and calcium independent for stability and activity would be even more suitable for this process (Satyanarayana et al., 2012).

Another important application of thermoalkalizable amylases is in textile industries: before dyeing and bleaching, textiles have to be treated to remove the size in a process named desizing; this is usually performed enzymatically by using α -amylases. Desizing is performed by prewashing and an impregnation of the textiles at 75–80 °C with a desizing liquor containing surfactants to wet starch and to make it available for hydrolysis. These lacunae can be addressed by bio-prospecting for high yielding strains as well as those producing amylases with better process performance characteristics.

6.3. CELLULASES

Extensive studies are going on globally for facilitating usage of cellulose-based substrates as renewable sources of bioenergy. Renewable agricultural residues and municipal wastes, cellulose and hemicellulose are not effectively utilized due to considerable costs associated with the conversion processes. Since lignocellulosic materials are more soluble at high-temperature and alkaline conditions, there is a need of lignocellulolytic enzymes which are active at high temperature and high pH. Several cellulases have been reported from thermophilic bacteria as recently reviewed by Klippel and Antranikian (2011); very few of them are having both alkalistability and thermostability simultaneously. In this panorama, cellulases from thermoalkaliphilic bacteria and archaea would be worthwhile to investigate. First, due to having intrinsic remarkable resistance to heat and alkali (Kevbrin et al., 2004) and other protein denaturants, their enzymes and proteins suit application in industrial processes. In addition, due to low complexity of the prokaryotic genomes, their genes are easily accessible and can be expressed and manipulated in heterologous hosts. Although cellulases from alkaline origin are being successfully utilized as detergent additives (Fukumori et al., 1985), thermoalkalizable cellulases find major applications in finishing of fabrics and clothes like biopolishing and biostoning of denim jeans (Pazarlioglu et al., 2005). Other major applications are deinking of wastepaper to improve the characteristics of recycled pulp (Bajpai and Bajpai, 1998). Recently Klippel and Antranikian (2011) have discussed in detail about thermostable cellulases and cellobioases produced by hyperthermophilic bacteria and archaea.

6.4. XYLANASES

Xylan is the major component of hemicellulose that contributes to 15–30 % of the total dry weight in angiosperms and 7–12 % in gymnosperms (Singh et al., 2003; Izydorczyk and Dexter, 2008). Owing to the heterogeneity and complex chemical nature of plant hemicelluloses, its complete breakdown requires the action of several hydrolytic enzymes with diverse substrate specificity, namely, β -1,4-endoxylanase, β -xylosidase, α -L-arabinofuranosidase, α -glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Singh et al., 2003; Collins et al., 2005). Among them, β -1,4-endoxylanase has a major role to play in industries like paper and pulp industry, biofuel industry, food and feed industries, and other biotechnological applications as reviewed recently by Satyanarayana et al. (2012). It has been established by so many workers in the past that xylanases from thermoalkaliphilic origin will be beneficial for such applications. Many *Bacillus* and *Geobacillus* spp. have recently been reported for the production of thermoalkaliphilic xylanases using lignocellulosic agricultural residues as well as commercial xylan sources in submerged fermentation (Anuradha et al., 2007; Sharma et al., 2007; Sanghi et al., 2009; Ko et al., 2010; Nagar et al., 2010; Kumar and Satyanarayana, 2011; Satyanarayana et al., 2012). Some recent xylanases produced by thermoalkaliphilic bacteria are cited in Table 2.

Other important enzymes produced by thermoalkaliphilic bacteria and archaea are pectinases used in degumming of ramie fibers (a novel thermophilic and alkaliphilic *Geobacillus thermoglucosidasius* growing optimally at 60 °C and pH 8.5 was isolated for production of pectinase (Valladares Juárez et al., 2009)); thermoalkaliphilic catalases and peroxidases can be used to remove residual hydrogen peroxide from effluent streams of the textile processing industry. Due to their versatile uses, thermoalkaliphilic enzyme-producing bacteria and archaea have received undue attention in the recent years (Wiegel and Kevbrin, 2004).

7. Conclusions and Future Prospects

Thermoalkaliphiles are an exciting group of extremophilic microorganisms that comprises representatives from both bacteria and archaea. Their adaptations to high pH and elevated temperature draw attention not only as a model for studying adaptive mechanisms to extreme environmental parameters but also as sources of industrially valuable enzymes. Some of the chemolithotrophic thermoalkaliphiles (e.g., CO-oxidizing iron reducers) have been considered as one of the earliest microbial life forms on Earth. In short, many opportunities exist for studying them to answer many questions regarding their adaptations and biotechnological applications. Future research directions including the refinement of culture media and enrichment isolation techniques, culture-independent metagenomic approaches for understanding their diversity and gene mining, strategies based on

cell-cell communication, high-throughput innovations, and a combination of these approaches with recombinant DNA technology will lead to novel insights into the world that once was thought to be hostile for any form of life.

8. References

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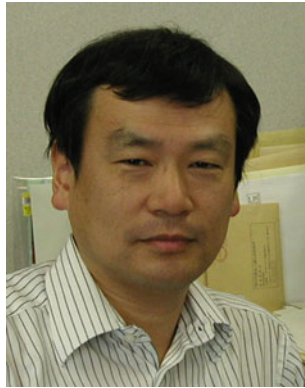
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ACIDO- AND THERMOPHILIC MICROORGANISMS: THEIR FEATURES, AND THE IDENTIFICATION OF NOVEL ENZYMES OR PATHWAYS

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1. Introduction to Acido- and Thermophilic Microorganisms

Numerous species of acidophilic and acidotolerant bacteria have been identified. For example, many lactic acid bacteria have been identified from traditional yogurt, and some species of *Helicobacter* are tolerant of acidic conditions. Most bacterial species with acidophilic features grow at normal temperatures, around 25–37 °C. Only few microorganisms from the domain *Bacteria* have been found to exhibit both acidophilic and thermophilic features. As shown in Table 1, only two genera in *Bacteria* have been identified as acido- and thermophilic microorganisms. The optimum growth temperature of these microorganisms is around 45–65 °C, which is slightly lower than optimum for similar microorganisms from the domain *Archaea*.

By contrast, a large number of genera in *Archaea* have been identified as acido- and thermophilic microorganisms, including members of the phyla *Crenarchaeota* and *Euryarchaeota* (Table 1). The optimum growth temperature of the acido- and thermophilic microorganisms within *Crenarchaeota* is around 65–85 °C. Moreover, some of these microorganisms are capable of growing under aerobic condition, as many of the microorganisms in *Crenarchaeota* were isolated from geothermal environments, like hot springs. In addition to being hot, geothermal environments typically contain high concentrations of sulfur or hydrogen sulfide, which make them acidic, so that microorganisms growing in these environments should have acido- and thermophilic features. It is thought that these aerobic organisms and the gene products derived from them or produced recombinantly in *E. coli* cells will be convenient for industrial application.

The typical features specific to the acido- and thermophilic microorganisms are extracted from the genus *Sulfolobus* as model microorganism and summarized in the following section.

2. Characteristics of Microorganisms in the Genus *Sulfolobus*

Among the acido- and thermophilic microorganisms in *Archaea*, those in the genus *Sulfolobus* are the most extensively characterized. In this section, therefore, the features of three *Sulfolobus* species will be summarized as typical of acido- and thermophilic microorganisms.

Table 1. List of acidothermophilic microorganisms.

Domain	Genus	Optimum growth temperature (°C)	Optimum growth pH	Phyla ^a
Archaea	<i>Acidianus</i>	70–85	0.8–2.5	C
	<i>Acidilobus</i>	80	3.5–3.8	C
	<i>Caldisphaera</i>	75	3.5	C
	<i>Caldivirga</i>	85	3.5	C
	<i>Metallosphaera</i>	65–75	2.5–4.0	C
	<i>Sulfolobus</i>	65–80	2.0–3.5	C
	<i>Sulfurisphaera</i>	75–80	3.5	C
	<i>Picrophilus</i>	55	1.0	E
	<i>Thermoplasma</i>	60	2.0	E
Bacteria	<i>Alicyclobacillus</i>	45–60	3.0–4.0	
	<i>Hydrogenobaculum</i>	65	3.0	

^aC Crenarchaeota; E Euryarchaeota.

Table 2. Characteristics of typical species in genus *Sulfolobus*.

	<i>S. acidocaldarius</i>	<i>S. solfataricus</i>	<i>S. tokodaii</i>
Origin	Thermal soils from Hot springs	Hot springs in Agnano, Napoli, Italy	Hot spring in Beppu, Japan
Morphology	Coccus	Coccus	Coccus
Temperature optimum	70–75	87	75–80
pH optimum	2–3	3.5	2–3
Growth conditions	Aerobic	Aerobic	Aerobic
Electron acceptor	S ⁰	S ⁰	S ⁰
Glucose metabolism	Modified ED pathway	Modified ED pathway	Modified ED pathway
Genome type	Circular DNA	Circular DNA	Circular DNA
Genome size	2,225,959	2,992,245	2,694,756

The characteristics of *S. acidocaldarius* (Brock et al., 1972), *S. solfataricus* (Millonig et al., 1975), and *S. tokodaii* (Suzuki et al., 2002) are summarized in Table 2. These species were isolated from geothermal environments (hot springs and hot soils) and, as is typical of such organisms, they exhibited aerobic features. Their optimum growth temperatures are around 70–80 °C, and their optimum growth pHs are between 1 and 3. These microorganisms are thus tolerant to both high temperatures and low pHs.

Electron microscopic observation revealed that all *Sulfolobus* species are cocci; i.e., they are round in shape (Millonig et al., 1975; Suzuki et al., 2002).

For energy production, glycolysis (degradation of glucose) is one of the most important pathways in any organism, and glucose is usually phosphorylated

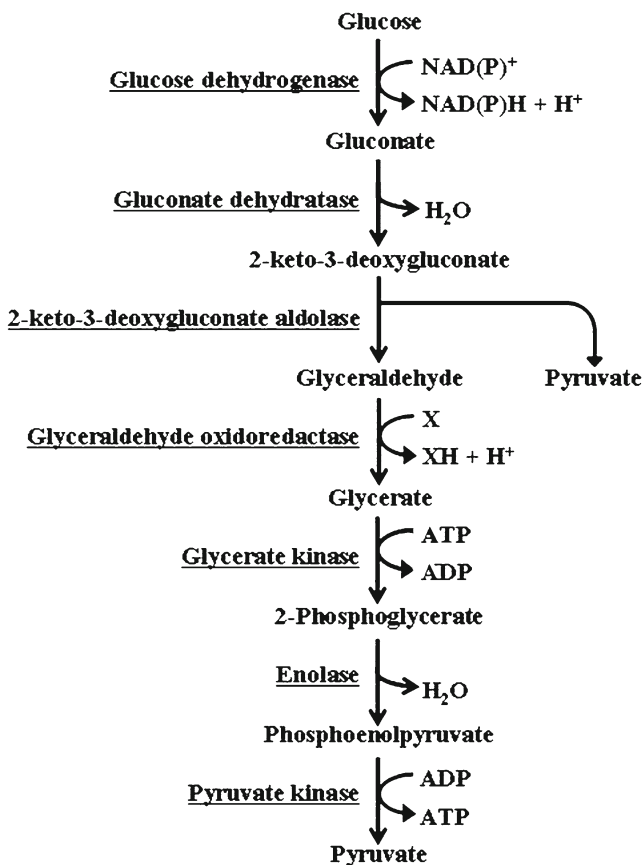


Figure 1. Nonphosphorylated Entner-Doudoroff pathway for metabolism of glucose identified in genus *Sulfolobus*. The enzymes catalyzing each reaction are underlined.

at the first step of the glycolytic pathway. However, the glycolysis pathway identified in *Sulfolobus* species is absolutely different from that identified in other organisms. These organisms in genus *Sulfolobus* utilized the nonphosphorylated Entner-Doudoroff pathway as the main glycolysis pathway (De Rosa et al., 1984). The enzymes catalyzing the reactions of this pathway and compounds converting through this pathway are shown in Fig. 1. In this pathway, glucose is converted into gluconate by glucose dehydrogenase (Lamble et al., 2003; Milburn et al., 2006) without phosphorylation of glucose. The gluconate is then converted to 2-keto-3-deoxygluconate by gluconate dehydratase (Lamble et al., 2004), which is in turn converted to glyceraldehyde by 2-keto-3-deoxygluconate aldolase (Theodossis et al., 2004) and then to glycerate by glyceraldehyde oxidoreductase. Glycerate is phosphorylated to 2-phosphoglycerate by glycerate kinase. In this

Table 3. List of *Sulfolobus* enzymes involved in the nonphosphorylated ED pathway.

Enzyme name	EC number	<i>S. acidocaldarius</i>	<i>S. solfataricus</i>	<i>S. tokodaii</i>
Glucose dehydrogenase	1.1.1.47	Saci_1079 (364 aa)	SSO3204(36 0 aa)	ST1704 (360 aa)
Gluconate dehydratase	4.2.1.39	Saci_0885 (395 aa)	SSO3198 (395 aa)	ST2366 (396 aa)
2-Keto-3-deoxygluconate aldolase	4.1.2.-	Saci_0225 (288 aa)	SSO3197 (308 aa)	ST2479 (290 aa)
Glyceraldehyde oxidoreductase	1.2.1.3	Saci_1700 (481 aa)	SSO3117 (478 aa)	ST1116 (490 aa)
Glycerate kinase	2.7.1.31	Saci_0113 (393 aa)	SSO0666 (400 aa)	ST2037 (399 aa)
Enolase	4.2.1.11	Saci_1377 (416 aa)	SSO0913 (419 aa)	ST1212 (416 aa)
Pyruvate kinase	2.7.1.40	Saci_1648 (441 aa)	SSO0981 (452 aa)	ST1617 (418 aa)

Numerals within parentheses indicate the length of amino acid residues in the corresponding enzyme.

pathway, two molecules of pyruvate and two protons are produced. The pyruvate, the final product of this pathway, is then utilized in the TCA cycle. All genes encoding each enzyme involved in the nonphosphorylated Entner-Doudoroff pathway were detected within the genomic data from three *Sulfolobus* species and are summarized in Table 3.

It is noteworthy that although microorganisms in genus *Sulfolobus* grow in an environment at an around pH 2.0, their cytoplasmic pH appears to be nearly neutral (Wakagi and Oshima, 1985). This means that protons entering the cells must be pumped out through the cytoplasmic membrane to maintain the neutral intracellular pH. As such, the cytoplasmic membrane from *Sulfolobus* species exhibits proton-pumping activity driven by respiration (Anemüller et al., 1985). Moreover, a novel ATPase comprised of three subunits, an α subunit with a molecular weight of 69,000, a β subunit with a molecular weight of 54,000, and a γ subunit with a molecular weight of 28,000, has been isolated from the membrane of *S. tokodaii* (Konishi et al., 1987). The characteristics of the ATPase include stability at high temperature, a pH optimum around 5, stimulation by bisulfite and inactivation by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, which is similar to other archaeal ATPases, though the presence of the γ subunit is exceptional (Konishi et al., 1987). The genes encoding the three subunits of the *S. tokodaii* ATPase have been isolated and their nucleotide sequences were determined (α subunit, Denda et al., 1988a; β subunit, Denda et al., 1988b; γ subunit, Denda et al., 1989). Analyses of the nucleotide sequences of the genes encoding each subunit confirmed that this ATPase belongs to a different family than the F_1 -ATPase.

The sequencing of the *S. solfataricus*, *S. tokodaii*, and *S. acidocaldarius* genomes was completed in 2001, 2001, and 2005, respectively (She et al., 2001; Kawarabayasi et al., 2001; Chen et al., 2005). The size of each genome is shown in Table 2. The genomic features and information extracted from the nucleotide sequences will be described in the next section.

3. Features Detected from the Genomic Information of Microorganisms in *Sulfolobus*

During the last decade, progress in genome sequencing has provided biological scientists with a huge amount of information that would be nearly unimaginable to traditional biologists. The entire sequencing of the *S. solfataricus*, *S. tokodaii*, and *S. acidocaldarius* genomes has brought to light previously unavailable information, which I have summarized in the following section.

One of the most important finding derived from the genomic sequences of *Sulfolobus* species is that *S. tokodaii* makes use of a eukaryotic-type tRNA system. Generally, the sequence CCA is present at the 3' end of tRNA molecule and is required for aminoacylation of tRNA molecules. In bacteria and archaea, the CCA sequence is usually present within tRNA genes and is contained within the tRNA transcripts themselves. By contrast, eukaryotic transcripts from tRNA genes do not contain the CCA sequence. Instead, the sequence is added after transcription in a reaction catalyzed by the tRNA nucleotidyltransferase. It was detected that most tRNA genes predicted on the genomic sequences of *S. tokodaii* do not contain a CCA sequence at their 3' ends (Kawarabayasi et al., 2001). Moreover, a gene encoding the tRNA nucleotidyltransferase was detected within the genomic information from this microorganism (Kawarabayasi et al., 2001). Because the tRNA system employed by *S. tokodaii* is very similar to the eukaryotic tRNA system, it has been proposed that this archaeal system is likely the origin of the eukaryotic tRNA system.

Among total 46 tRNA genes predicted in the *S. tokodaii* genomic sequence, 24 were detected as the interrupted genes in which intron was present within tRNA coding region (Kawarabayasi et al., 2001). This means that more than 50 % of tRNA genes predicted from the archaeal genomic sequence contain an intron within their tRNA coding regions. It was therefore of interest to know whether these introns found within tRNA genes predicted from the *S. tokodaii* genome are actually removed during their maturation.

Splicing sites in the tRNA genes from *Aeropyrum pernix* were easily determined by comparing immature and mature forms of cDNA molecules (Yamazaki et al., 2005). However, when cDNAs were constructed from mature and immature tRNA molecules and compared, this approach did not yield conclusive results (Yamazaki et al., 2011). To determine the correct digestion site for introns in *S. tokodaii* tRNA molecules, immature tRNA molecules were digested in vitro using recombinant stEndA, a tRNA intron endonuclease from *S. tokodaii* (Yoshinari et al., 2005). The results indicated that all predicted tRNA molecules were expressed in *S. tokodaii* and that all of the predicted introns were correctly spliced. Furthermore, analysis of the structures surrounding the splicing sites indicated that the common archaeal bulge-helix-bulge structure was detected at all intron-surrounding sites in *S. tokodaii* tRNA genes (Yamazaki et al., 2011). This means that although the unusually sized and positioned introns were detected within *S. tokodaii* tRNA genes, the microorganism makes use of a common splicing mechanism to remove those introns.

Table 4. List of genes related to sulfur metabolism.

ORF ID	Length (aa)	Predicted product	Reaction predicted
ST0615	384	Sulfide dehydrogenase	$H_2S \rightarrow S + H_2$
ST0971	390	Sulfide dehydrogenase	$H_2S \rightarrow S + H_2$
ST1010	208	Sulfite oxidase	$SO_3^{2-} + O_2 + H_2O \rightarrow SO_4^{2-} + H_2O_2$
ST1839	270	Thiosulfate reductase electron transport protein	$H_2S \rightarrow S_2O_3^{2-}$
ST2564	293	Thiosulfate sulfurtransferase	$S_2O_3^{2-} + \text{cyanide} \rightarrow SO_3^- + \text{thiocyanide}$
ST2566	628	Sulfite reductase	$H_2S + 3Fe\text{-oxy} + 3H_2O \rightarrow SO_3^{2-} + 3Fe\text{-Red}$
ST2567	239	Phosphoadenosine phosphosulfate reductase	$SO_4^{2-} + H_2 \rightarrow SO_3^{2-} + H_2O$
ST2568	412	Sulfate adenylyltransferase	$ATP + SO_4^{2-} \rightarrow PPi + AMP\text{-}SO_4^{2-}$

Table 5. Repetitive sequences detected in the *S. tokodaii* genome.

Type of repetitive sequence	Length (nt)	Number of repetitions
Tn-like element	1,779	7
Truncated Tn-like element	345	9
<i>Dispersed repetitive sequence</i>		
Type I	1,459	2
Type II	1,322	4
Type III	844	5
Type IV	518	2
<i>LR-SR-type repetitive sequence</i>		
Type LS-I	SR unit: 24 bp	3
Type LS-II	SR unit: 25 bp	2

S. tokodaii strain 7 is known to produce energy by oxidizing hydrogen sulfide to sulfate. A search for genes related to this reaction yielded a total of eight open reading frames (ORFs) involved in sulfide metabolism (Kawarabayasi et al., 2001). The predicted enzymes and the reactions they catalyzed are summarized in Table 4. These enzymes appear to be sufficient to oxidize hydrogen sulfide to sulfate. But to confirm their activities, heterologous expression in *E. coli* and functional analysis of the enzymes will be required.

Three types of repetitive sequences were detected in the *S. tokodaii* genome (Kawarabayasi et al., 2001). As shown in Table 5, seven full-size transposon (Tn)-like repetitive sequences and nine truncated Tn-like repetitive sequences were detected. This result indicates that Tn-like mobile elements did and do move within the *S. tokodaii* genomic DNA and that nine truncated forms of Tn-like elements were constructed through recombination after transfer of the

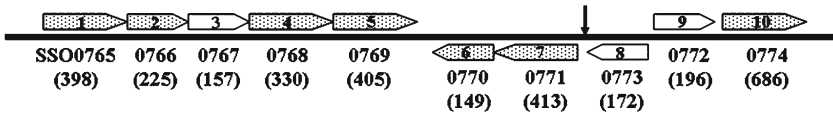
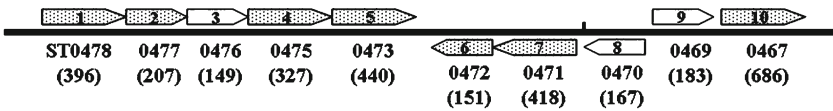
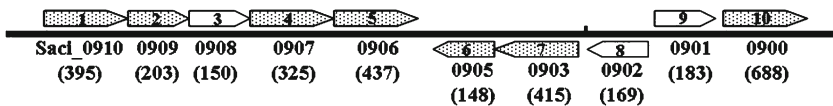
Sulfolobus solfataricus***Sulfolobus tokodaii******Sulfolobus acidocaldarius***

Figure 2. Structures surrounding the predicted replication origin of three species in the genus *Sulfolobus*. Numbers indicate the corresponding ORF ID in each species. In parentheses are the numbers of amino acid residues encoded in each ORF. Numbers within arrows are defined in Table 6 and indicate the predicted function of each ORF. Vertical arrow indicates the assigned replication origin.

elements. The second type of repetitive sequence was the dispersed repetitive sequence without Tn-like features, which consisted of four subtypes (types I–IV) with different sequences. The longest one repeated two times, the second longest repeated four times, the third longest five times, and the shortest two times. The third type of typical repetitive sequence was the LR-SR-type repetitive sequence. Two structurally similar but sequentially different LR-SR-type repetitive sequences were detected. One was an LS-I type and contained from 47 to 117 repeats of a 24-bp-long short repetitive segment following a 223–310-bp-long repetitive unit. The other was an LS-II type, which contained 73–113 repeats of a 25-bp-long short repetitive segment following a 228-bp-long repetitive unit.

The origin for genomic replication was well analyzed in *S. solfataricus*, and it was found that multiple regions throughout the genome are utilized as the origin for genomic replication (Robinson et al., 2004). In one instance, the region surrounding the replication origin contained ten genes, including MCM and Cdc6. The genes from *gatA* to MCM and the ordering of those genes within the region surrounding the replication origin are conserved among the three *Sulfolobus* species studied, as shown in Fig. 2 and Table 6. This structural feature appears to be unique to microorganisms in the genus *Sulfolobus*. Even other archaea from different genera do not conserve this structure of the replication origin. Apparently, the structure of the replication origin conserved among *Sulfolobus* species is not necessary for other archaeal species, though it is thought that this structure plays a key role during DNA replication in *Sulfolobus* species.

Table 6. List of *Sulfolobus* genes involved in the predicted replication origin.

	Predicted products	Gene name	<i>S. acidocaldarius</i>	<i>S. solfataricus</i>	<i>S. tokodaii</i>
1.	Glutamyl-tRNA(Gln) amidotransferase	gatA	Saci_0910	SSO0765	ST0478
2.	Proteasome β subunit		Saci_0909	SSO0766	ST0477
3.	Hypothetical protein		Saci_0908	SSO0767	ST0476
4.	Replication factor C small subunit	rfcS	Saci_0907	SSO0768	ST0475
5.	Replication factor C large subunit	rfcL	Saci_0906	SSO0769	ST0473
6.	Molybdenum cofactor biosynthesis protein	moaC	Saci_0905	SSO0770	ST0472
7.	Cell division control protein 6	Cdc6	Saci_0903	SSO0771	ST0471
8.	Hypothetical protein		Saci_0902	SSO0773	ST0470
9.	Hypothetical protein		Saci_0901	SSO0772	ST0469
10.	Mini-chromosome maintenance protein	MCM	Saci_0900	SSO0774	ST0467

4. Resources Identified from *Sulfolobus* Species

In the following section, the identification of two novel enzymatic activities from the acido- and thermophilic archaeon *S. tokodaii* is described. In one example, a novel enzyme that was not predicted from the genomic data was isolated using traditional purification techniques, after which its gene was isolated. In the second example, unexpected novel activity not predicted from the genomic information was detected through activity analysis of proteins heterologously expressed in *E. coli*.

4.1. HEXOSE KINASE

As mentioned, *Sulfolobus* species mainly utilize the nonphosphorylated Entner-Doudoroff pathway to derive energy from glucose (Fig. 1). However, ATP-dependent glucose-phosphorylating activity was detected in the cell extracts of *S. solfataricus* (De Rosa et al., 1984), though the enzyme responsible for the activity was not isolated until much later. An enzyme catalyzing the ATP-dependent glucose phosphorylation was purified from extracts of *S. tokodaii*, after which the N-terminal amino acid sequence was used to determine the gene encoding this enzyme to be ST2354, the original annotation of which was just as a hypothetical protein (Nishimasu et al., 2006). The isolated ATP-dependent hexokinase accepted a broad range of sugar substrates, including glucose, mannose, 2-deoxyglucose, glucosamine, and *N*-acetylglucosamine (Nishimasu et al., 2006). Furthermore, the four resolved crystal structures of this hexokinase showed that large conformational changes are induced by the binding of sugars

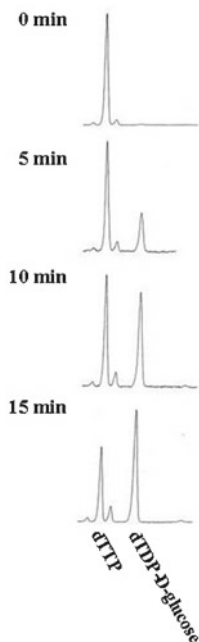


Figure 3. HPLC elution profile of the products of the glucose-1-phosphate nucleotidyltransferase activity. Substrates were incubated with the ST0452 protein for the indicated times at 80 °C. The scale was automatically adjusted depending on the amount of material detected.

to the enzyme (Nishimasu et al., 2007), which may provide an explanation for the enzyme's substrate specificity.

4.2. ST0452 PROTEIN AS A SUGAR-1-PHOSPHATE NUCLEOTIDYLTRANSFERASE AND AN AMINO-SUGAR-1-PHOSPHATE ACETYLTRANSFERASE

The ST0452 gene was annotated as a sugar-1-phosphate nucleotidyltransferase gene based on its similarity to the bacterial enzyme glucose-1-phosphate thymidyltransferase. However, the ST0452 protein contains a long extra region that is not present in the other similar genes, which encodes a segment at the C-terminus of the ST0452 protein. To characterize the activity of this protein and determine the function of the extra C-terminal region, the ST0452 gene was successfully expressed using a pET vector system in *E. coli* BL21codonplus RIL cells. This system yielded a soluble form of the ST0452 protein, which was stable for 30 min at 80 °C.

Initially, the ability of the ST0452 protein to catalyze the synthesis of nucleotide-sugar molecules from thymidine triphosphate (TTP) and glucose-1-phosphate was examined. As shown in Fig. 3, incubation of TTP and glucose-1-phosphate with the ST0452 protein yielded a nucleotide-sugar product, TDP-glucose, in a

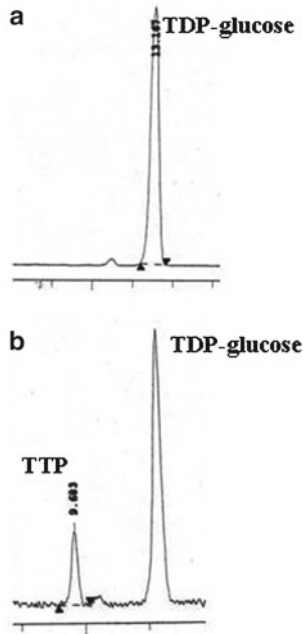


Figure 4. HPLC elution profile of the products of the reverse direction of glucose-1-phosphate nucleotidyltransferase activity of the ST0452 protein. The HPLC elution profiles show the products before (a) and after (b) 20 min of incubation at 80 °C with the ST0452 protein. The TDP-glucose and PPi were added as substrates for the primary reaction.

time-dependent manner. This confirms that the ST0452 protein possesses glucose-1-phosphate thymidyltransferase activity. The reverse reaction was also analyzed. As shown in Fig. 4, the ST0452 protein catalyzed the production of TTP using TDP-glucose and pyrophosphate as substrates. Thus the ST0452 protein appears capable of catalyzing both directions of the sugar-1-phosphate nucleotidyltransferase reaction (Zhang et al., 2005).

The substrate specificity of the ST0452 protein is summarized in Table 7. The ST0452 protein was capable of combining all dNTPs with glucose-1-phosphate. Interestingly, the ST0452 protein also catalyzed the synthesis of UDP- or TDP-*N*-acetyl-*D*-glucosamine from UDP or TTP and *N*-acetyl-*D*-glucosamine-1-phosphate. Moreover, this activity was three or five times greater than the glucose-1-phosphate thymidyltransferase activity. This observation suggests the ST0452 protein may catalyze the last reaction in the UDP-*N*-acetyl-*D*-glucosamine biosynthetic pathway. Therefore, the C-terminal region was carefully searched again, and a motif that repeated 24 times and was originally identified in acyl- and acetyltransferases was detected (Zhang et al., 2010). Because it was expected that the ST0452 protein would also be capable of catalyzing the acetyltransferase reaction for synthesis of *N*-acetyl-*D*-glucosamine-1-phosphate from glucosamine-1-phosphate, this

Table 7. Substrate specificity of the sugar-1-phosphate nucleotidyltransferase activity of the ST0452 protein.

Substrate A (0.1 mM)	Substrate B (10 mM)	Relative activity
dTTP	α -D-Glucose-1-phosphate	100
dATP		35
dCTP		7
dGTP		1
UTP		130
ATP/CTP/GTP		ND ^a
dTTP	<i>N</i> -Acetyl-D-glucosamine-1-phosphate	320
UTP		540

^aND not detected.

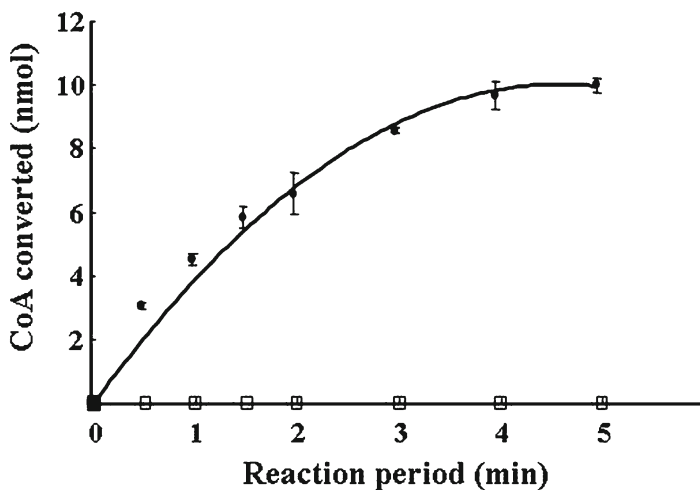


Figure 5 Time course of CoA production catalyzed by the ST0452 protein. Ellman's reaction was used to monitor CoA production. The amount of CoA was calculated from the absorbance measured at 412 nm. The reaction was run at 80 °C in the presence (*closed circles*) or absence (*open squares*) of the ST0452 protein.

acetyltransferase activity was examined. In that reaction, acetyl-CoA usually served as the acetyl group donor, yielding free CoA. Since CoA exhibits absorbance at 412 nm by using Ellman's reaction (Riddles et al., 1983) but acetyl-CoA does not, acetyltransferase activity of the ST0452 protein can be estimated based on the absorbance at 412 nm (Zhang et al., 2010).

As expected, within a reaction mixture containing glucosamine-1-phosphate, acetyl-CoA, and the ST0452 protein, there was a time-dependent increase in the absorbance at 412 nm (Fig. 5), indicating just the release of the acetyl group from acetyl-CoA. However, this observation does not indicate production of the

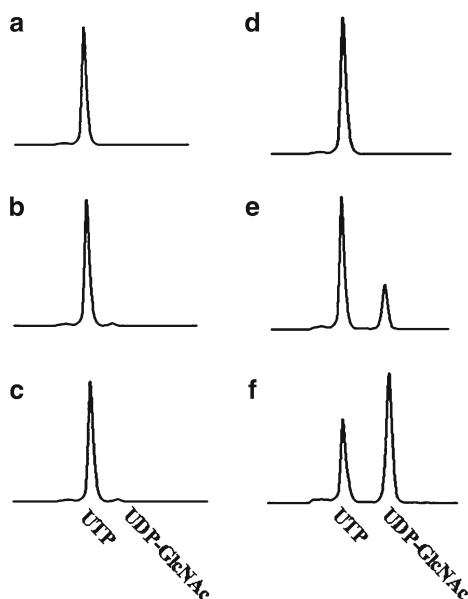


Figure 6. HPLC elution profile of the product of the coupling reaction catalyzed by the ST0452 protein. The products were eluted on a Wakosil 5C18-200 column. The reaction was run at 80 °C with 0.1 mM UTP and 2 mM glucosamine-1-phosphate (a–c) or with 0.1 mM UTP, 2 mM acetyl-CoA, and 2 mM glucosamine-1-phosphate (d–f). Shown are the elution patterns for compounds in the reaction mixture before starting the reaction (a, d) and after running the reaction for 5 min (b, e) or 10 min (c, f). The elution positions of standard UTP and UDP-*N*-acetylglucosamine are indicated by “UTP” or “UDP-GlcNAc” under panels c and f. The scale was automatically adjusted depending on the amount of material detected.

expected product. Thus, the coupling reaction, in which the sugar-1-phosphate nucleotidyltransferase activity of the ST0452 protein was used, was designed to detect the product of the acetyltransferase activity, because the facility of direct detection of modified sugar-1-phosphate was not available in my laboratory. The sugar-1-phosphate nucleotidyltransferase activity of the ST0452 protein was supported by *N*-acetyl-*D*-glucosamine-1-phosphate, the expected product of the acetyltransferase reaction, but not by glucosamine-1-phosphate, the substrate added into the acetyltransferase reaction mixture. Therefore, the acetyltransferase reaction was run in the presence of UTP. As shown in Fig. 6a–c, when acetyl-CoA was not added to the reaction mixture, only UTP was detected after incubation. However, the final product, UDP-*N*-acetylglucosamine, was detected when the reaction was run with glucosamine-1-phosphate; UTP and acetyl-CoA were added to the reaction mixture (Fig. 6d–f). It thus appears that the ST0452 protein can catalyze transfer of the acetyl group from acetyl-CoA to glucosamine-1-phosphate to produce *N*-acetylglucosamine-1-phosphate (Zhang et al., 2010).

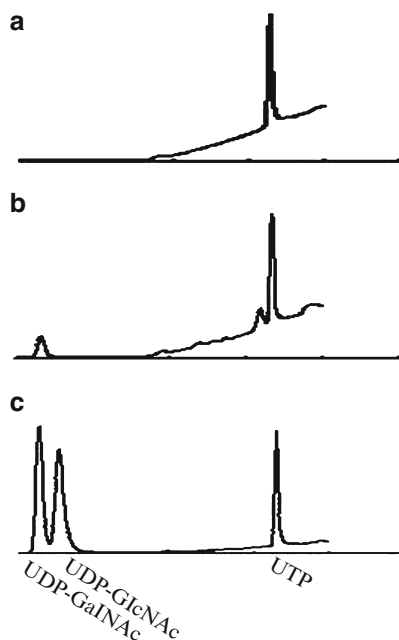


Figure 7. HPLC elution profile of the standard molecules and the product by the activities of the ST0452 protein. Standard UTP, UDP-acetyl-aminosugar, and the products were eluted on a CarboPac PA1 column. The reaction was run at 80 °C for 10 min with 0.1 mM UTP and 2 mM galactosamine-1-P (a) or with 0.1 mM UTP, 2 mM acetyl-CoA, and 2 mM galactosamine-1-P (b). Elution profile of a mixture of the standard UTP, UDP-*N*-acetylglucosamine, and UDP-*N*-acetylgalactosamine (c). Each elution position is indicated by UTP, UDP-GlcNAc, and UDP-GalNAc, respectively.

When the substrate specificity of the acetyltransferase activity of the ST0452 protein was analyzed, increases in absorbance at 412 nm indicating release of the acetyl group were observed upon addition of galactosamine-1-phosphate and acetyl-CoA to the reaction mixture as acetyl acceptor and donor, respectively. The product of this reaction was then analyzed using the same coupling reaction used to detect glucosamine-1-phosphate activity. Only when acetyl-CoA and UTP were added to the reaction mixture, UDP-*N*-acetylgalactosamine, the final product of this reaction was detected (Fig. 7). This confirms that the ST0452 protein is capable of acetylating galactosamine-1-phosphate to *N*-acetyl- β -galactosamine-1-phosphate as well as glucosamine-1-phosphate to *N*-acetyl- β -glucosamine-1-phosphate and of catalyzing the synthesis of UDP-*N*-acetylgalactosamine from *N*-acetyl- β -galactosamine-1-phosphate and UTP. Notably, the detection of the ST0452 protein's galactosamine-1-phosphate acetyltransferase activity was the first discovery of this activity in any organism.

These findings indicate that although enzymatic activity may be predicted from the genomic information based on similarity to known genes, unexpected activity is often hidden behind the predicted activity. It can therefore be said that the genomic data often contains a large number of hidden gems waiting to be discovered by biologists.

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MICROBIAL DIVERSITY IN ACIDIC HIGH-TEMPERATURE STEAM VENTS

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1. Introduction

Volcanic areas release heated water in a number of different ways. Heated water exits slowly in seeps or more rapidly in flowing springs (Boyd et al., 2007). Hot water can also enter pools with outflow channels forming flowing springs, or water can recirculate from the pool back into the subsurface (Brock and Mosser, 1975). Fumaroles represent a largely ignored area likely comprising a major source of heated water in volcanic regions (Bonheyo et al., 2005; Ellis et al., 2008). We have begun a series of studies on this area of heated water. Steam can leave the subsurface directly upward in vents or crevices or can exit laterally through passageways (Benson et al., 2011). Heated water vapors will rise up and hit a cooler ceiling often deep within an enclosed area. We have designated these as steam caves and believe they represent a unique and largely unexplored portal to the subsurface that provides an opportunity to increase our understanding of microbial life in extreme habitats as more information on these steam associated caves/vents is obtained. In this study, we report and summarize our findings and explain how we have examined this area and what approaches and instrumentation we have used as part of our investigation.

Fumaroles and deposits from rising steam vapors that collect on cave ceilings and walls or vent surfaces form in a number of different ways. The contact sites may be cooler than the rising steam, resulting in the concentration of material of several types: (1) Steam contacting a solid surface changes state from a vapor to a liquid, which evaporates and leaves a residue of soluble components including nitrate, sulfate, or others as *evaporites*; (2) iron in the form of Fe(II) belowground can change its state and deposit as Fe(III) on surfaces in multiple complex oxyhydroxides, oxides, or other complex iron *precipitates*; (3) *particulates* carried by steam away from the subsurface crevices, passageways, or even biofilms deposit in matrix material by selective adhesive or trapping mechanisms; and (4) gases such as H₂S and SO₂ will exist in a highly soluble state belowground at high temperature and pressure, but as they approach the surface, reduced solubility and oxygen force *sublimates* to form as solids such as sulfur crystals as they undergo oxidation. We use the term “steam deposits” to refer to material collected from steam cave/vent surfaces because it describes the four processes above, but does not include sediments or sedimentation, a term commonly used with flowing springs or bubbling pools.

These steam-bathed habitats have deposits carrying a variety of metals and elements, and many are dominated by sulfur (Mayhew et al., 2007) or iron (Benson et al., 2011). Although steam caves/vents have both high temperatures and low pH in the extreme, some fumaroles may present milder conditions (Portillo and Gonzalez, 2008), allowing for interesting comparisons. The extremes in temperature and pH make steam caves and chemical diversity useful for the discovery of new extremophile life. The challenge in these types of volcanic steam settings is twofold. Firstly, extracting high-quality environmental DNA has been difficult, despite the finding that extremophiles are abundant in many other extreme settings (Herrera and Cockell, 2007). Secondly, obtaining purified DNA suitable for amplification of 16S rRNA gene sequences of Archaea has been especially difficult, leading most investigators of volcanic settings to abandon studies of Archaea altogether. There has been only one study of Bacteria (Mayhew et al., 2007) and one study of Archaea and Bacteria using steam deposit samples (Benson et al., 2011). Volcanically heated soils near steam vents, on the other hand, have been successfully investigated for Bacteria by several groups (Dunfield et al., 2007; Stott et al., 2008; Costello et al., 2009; Soo et al., 2009) and provide potential methods for DNA extraction from high-pH heavily mineralized environments. Despite the multiplicity of kit types and approaches to sample processing (Henneberger et al., 2006; Herrera and Cockell, 2007), routine extraction of useful environmental DNA from steam deposit samples has proven to be challenging (Mayhew et al., 2007; Benson et al., 2011).

2. Steam Cave and Vent Sites

As previously stated, fumaroles are abundant thermal features in many volcanic areas. Consider, for example, the geothermal site called Roaring Mountain at Yellowstone National Park. Here, there is only one flowing spring, the southern effluent, yet many hundreds of fumaroles, steam vents, and steam caves are dispersed throughout the thermal site. In Hawaii, most of the thermal sites remain unexamined, and similarly, there are only a few “hot springs,” but hundreds of fumaroles exist as steam-filled caves and vents that carry heated water vapors from the subsurface through fissures and cracks until they exit into the atmosphere. The exit portal of a cave often travels horizontally either for a short distance less than a meter or a longer distance as a deep steam cave in Hawaii or Roaring Mountain in Yellowstone for several meters. Steam caves can also be vertical in their orientation and depending on the flow rate either produce a continuous thick or reduced flow of steam. In both cases, steam does not return belowground to recycle as it may do with flowing hot springs or pools (Brock and Mosser, 1975). These steam sources are essentially artesian in their flow of vapors to the surface.

Our first studies of fumarole ecosystems began with the design of a small portable collection device that could be used to aseptically collect rising steam vapors from caves and vents. We prepared a mock-up model in our lab using an

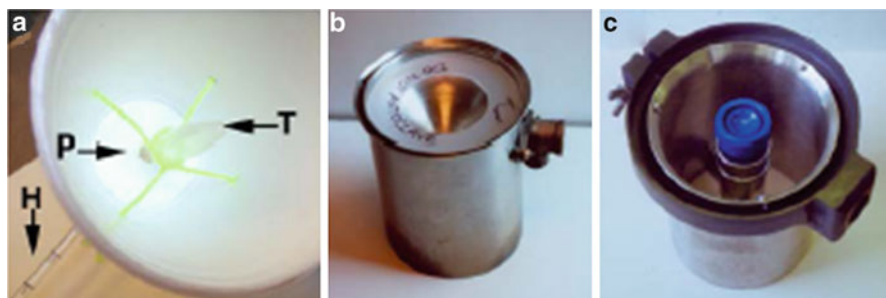


Figure 1. Steam sampler. (a) Mock-up collector. Handle *H*, pipet tip *P*, collection tube *T*, suspended with neon yellow string inside cup. (b) Working model with condenser. (c) Working model with windscreen.

upside-down Styrofoam® coffee cup, a disposable lab pipet as a handle, and an attached Eppendorf tube to collect vapors that condensed and were guided by a 200 μ l plastic pipet tip. It worked surprisingly well. When we ran field studies on steam vapors from our heating vents on the SDSU campus, in 15 min we obtained more than 1 ml of condensed steam water (Fig. 1a). In Fig. 1a, the handle is used to hold the steam collector over rising steam. The steam condenses when it hits the closed Styrofoam® cup. The pipet tip guides condensed steam, i.e., liquid, into the collecting Eppendorf tube. We built a larger working model out of stainless steel and substituted a funnel for the pipet tip and a 50 ml polypropylene tube for collecting the condensed vapors and increased our collection efficiency in natural steam vents at Yellowstone National Park, using water as a condensing medium (Fig. 1b). We later added a windscreen that surrounded the collector to eliminate potential airborne contamination (Fig. 1c). Our collection of steam averaged about 1 ml per minute but was much higher if a fumarole produced greater amounts of water vapors and gases, and the steam temperature was higher (Ellis et al., 2008).

3. Fumarole Characteristics and Sample Collection

Sites were selected from national parks at Hawaii, Yellowstone, and Lassen for their physical and chemical properties. Table 1 shows the properties of the sites sampled.

The three dominant chemistries indicated in Table 1 are illustrated in Fig. 2, nonsulfur, sulfur, and iron. Most of the steam caves and vents presented a continuous flow of steam. Visualization of vapors in the images that follow was dependent on the ambient temperature in addition to the flow of steam. Even when the vapors were not readily visible in a given image, steam flow was uninterrupted in the selected site. Physical features, such as size of the cave opening, physical orientation, and our ability to sample aseptically, were also part of the process for selecting the sites, in addition to fundamental features such as pH and temperature

Table 1. Physical conditions of sites sampled.

Location	pH	Temp (°C)	Eh (mV)	Type
Hawaii Volcanoes, National Park				
Hawaii (H1)	6.4	65.0	ND	Nonsulfur cave
Hawaii (H2)	3.1	82.0	ND	Sulfur cave
Yellowstone National Park				
Amphitheater Springs (AS1)	3.2	76.0	ND	Sulfur vent
Amphitheater Springs (AS2)	4.8	92.0 ± 0.4	ND	Sulfur cave
Norris Geyser Basin (NGB1)	4.5	61.0	ND	Nonsulfur cave
Norris Geyser Basin (NGB2)	5.5	93.0	ND	Iron vent
Roaring Mountain (RM1)	4.8	93.0 ± 0.1	+185	Nonsulfur cave
Roaring Mountain (RM2)	3.01	87.0	+368	Sulfur vent
Lassen Volcanic, National Park				
Sulfur Works (SW1)	5.0	92.0 ± 0.1	ND	Nonsulfur cave
Sulfur Works (SW2)	4.0	93.0 ± 0.8	+272	Sulfur vent
Sulfur Works (SW3)	4.2	91.2 ± 1.0	ND	Sulfur cave
Sulfur Works (SW4)	3.0	90.0	ND	Iron vent
Sulfur Works (SW5)	2.5	84 ± 2.8	ND	Iron cave
Valles Caldera, National Preserve				
Sulfur Springs, NM (SS)	2.0	89.0	ND	Sulfur cave (vertical)
Kamchatka, Russia				
Mutnovsky Volcano (MV)	3.9	94.0	ND	Sulfur vent

Averages and standard deviations are reported for vents in which 3 or more measurements of temperature were taken at least 3 months apart between 2005 and 2009.

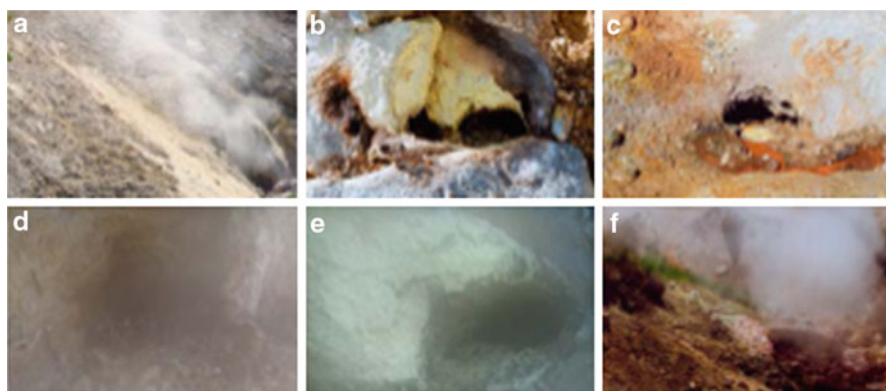


Figure 2. Steam caves and vent at Lassen Volcanic National Park. (a) Nonsulfur steam cave, Sulfur Works. (b) Sulfur steam cave, Sulfur Works. (c) Iron steam cave, Upper Sulfur Works. (d) Enlargement of nonsulfur steam cave (a), Sulfur Works. (e) Close up of steam from sulfur steam cave (b), Sulfur Works. (f) Iron steam vent, Upper Sulfur Works.

listed in Table 1. Our ability to sample was dependent on how deep into a cave we could sample and the type of sampling device, i.e., the length of the extension pole used with larger caves. Smaller caves, less than a meter deep, were also important sources for our sampling since we could more easily visualize our sampling as we were collecting and direct our sampling tools to the most critical sites. In short, it was a site-dependent process. Once we obtained our samples, they were immediately chilled if they were to be used for cloning, while for culturing they remained at ambient temperature (20–30 °C).

To chemically characterize our collected steam deposits, we used several approaches. First, we collected steam wherever the flow was adequate to allow condensation. Next, we extracted the deposits with nanowater to release soluble elements that might influence microbial growth. In both cases (condensed steam and deposit extracts), we filtered the water (0.2 µm) and analyzed the samples for elements by inductively coupled plasma optical emission spectroscopy, ICP. We also analyzed our samples for selected nutrients. The results are shown in Table 2. In our analysis, we concentrated on the three basic chemical types described in Table 1. Sulfur-dominated sites derive their vapors from deep within the subsurface through passageways that reach to magmatic gases, while nonsulfur caves are supplied vapors that originate from heated meteoric water and possibly explains why some sites provide ideal places for the development of unique microbial communities. Iron-dominated sites are encrusted with iron oxides or oxyhydroxides. In Hawaii, iron was occasionally deposited on top of sulfur, providing a chemically rich steam deposit for the resident microorganisms.

3.1. STEAM SAMPLE COLLECTION

We collected steam with our portable steam collector (Ellis et al., 2008) as it exited from a cave or vent. Our steam collector was efficient (~14 %) and successful in every cave or vent we examined, yielding collection rates between 30 and 90 ml in about 30 min. Phase-contrast microscopy and 4',6'-diamidino-2-phenylindole (DAPI) staining for dsDNA showed that all steam condensed from caves/vents contained recognizable microorganisms. No cells were seen in adjacent collected control samples, indicating that the organisms were present in the steam and not derived from airborne contamination. Even with small volumes of condensed steam examined, samples revealed a wide morphological variety of cells that varied in shape, diameter, length and spherical or rod-shaped appearance. The concentration of organisms in steam was low and varied from 100 to 1,200 cells/ml, although the steam flow was continuous, indicating that over time many organisms were released from the subsurface into the air above and dispersed as the steam exited caves/vents. In order to analyze our samples, we found it was necessary to use a GenomiPhi[®] kit to increase the yield of DNA so we could obtain clones and sequences from PCR-amplified environmental DNA. For the steam samples, this method was successful in many cases and revealed that steam from the subsurface

Table 2. Steam deposit chemistry of seven Yellowstone and Lassen fumaroles based on ICP and nutrient analysis

Analyte (mg/l)	RMNS°CST	RMNS°C	SWS°VST	SWS°V	ASS°VST	ASS°V	USWFeV
Na	0.666	2.421	1.6995	2.356	3.463	0.634	3.453
Ca	0.00	0.596	0.876	1.240	3.592	0.00	2.870
Al	0.00	4.432	0.307	8.431	1.082	0.0389	2.006
Fe (total)	0.00	3.084	0.434	1.674	0.998	0.283	0.997
Si	0.0676	19.311	0.342	9.733	29.222	0.207	25.706
B	0.00	2.332	0.441	1.036	0.700	0.00	0.760
K	0.00	0.371	1.747	0.367	2.519	0.557	3.401
Mg	0.00	0.0245	0.0432	0.256	1.434	0.00	0.945
Zn	0.127	0.0598	0.0115	0.0619	0.0195	0.316	0.0362
Mn	0.00	0.00	0.0187	0.0157	0.00646	0.00	0.0256
Mo	0.0270	0.00	0.00	0.00	0.00	0.0265	0.00
Se	0.00	0.0171	0.00	0.0191	0.00	0.00	0.0251
Ni	0.00	0.0173	0.00289	0.0416	0.00	0.0272	0.00
Pb	0.00165	0.00	0.00	0.00	0.00	0.00435	0.00
Cr	0.00535	0.00	0.00612	0.00	0.00	0.0362	0.00
Cd	0.00	0.0229	0.0138	0.00874	0.00215	0.00	0.00236
Cu	0.00	0.498	0.00	0.000994	0.00	0.00	0.00
Hg	0.00	0.00	0.00	0.00	0.00	0.00	0.00
As (total)	0.00059	0.0109	0.00	0.0194	0.0266	0.0112	0.0209
S	5.181	50.763	1.820	250.428	0.00	9.767	17.833
Sr	0.0311	0.00459	0.00	0.0832	0.00858	0.0320	0.0387
NH ₄ ⁺ , N-NH ₄ (µM)	0.011	18.391	0.23	66.573	79.023	0.473	5.780
PO ₄ ³⁻ , P-PO ₄ (µM)	0.067	5.593	0.015	74.493	27.372	0.074	0.00
SO ₄ (µM)	781.3	355.0	260.4	175	0.00	833.3	222.0
Conductivity (µS/cm)	47.6	1,420.0	139.3	8,970.0	29.3	284.0	516.0
Eh (mV)	ND ^a	+185.0	ND	+272	ND	ND	ND

^aND not determined, RMNS°CST Roaring Mountain nonsulfur cave steam, RMNS°C Roaring Mountain nonsulfur cave, SWS°VST Sulphur Works sulfur vent steam, SWS°V Sulphur Works sulfur vent, ASS°VST Amphitheater Springs sulfur vent steam, ASS°V Amphitheater Springs sulfur vent, USWFeV Upper Sulphur Works iron vent.

carried halophilic Archaea (Ellis et al., 2008). We were able to grow one of these organisms in culture from Amphitheater Springs vent samples, extract the DNA, and obtain a 16S rRNA gene sequence. We also successfully subcultured this halophilic archaeon. We obtained clones and sequences from high-temperature acidic fumaroles in Kamchatka, Hawaii, New Mexico, California, and Wyoming. All of the Archaea from these widely dispersed collection sites were related to halophiles in a variable collection of saline habitats that included brine pools, salt lakes and soils, a subglacial hypersaline lake (Antarctica), and Dead Sea waters (Arahal et al., 1996; Ihara et al., 1997; Oren et al., 1999; Bowman et al., 2000).

3.2. STEAM DEPOSIT COLLECTION

Steam deposit samples differ from condensed steam in having a solid substrate where the steam contacts the surface of a cave and leaves a deposit over time. We began our study by collecting samples at the site of steam contact with the cave or vent surface and initiated an analysis of the deposit samples to obtain information on the solid surface chemistry of the collected deposits. Steam deposits were adhered to a sample stub with a conductive carbon tab and analyzed in an FEI Quanta 450 SEM for elements using an Oxford X-Max 50 energy dispersive spectroscopy detector and Inca software. The results of three chemical types of steam cave deposits are shown in Fig. 3a–c, which include X-ray spectra of (a) nonsulfur steam cave deposit Sulphur Works, Lassen; (b) sulfur steam cave deposit Amphitheater Springs, Yellowstone; and (c) iron steam cave deposit Upper Sulphur Works, Lassen. Solid surfaces can concentrate and entrap particles, gases, compounds, and other substances rising from the magma and make them available as metabolites for microbial growth.

In the examples of steam deposits shown in Fig. 3a–c, nonsulfur cave deposits (a) showed the highest peak for silicon (Si). A smaller peak for sulfur (S) was always present as well as peaks for oxygen (O) and titanium (Ti). Although Ti was always found in nonsulfur cave deposits, the exact metabolic role of this element is unknown. Sulfur cave deposits (b) had S as the main component with Si as a minor peak along with O. Iron steam cave deposits (c) had Si and O as prominent elements, a small peak of S and moderate peaks with both K and L lines for iron, Fe. Phosphorus, P, was also found in the iron cave steam deposit sample as a minor peak.

4. Steam Deposit DNA Extraction

We had already seen organisms in collected steam samples from widely distant sites around the world. Our goal was to develop a method to successfully isolate DNA from the site where steam directly contacted cave or vent surfaces and learn more about the subsurface and the organisms harbored or growing there by using caves and vents as a portal to this area. Earlier studies failed to isolate archaeal

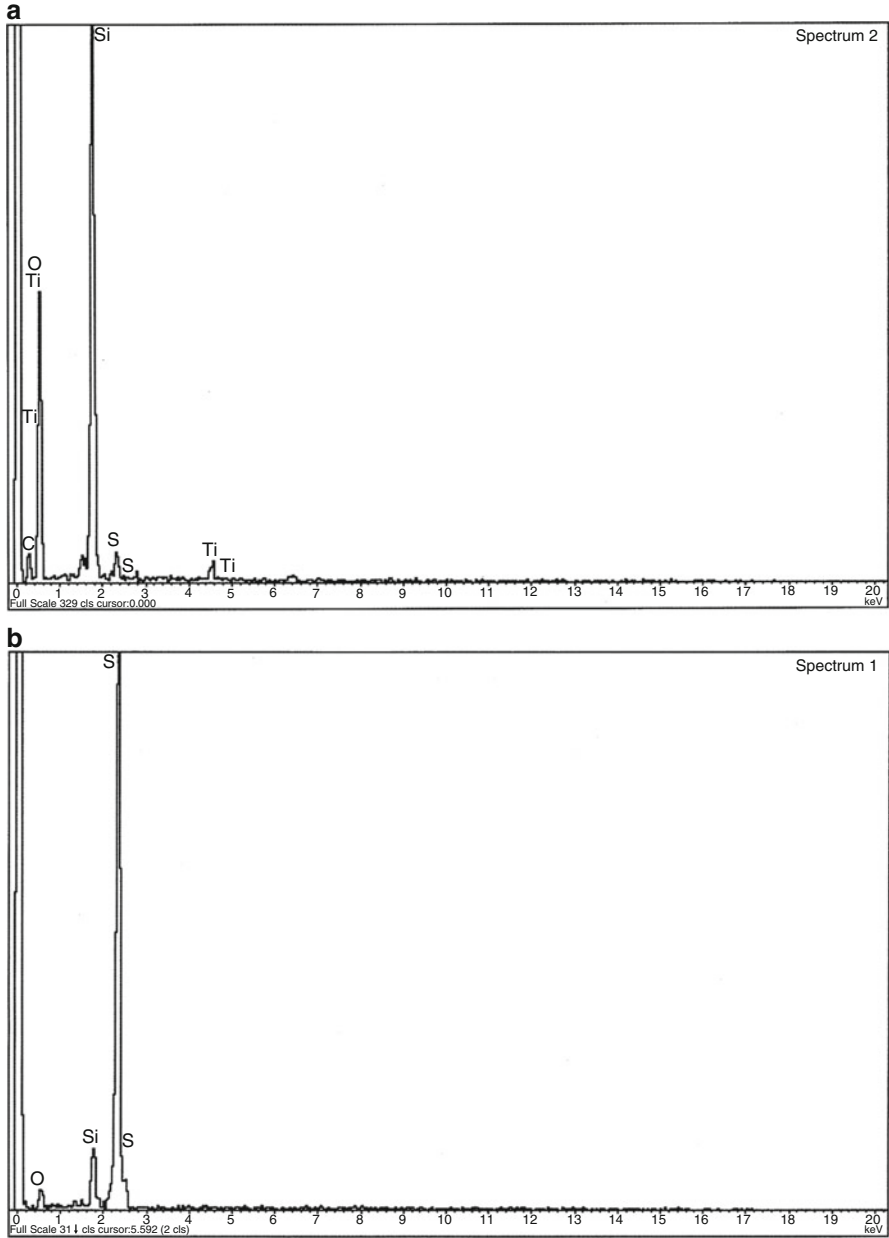


Figure 3. X-ray spectra: (a) nonsulfur, (b) sulfur, and (c) iron deposits.

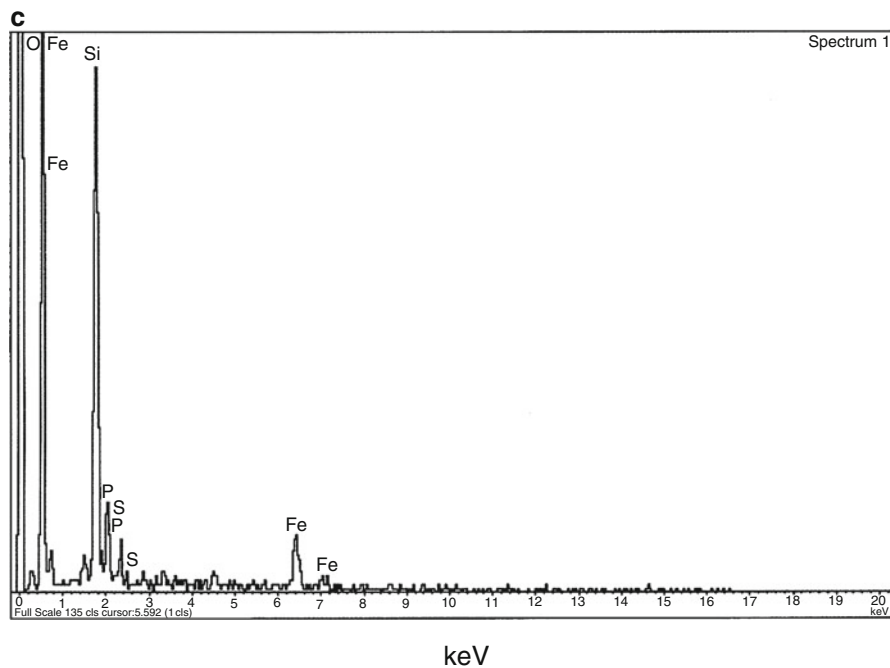


Figure 3. (continued)

DNA from volcanic vent areas and had concentrated on bacterial or eukaryotic DNA extractions (Ackerman et al., 2007; Mayhew et al., 2007). We recognized that damage to isolated DNA can occur in low-pH sulfur and iron habitats if the DNA is exposed to acidic conditions during isolation. This does not present a problem in nonsulfur caves with near-neutral pH. Still, we considered this aspect and pH-neutralized samples before we developed a general method to obtain purified DNA from all of our samples. Direct microscopic analysis showed that cells were attached to sulfur in sulfur caves and to iron in the iron cave deposits as well as to silica particles in all three steam cave types. We determined that flowing steam resembled a flowing hot spring, and the deposits seemed similar in some respects to spring sediments. We had used lysozyme successfully with flowing springs and had obtained high-quality DNA with this method, but had no success with the steam cave/vent deposit samples.

Understanding the need for a new approach, we turned to UltraClean[®] DNA isolation kits. Previous studies of volcanic soils had included various animal protein sources such as BSA or skim milk (Takada-Hoshino and Matsumoto, 2004) to improve the quality of their isolated DNA (Ikeda et al., 2008), but PCR revealed that contaminating bacterial DNA was present in added milk products (Ikeda et al., 2008). Plant proteins had not been evaluated as a potential additive to improve DNA quality. Benson (2010) found that this, along with a step-up in

kit selection from UltraClean® to the PowerSoil® isolation kit, yielded high-quality DNA suitable for PCR and sequencing. Using this newly developed method, DNA was obtained from a number of samples taken from steam cave/vent deposits in our study (Table 1). Throughout the process of applying our selected plant-based protein within the PowerSoil® kit, we met with some successes and some failures at various levels. In one case, a precipitate developed, whereas at other times low-quality DNA was obtained, or even with high-quality DNA, samples failed to amplify or bad sequences were returned. No single step in the process from DNA isolation to sequences returned consistently represented a blockade point in obtaining success with the Power Soil/Protein method.

5. Clones Obtained and Phylogenetic Analysis

The protein-based DNA extraction method allowed us to successfully obtain archaeal PCR clone libraries from all the Hawaii steam deposit samples and one Yellowstone site. The sulfur sediments were comprised of *Sulfolobus* species and uncultured Archaea (Benson et al., 2011). Interestingly, all of the Yellowstone sites and Lassen sites that failed to return sequences were successful in obtaining archaeal cultures. Archaeal sequences returned were *Acidianus*, *Sulfolobus*, and uncultured *Sulfolobus* species. A worthy finding in the nonsulfur caves was the presence of high ammonia concentrations in Hawaii (Table 1, H1) and that both Hawaii (H1) and Yellowstone (NGB1) nonsulfur sites contained undescribed Archaea; the NGB1 organisms were related solely to clones from thermal soils. The Hawaiian site supported high-temperature ammonia-oxidizing archaea (AOA) related to a newly cultured archaeon in “group” 1 *Crenarchaeota* and closely related to the cultured ammonia oxidizer, “*Candidatus Nitrosocaldus yellowstonii*” (Könneke et al., 2005; de la Torre et al., 2008). Of importance here is that Könneke and coworkers found the first instance of an archaeon carrying out ammonia oxidation in the marine environment. Marine AOA appear to be significant worldwide members of the ocean habitat. The Hawaii nonsulfur steam cave contained members closely related to AOA, representing the first discovery of AOA in this habitat. The AOA *Crenarchaea* have been considered in a proposal for a third phylum in the *Archaea*: *Crenarchaeota*, *Euryarchaeota*, and proposed *Thaumarchaeota* to include marine plankton and closely related AOA (Brochier-Armanet et al., 2008). The protein-based DNA extraction method also succeeded in returning a large collection of bacterial clones from steam cave/vent deposits in Hawaii, Yellowstone, and Lassen. Typically, these included *Chloroflexi*, *Bacillus*, *Alicyclobacillus*, and *Proteobacteria*, among others. Bacteria were cultured from most sites with the exception of a single site in Hawaii with iron/sulfur deposits.

6. Steam Deposit Cultures

Both Bacteria and Archaea were enriched in liquid medium. We initially incubated all of our steam deposit enrichments at 55 °C, pH 3 or 4.5. Liquid cultures commonly arose from an inoculum of 1–5 % steam deposit material in 5–12 days. Bacteria grew readily in liquid medium before transfer to agar or Gelrite® plates to obtain isolated cultures. Archaea grew readily in liquid enrichments and then grew only as mixed cultures on Gelrite® overlay plates. Figure 4 shows representative archaeal cultures. We successfully enriched Bacteria and Archaea from most of the steam cave/vent deposit sites. In liquid culture, Bacteria and Archaea grew optimally at the pH of steam, pH 4.5. To obtain isolates, cultures were incubated at 55 or 70 °C and most isolates of both Bacteria and Archaea grew best at 55 °C, as reported earlier (Ellis et al., 2008). We next selected two high-temperature sites, the Sulphur Works iron vent and sulfur cave (Fig. 2e, f), and established cultures at 85 °C, pH 4.5. Iron vent biofilms had mostly spheres, a few rods, and fewer thin filaments. We found that the enrichment cultures from the sulfur cave (SW3) were distinct from those in Yellowstone, in that most cells were closely associated with the sulfur granules.

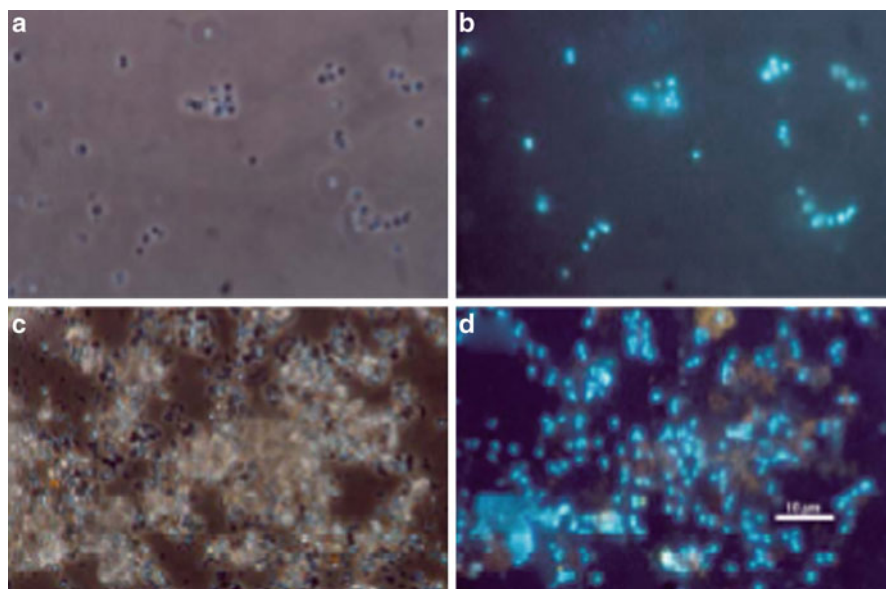


Figure 4. Cultures enriched from steam deposit samples in Yellowstone National Park. Cells in phase-contrast images are dark irregular spheres. DAPI-stained cells appear as a *blue color* identifying dsDNA in phase-dense cells. (a, b) Amphitheater Springs sulfur vent steam deposit culture, (a) phase contrast. (b) DAPI fluorescence. (c, d) Amphitheater Springs sulfur steam cave deposit culture, (c) phase contrast. (d) DAPI fluorescence. Cells attached to sulfur surface in c, d.

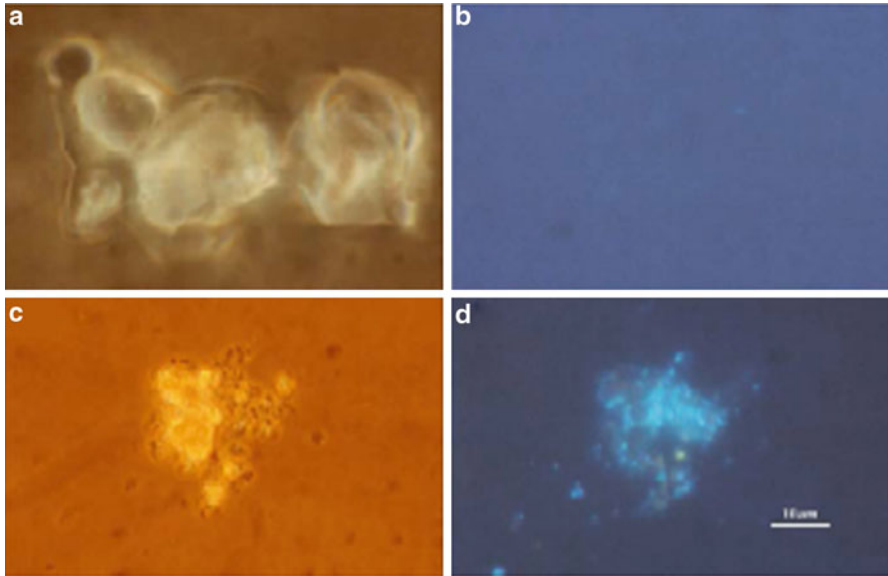


Figure 5. Phase-contrast and DAPI fluorescence microscopy of uninoculated sulfur and cultures following incubation at 85 °C, pH 4.5 for 24 h. **(a, b)** Uninoculated sulfur. **(a)** Phase contrast. **(b)** DAPI fluorescence. **(c, d)** Sulphur Works sulfur cave deposit culture. **(c)** Phase contrast. **(d)** DAPI fluorescence. The sulfur in **c, d** appears with most of the DAPI-stained cells buried inside the individual grains. Compare to Fig. 4c, d.

To show this feature, Fig. 5 presents a comparison of uninoculated and inoculated lab sulfur using the steam cave deposit sample from Sulphur Works, Lassen. Uninoculated controls (Fig. 5a) showed a smooth surface on the sulfur grain incubated at 85 °C. No cells were seen with DAPI staining in Fig. 5b. In contrast, cells grown in a culture inoculated with a steam deposit from the Sulphur Works sulfur cave displayed sulfur with a particularly irregular rough surface after incubation for 24 h at 85 °C (Fig. 5c). DAPI-stained cells appeared to be associated with the sulfur grains as shown in Fig. 5d. Cultures established from the nonsulfur cave (NGB1) at the Norris Geyser Basin in Yellowstone showed almost exclusively spherical cells; similar images were seen with steam deposit cultures obtained from the nonsulfur cave at Sulphur Works, Lassen (SW1). Here, the cells grew in clusters that resembled microcolonies (Fig. 6a, b). Figure 3a shows the nonsulfur steam deposits (SW1) to contain mainly siliceous material, a small amount of sulfur and some titanium. These cells (SW1) resemble spheres in a mixed culture containing abundant thin filaments grown from steam deposits at Sulphur Works sulfur steam cave (Fig. 6c, d). The sulfur cave spheres (SW3) were identified as *Sulfolobus* by BLAST analysis of 16S rRNA gene sequences.

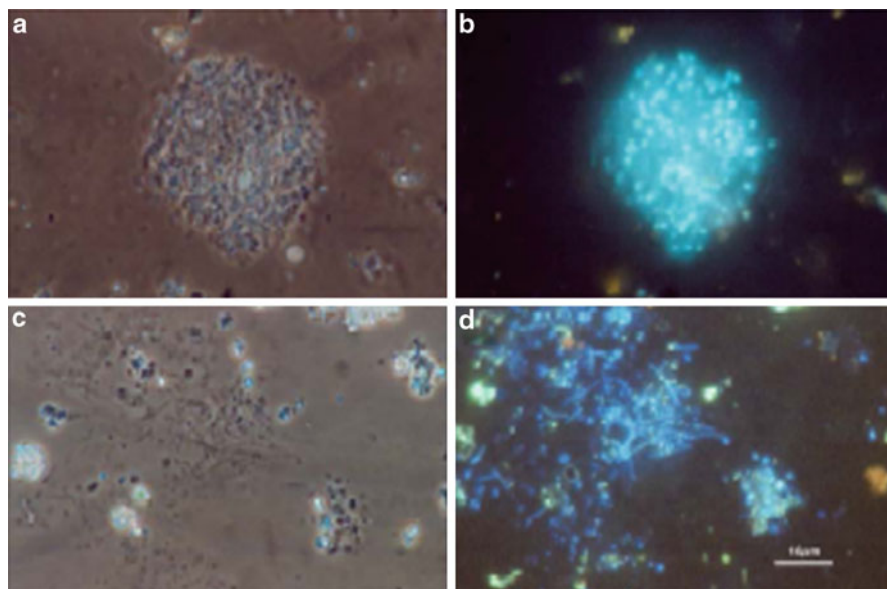


Figure 6. Comparison of cultures of unknown or undescribed organisms enriched from steam cave deposits in Lassen Volcanic National Park. (a, b) Sulphur Works nonsulfur steam cave deposit culture. (a) Phase contrast. (b) DAPI fluorescence. Undescribed cells are mostly spherical with only one or two rod-shaped cells seen in the culture. (c, d) Sulphur Works sulfur steam cave deposit culture. (c) Phase contrast. (d) DAPI fluorescence. Cells represent a mixture of unknown thin filaments and spherical cells identified by BLAST as an uncultured *Sulfolobus* sp.

Similar very thin filaments were seen in enrichment cultures and occasionally in natural samples from several steam cave deposits examined in the present study, including a small unidentified Roaring Mountain sulfur cave, an iron vent (SW4) and nonsulfur caves SW1 and H1. We were able to grow and subculture these ultrathin filaments in liquid cultures at 55 °C. With subculture we were unable to obtain cell concentrations that exceeded $2\text{--}4 \times 10^6$ cells/ml, although the cells were easily grown and subcultured in liquid medium. All of our attempts to isolate DNA from the filamentous cultures obtained from several steam deposit sites met with failure. Consequently, we were not able to obtain 16S rRNA gene sequences and do not know whether the cultured cells are Bacteria or Archaea. The finding of extremely thin filaments in steam cave deposits was unexpected. High-temperature low-pH sites might preferentially support spherical organisms such as *Sulfolobus* or *Acidianus*, depending on the available oxygen. Other organisms may exist within mixed communities, providing they are supplied with essential nutrients or conditions to support their growth. As an example, *Halorubrum luteum* and uncultured haloarchaea were grown at 37 °C from most of the Hawaii samples. In one Lassen site where we failed to obtain environmental DNA for identification, we used a more direct method, fluorescence in situ

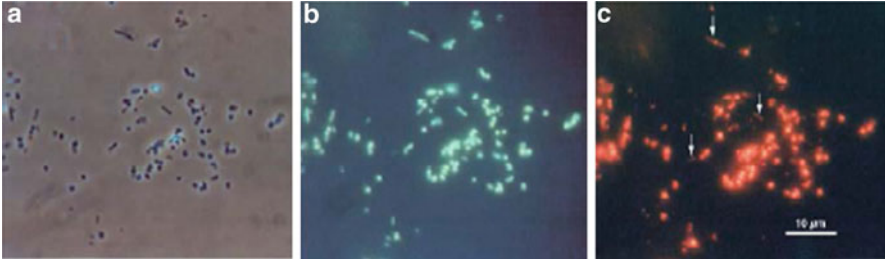


Figure 7 FISH-labeled culture from iron vent, Sulphur Works, Lassen. (a) Phase-contrast image. (b) DAPI stain. (c) Fluorescent image of cells labeled with Archaea-specific probe 915-CY3 probe. Labeled cells are *bright orange* archaeal spheres. *Arrows mark 3 separate nonlabeled rod-shaped cells.*

hybridization (FISH), to identify cells as Bacteria or Archaea. We were unable to PCR amplify DNA from the Upper Sulphur Works iron steam vent deposit culture. An example of a FISH-labeled culture from this site is shown in Fig. 7. Controls consisted of a nonsense probe, NON338, and no probe added to the sample; all three samples were processed identically and side by side. No labeling was seen with either of the controls indicating that the Arc915 probe labeling, if present, was specific for Archaea. In Fig. 7, only spherical cells were labeled, identifying them as Archaea. Several rod-shaped cells were not labeled (arrows, Fig. 7c), indicating they were not Archaea.

7. Final Considerations

The existence of an abundant subsurface biosphere “seeding” geothermal features has been strongly debated. The present study adds new information to that controversy. Artesian steam caves preclude airborne entry of contaminating microbes, and steam deposits – formed by continuous steam cave ceiling contact – likely originate from the near subsurface or deeper underground, implying that the microorganisms recovered from steam deposits arise from these depths as well.

We modified our approach to DNA isolation with the protein, VeggieTones GMO-Free soya peptone, added to the Power Soil/Protein method. Yet, more changes are needed. We should explore pulverizing at LN₂ temperatures, adding chelating agents and/or Fe(III)-reducing agents, and investigating potential silica inhibition of DNA isolation. In geothermal settings silica coats sulfur crystals, and at ambient temperature silica sheets form and bind cells. Further, Fe(III) leads to sample precipitation with the Power Soil/Protein method, and this problem needs to be resolved before iron vent sequences can be recovered from the NGB2 site in Yellowstone National Park.

The way ammonia concentrates in near-neutral steam deposits (Jones, 1963; Nordstrom et al., 2005) is similar to the way H₂S concentrates in acidic systems. We recovered sequences related to AOA from the high-ammonia H1 site. If we

can demonstrate by enrichment-isolation or by *amoA* gene sequences that these H1 organisms represent AOA, then this represents a newly uncovered AOA habitat, strengthening the proposal for a third archaeal phylum, *Thaumarchaeota* for marine *Crenarchaea* and close relatives – AOA (Brochier-Armanet et al., 2008).

Our follow-up studies of bacterial diversity in Hawaii show that this diversity is far greater and more complex than we initially anticipated and suggest steam deposits and fumarole-derived soils represent a “hot spot” for microbial biodiversity. Following up on our studies with a concentration on Hawaiian bacterial diversity represents an important area that deserves further investigation.

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**PART IV:
PSYCHROPHILES**

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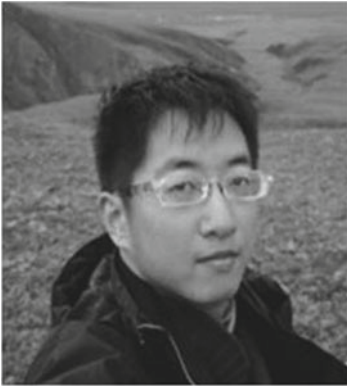
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LEFT OUT IN THE COLD: LIFE IN CRYOENVIRONMENTS

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1. Introduction

Cryoenvironments are generally defined as environments that exist continuously and predominately at subzero temperatures. They exist primarily in polar and alpine regions and consist of large-scale geomorphological features such as permafrost, glaciers, ice caps, and sea ice. Cryoenvironments also include relatively rare subzero habitats such as cold lakes and ponds, which can be permanently ice covered, and subzero saline springs, which flow throughout the year, warmed by geothermal gradients, and maintained liquid due to their high salinity (Andersen et al., 2002; Doyle et al., 2012). The primary constraint to life in cryoenvironments is the availability of liquid water; life needs liquid water to survive, mediate biochemical reactions, provide transport of molecules, and act as a solvent. It is not necessarily subzero temperatures that constrain life in cryoenvironments but rather the conditions that are typically found associated with subzero temperatures, which include freezing, desiccation, or high osmolarity. Microorganisms in subzero environments must, however, be able to cope with the thermodynamic effects of low temperatures including lower reaction rates, increased molecule stability, and conformational changes of proteins (Bakermans, 2008). Because the presence of liquid water in cryoenvironments is often facilitated through the freezing-point depression properties of various solutes, microorganisms must also be able to tolerate osmotic stress, usually in the form of high salinity. Despite these harsh environmental conditions, there is a recent and growing body of evidence that cryophilic microorganisms (those able to reproduce at <0 °C) exist and are metabolically active in these cryoenvironments at ambient temperatures.

2. Microbial Habitats in Polar Cryoenvironments

2.1. PERMAFROST

Permafrost is defined as soil that remains below 0 °C for at least 2 consecutive years and also contains multiple unique geomorphotypes such as ice wedges, massive ground ice, cryopegs, pingos, and taliks. Permafrost is typically overlain

with an “active layer” that seasonally rises above 0 °C and which can extend several meters down. Permafrost regions cover 27 % of the terrestrial surface of Earth and occur in polar and high altitudes. Any microbial life present in permafrost must cope with long-term exposure to subzero temperatures, background radiation, limited liquid water availability, and often oligotrophic conditions (Steven et al., 2006, 2009). While these extreme conditions are lethal for some cells, there is mounting evidence indicating that permafrost microbial communities may be active in situ (see Table 2).

Liquid water available for microbial life in permafrost should be present in small amounts, despite the subzero temperatures. Concentrated solutes in frozen soils may reduce the freezing point of water allowing the occasional presence of briny veins (Anderson, 1967). The ordering effects of clay minerals are also known to stabilize liquid water into thin films adsorbed to the mineral grain and may be the only available water below certain temperatures (Anderson, 1967; Jakosky et al., 2003). Due to surface area differences, particle size greatly influences the fraction of unfrozen water present in permafrost. Whereas Arctic loamy soils can contain up to 2–10 % unfrozen water down to –12 °C, sandy soils contain virtually no liquid water due to their larger particle size (Gilichinsky et al., 1993; Steven et al., 2006). While these films may be too thin to allow microbial mobility, they are thought to be sufficient to transport wastes and nutrients and to sustain microbial life (Price and Sowers, 2004).

The Upper McMurdo Dry Valleys of Antarctica are a particularly harsh and low water activity permafrost environment, characterized by hyperaridity in addition to the cold. The polar deserts are the only place on Earth where a layer of dry soil overlays ice-cemented ground permafrost (McKay, 2009) and, in some places, lack an active layer which rises above 0 °C (Marinova et al., 2011). Water exchanges between the dry soil and ice-cemented permafrost via vapor diffusion rather than liquid water. Despite being in the vapor phase, this water is thought to be available to microbial cells (Stomeo et al., 2012).

Within permafrost, unique geomorphological features may additionally be present (Steven et al., 2006). For example, polygon-patterned ground is common in permafrost-affected terrain. Depressions forming the polygon boundaries are underlain with V-shaped ice wedges; they are formed over thousands of years by repeated seepage and freezing of water through cracks in the soil created by thermal contraction (Wilhelm et al., 2012). Ice wedges in wet permafrost environments such as those found in the Canadian High Arctic can range in size from 2 to 4 m in width at the top and can extend to depths of 5–10 m (Wilhelm et al., 2012). In the Upper McMurdo Dry Valleys, where dry active layer soils predominate, the trough-like depressions may be underlain by sand wedges instead of ice (Singleton et al., 2010). Massive ground ice refers to horizontally extensive bodies (>2 m thick) of ice also found embedded within permafrost (Steven et al., 2008). Permafrost environments can also contain supercooled (–9 to –11 °C) anaerobic brine lenses (6–30 % NaCl) known as cryopegs. These relatively rare liquid permafrost habitats are ~1–2 m in diameter and can remain isolated on geological timescales.

Cryopegs were formed from ancient marine sediments 100–120,000 years ago after the Arctic Ocean regression, when sediments surrounding the remaining pockets of water froze (Gilichinsky, 2003; Gilichinsky et al., 2003, 2005).

2.2. SEA ICE

Formed of frozen seawater, sea ice contains extensive channels of liquid brines. As water freezes, salts, solutes, and impurities present in ice (including microbial cells) are extruded from growing crystals and concentrated into inclusion veins and brine pockets. Dictated by in situ temperature, the salinity and volume of these brines are subject to continuous fluctuations that vary seasonally. Brine veins can range in size from several millimeter thick channels with near-seawater salinity (~3 %) in the summer to micrometer-sized veins or inclusions that can reach 23.7 % salt in winter sea ice (Junge et al., 2004; Mock and Junge, 2007; Collins et al., 2010). A vertical gradient is also observed in sea ice with saltier, thinner veins normally found at the colder ice-air interface as opposed to underlying ice sections in contact with warmer ocean water (Skidmore et al., 2012). These changes in ice vein dynamics consequently expose sea ice communities to both temperature and salinity extremes that vary on an annual basis.

In spite of the harsh nature of sea ice, the veins are still considered microbial habitats. The habitability of sea ice was clearly demonstrated by Junge et al. (2001) via microscopy showing that bacteria populate ice veins and brine pockets in temperatures as low as -15°C (Fig. 1).

2.3. GLACIAL ICE

Similar to sea ice, glacial ice also bears habitable vein systems in between ice crystals (Price, 2000, 2007). The extent of these veins, however, is thought of as being smaller than their sea ice counterparts, considering glacial ice's lower dissolved solute content (Doyle et al., 2012). The smaller-sized veins consequently restrict biological colonization to small cells. Mader et al. (2006) showed how small-sized particles will preferentially be extruded over larger ones from growing ice crystals. Whereas nearly all particles smaller than $2\ \mu\text{m}$ (including bacteria) partitioned in the liquid portion of artificial polycrystalline ice at -10°C , virtually all particles larger than $5\ \mu\text{m}$ ended up trapped within the bulk ice crystals. Consistent with the constrained vein environment of glacial ice, the Greenland ice sheet has been found to be dominated by microorganisms smaller than $0.1\ \mu\text{m}^3$; these cells may represent small microbes or starved miniature cells (Miteva and Brenchley, 2005).

The nature of the ice will also greatly influence its liquid water content. For example, Antarctic ice is known to be acidic, with SO_4^{2-} being the most prevalent ion (Price, 2000). Considering the eutectic point of sulfuric acid to be of -73°C ,

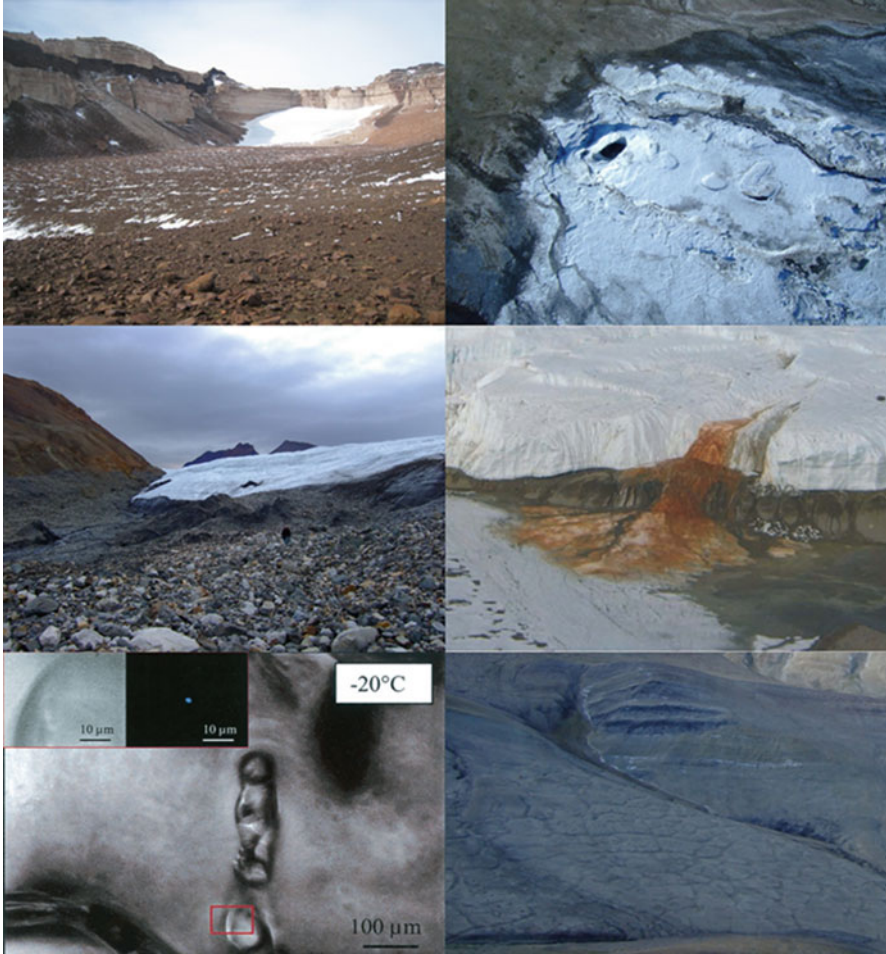


Figure 1. Microbial habitats in cryoenvironments. *Top left:* McMurdo Dry Valleys (M. Marinova). *Top right:* Lost Hammer spring (C.-Y. Lay). *Middle left:* White Glacier (C.-Y. Lay). *Middle right:* Blood Falls (Adapted from Mikucki et al., 2009). *Bottom left:* Sea ice DAPI-stained bacteria in a sea ice brine pocket at -20°C (Adapted from Junge et al., 2004). *Bottom right:* Polygon structures in permafrost (C.-Y. Lay).

it is thought that even the coldest recorded Antarctic ice (-56°C) should contain liquid veins (Price, 2012). For life to exist in such extreme conditions however, it not only needs to be able to cope with extreme cold and restrained environments but also concentrated acid.

Vein systems are not the only possible habitat present in ice. Much like permafrost environments, thin films of liquid water can also persist due to the ordering effects of mineral grains such as clays found in dirty ice. Consequently, the bottom of the Greenland ice sheet, which overlays clay-rich wetland sediments,

has been found to contain higher concentrations of cells attached on glacial clay minerals (Table 1) compared with the relatively low biomass present in the overlying clean ice from the same core (Tung et al., 2006). Lastly, Rohde and Price (2007) proposed a third ice habitat where cells could potentially metabolize within the ice crystal itself via redox reactions of small molecules diffusing through crystal grains.

2.4. COLD AND SALINE WATER BODIES

There are several types of aquatic systems in cold environments in which microorganisms must face multiple stressors. For example, alkaline freshwater lakes predominate in the Antarctic, some of which are permanently ice covered. In spite of the cold, minimal photosynthetically active radiation (PAR), oligotrophic conditions, salinity, and high pH reaching 10 in some locations, diverse microbial assemblages have been found in these environments (Sattler and Storrie-Lombardi, 2010). Meromictic (permanently stratified) saline lakes are those in which a proportion of the water remains perpetually unmixed with the remainder of the lake. These lakes have a mixed upper oxic layer, an oxycline, and then a lower stagnant anoxic layer, which never mixes with the upper ones. The Vestfold Hills in the Antarctic contain several meromictic lakes and have been the target of most of the microbiology-related studies of Antarctic lakes to date (Bowman et al., 2000a).

In the Antarctic McMurdo Dry Valleys, a subglacial outflow (Blood Falls) flows from the Taylor Glacier, an outlet glacier of the East Antarctic Ice Sheet. Blood Falls discharges a cold (~ 7 °C), ancient marine brine, rich in sulfates and iron-oxides, resulting in the reddish hue that gives it its name (Mikucki and Priscu, 2007; Postberg et al., 2011). Cold saline springs can also be found in the high Arctic arising through several-hundred-meter thick permafrost on the Norwegian archipelago of Svalbard and Axel Heiberg Island (AHI) of Canada (Andersen et al., 2002; Reigstad et al., 2011). The coldest springs reported to date discharge at several locations on AHI (AHI) in an area with an average annual air temperature of -15 °C and winter minima reaching -50 °C. These springs are linked to subpermafrost groundwater flow through carboniferous evaporites in areas of diapiric uplift (Pollard et al., 2009; Niederberger et al., 2010). The AHI Gypsum Hill and Color Peak springs flow perennially with constant discharge temperatures ranging from -0.7 to 6.9 °C and discharge waters that are moderately saline (7.5–15.8 % salts), anoxic (mean oxidation-reduction potential (ORP) of -325 mV), and rich in both sulfate and sulfide (Perreault et al., 2007, 2008; Niederberger et al., 2009; Pollard et al., 2009). A third AHI perennial spring, Lost Hammer spring, is characterized by a cone-shaped tufa structure and is the only known perennial subzero (-5 °C) and hypersaline (~ 24 %) terrestrial methane seep on Earth (Niederberger et al., 2010). Lost Hammer spring sediments and water are near neutral pH, reducing (ORP is ~ -165 mV), microaerophilic,

Table 1. Microbial diversity and abundance in cryoenvironments.

Location	Biomass	Major taxa	Functional groups reported	References
Permafrost				
Permafrost	10 ³ (CFU)	Actinobacteria	Methanogens, methanotrophs	Steven et al. (2006, 2009)
Eureka	10 ⁸ (microscopy)	Bacteroidetes		and Yergeau et al. (2010)
Canadian High Arctic	10 ⁶ (qPCR)	Firmicutes ^{s,d}		
Permafrost	<10–10 ³ (CFU)	Actinobacteria	N.D.	Aislabie et al. (2006)
Miers Valley, McMurdo	10 ⁷ (microscopy)	Proteobacteria		and Cowan et al. (2002)
Dry Valleys	10 ⁸ (ATP)	Acidobacteria ^a		
Permafrost	10 ⁴ dry surface soils	Proteobacteria	N.D.	Gilichinsky et al. (2007)
Beacon Valley	10 ⁶ permafrost (microscopy)	Actinobacteria		
McMurdo Dry Valleys		Firmicutes ^b		
Ice wedge, Canadian High Arctic	10 ⁵ CFU	Proteobacteria	AOA, denitrifiers	Wilhelm et al. (2012)
	10 ⁸ (microscopy)	Actinobacteria		
Massive ground ice, Canadian High Arctic	10 ⁴ (microscopy)	Firmicutes	N.D.	Steven et al. (2008)
	0 CFU	Actinobacteria		
Cryopegs, Siberian permafrost	10 ⁷ cells/mL (microscopy)	Proteobacteria	SRBs, methanogens, acetogens, yeasts	Gilichinsky et al. (2003, 2005)
		Actinobacteria ^d		
Ice environments				
Bottom of Greenland ice sheet (silty)	10 ⁸ cells/mL (microscopy)	N.D.	Methanogens, Fe-reducers	Tung et al. (2006)
Arctic sea ice	10 ³ upper winter ice 10 ⁷ summer bottom ice	N.D.	Psychrophilic and halophilic heterotrophs, AOA	Deming (2010), Collins et al. (2008)

Aqueous cryoenvironments

Lost Hammer spring, Canadian High Arctic	10 ⁷ (microscopy)	Bacteroidetes Proteobacteria Actinobacteria ^a	Methanogens, AOA, SRB, methanotrophs, methylotrophs, halophiles	Niederberger et al. (2010), Lay et al. (2012)
Expedition Fiord springs, Canadian High Arctic	10 ⁷ (microscopy)	Proteobacteria Bacteroidetes Firmicutes ^a	Methanogens, methanotrophs, sulfur oxidizers, halophiles	Perreault et al. (2007)
Don Juan Pond, Antarctica	0	N.D.	No evidence of life in pond water	Samarkin et al. (2010), Wright and Burton (1981)
Blood Falls, Antarctica	10 ⁵ cells/mL	Proteobacteria (<i>Thiomicrospira</i>) Bacteroidetes ^a	Sulfur oxidizer, SRB, denitrifiers, halophiles	Mikucki and Priscu (2007)
Ace Lake, Antarctica	10 ⁵ cells/mL	Proteobacteria Actinobacteria Cyanobacteria ^c	Halophiles, cyanobacteria, SRB, methanotrophs,	Lauro et al. (2011)

CFU colony forming units, *AOA* ammonia-oxidizing archaea, *SRB* sulfate reducing bacteria, *N.D.* no data.

Methods of assessing microbial community composition: ^aclone library; ^bpyrosequencing; ^cmetagenome; ^dculturable isolates.

oligotrophic, and also rich in sulfates and sulfides and known to host indigenous cryophilic halophiles (Table 1). An extreme example of a saline water body is the Don Juan Pond, the most saline water body on earth with a salinity of 42 %, a salinity which may overreach the limits of life, as it is likely the pond water itself contains no life or evidence of microbial activity (Wright and Burton, 1981; Samarkin et al., 2010).

3. Diversity of Microorganisms in Cryoenvironments

Biomass and diversity in cryoenvironments are as heterogeneous as the habitats themselves. In spite of the multiple stressors associated with cold environments, there can be significant biomass and diversity present. For example, permafrost from both the Arctic and the Antarctic have been observed to have a microbial load of up to 10^8 cells/g (Cowan et al., 2002; Steven et al., 2007a), and 10^7 cells/mL have been found in hypersaline springs from the Arctic and in cryopegs found in Siberian permafrost (Gilichinsky et al., 2003; Niederberger et al., 2010). In contrast, microbiological studies on sites like Blood Falls in Antarctica, winter glacial ice, and massive ground ice have reported biomasses as low as 10^3 – 10^4 cells/mL (Table 1). Early microbial biodiversity studies of many cold environments typically involved culture-dependent methodology, followed by clone libraries or pyrosequencing to identify microbial composition. More recently, the use of next-generation sequencing is allowing researchers to identify microbial inhabitants of cold environments in depths never accessible before (Bartram et al., 2011).

3.1. PERMAFROST

Molecular surveys of permafrost from the Arctic and the Antarctic both include aerobic and anaerobic bacteria and show a predominance of phylotypes belonging to *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Gilichinsky et al., 2007; Steven et al., 2007a, 2009; Hinsä-Leasure et al., 2010; Yergeau et al., 2010). These bacteria may be selected for in the permafrost environment; *Actinobacteria* are known to be able to metabolize at low temperatures, and spore-forming *Bacteroidetes* and *Firmicutes* may better resist the permanently frozen conditions. Accordingly, *Actinobacteria* were found to dominate active layer samples in a Canadian High Arctic core, while *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* co-dominated the permafrost samples (Steven et al., 2008; Yergeau et al., 2010).

The desiccating mineral soils of the McMurdo Dry Valleys have been found to harbor high levels of diversity (Smith et al., 2006), often with a microbial composition of many species occurring in low abundance (Takacs-Vesbach et al., 2010). Microbial composition and biomass vary with the environment. Maritime-influenced Dry Valley soils have been found to contain a surprisingly high biomass on the order of 10^8 cells/g (Cowan et al., 2002) compared to the

inland Beacon Valley, which were shown to support minimal populations, up to 10^4 cells/g in the dry surface soils and 10^6 cells/g in the permafrost (Gilichinsky et al., 2007).

Biodiversity found in cryopegs (isolated pockets of saline water located within permafrost) has been assessed primarily through culturing viable microbial communities, and thus, it is difficult to quantify bacterial diversity reflective of in situ abundances (Gilichinsky et al., 2003, 2005). Microorganisms that have been isolated from cryopegs were related to common soil organisms as well as species that have also been isolated from cold aqueous environments such as *Psychrobacter*, *Frigoribacterium*, *Paenibacillus*, and *Rhodococcus*. *Psychrobacter* was the most commonly isolated bacterium from cryopeg environments (Gilichinsky et al., 2005).

There are to date a limited number of microbiological studies on ice wedges and massive ice bodies. Culturable microorganisms from massive ice deposits are low (0 CFU) (Steven et al., 2008) compared to both 25,000-year-old ice wedges from Alaska and younger ice wedges (>4,000 years old) from the Canadian High Arctic (10^5 – 10^6 cell/g) (Katayama et al., 2007; Wilhelm et al., 2012). Interestingly, these values from ice wedges are high compared to the culturable cell counts typical of permafrost (Table 1). Ice wedge bacterial diversity was previously considered to be low (Katayama et al., 2007); however, pyrosequencing techniques have recently shown a high bacterial diversity in a high Arctic ice wedge sample, dominated by *Proteobacteria* and *Actinobacteria* (Wilhelm et al., 2012). The archaeal community was also surveyed but found to have a low diversity, composed primarily (>90 %) of ammonia-oxidizing *Thaumarchaeota* (Wilhelm et al., 2012). The source of the high bacterial diversity was likely the surrounding soils, as community structure was reflective of the surrounding tundra permafrost, surveyed in a previous study (Wilhelm et al., 2011). Conversely, community composition of massive ground ice sampled from the Canadian High Arctic did not reflect surrounding permafrost, being dominated by *Firmicutes*, which were poorly represented in clone libraries from the same permafrost core (Steven et al., 2008).

This absence of overlapping communities was likely because massive ground ice is relatively stable within permafrost when compared to ice wedges, which undergo cyclic thermal contraction and possible influx of sediments (and microbiota) from surrounding soils.

3.2. GLACIAL ICE AND SEA ICE

Sea ice microbial communities can be complex and diverse, supporting a microbial loop which includes protists and metazoa, with much of the biomass being concentrated at the sea ice-water interface (Brown and Bowman, 2001) and a majority of the microbial community being composed of heterotrophic bacteria, diatoms, and unicellular algae (Brown and Bowman, 2001; Mock and Thomas, 2005).

Relative to other cryoenvironments, bacteria from sea ice are highly culturable; for example, 62 % of the biomass from Arctic sea ice was found to be culturable (Junge et al., 2002). Minimal change in microbial community structure and diversity is seen over time during the winter season, although bacterial numbers are found to decrease, implying little selection of specific microbial groups during the colder winter season (Collins et al., 2010). Similarly, the microbial diversity seen in a Greenland ice sheet core is thought to be primarily reflective of microorganisms deposited within the glacier over time rather than selected for within the ice environment. Microbial diversity was found to vary with the type of ice (silty or clear) but also among clear samples tested, though bacterial numbers were consistently higher in the silty ice (Miteva et al., 2009). Anaerobic methanogens, acetogens, and sulfate/sulfur reducing bacteria have been identified in glacial ice, especially associated with silty particles (Tung et al., 2006; Miteva et al., 2009; Simon et al., 2009).

3.3. COLD AND SALINE WATER BODIES

In hypersaline spring and lake systems, such as the AHI springs in the Arctic or the Vestfold Hills meromictic lakes of the Antarctic, halophilic/halotolerant bacteria and archaea are commonly detected (Bowman et al., 2000a, b; Naganuma et al., 2005; Niederberger et al., 2010; Lay et al., 2012). For example, the majority of the culturable isolates from the AHI Lost Hammer spring are psychrotolerant and halotolerant, and molecular surveys of its microbial community reflect the geochemistry of the spring. Accordingly, methane and sulfur metabolizers have been found in this methane- and sulfur-rich spring (Niederberger et al., 2010; Lay et al., 2012). The runoff channels of the Gypsum Hill (GH) and Color Peak (CP) spring systems (anoxic, sulfate and sulfide-rich brines) harbor hetero- and chemolithotrophic microbial populations (Perreault et al., 2007, 2008). Of special interest are the GH spring runoff channels which support chemolithoautotrophic, phototrophic-independent metabolism by *Thiomicrospira* streamers only in the winter months when the channels are covered in snow, trapping gases, while in the summer months, the streamers disappear (Niederberger et al., 2009). Sulfur-oxidizing bacteria are also found in other sulfur-rich springs such as those found on Svalbard, as well as Blood Falls in the Antarctic, where *Thiomicrospira* is the dominant species (Mikucki and Priscu, 2007).

Similar to springs, polar lakes are important cryohabitats for microorganisms due to availability of liquid water. Lake Untersee is a permanently ice-covered, alkaline pH (10.4), oxygen-supersaturated freshwater lake, which contains pinnacle and conical stromatolitic microbial mats which can reach up to 0.5 m from the lake floor. The two stromatolite morphotypes are composed of different assemblages of cyanobacteria, though it is uncertain how exactly the species composition affects morphology of these structures as no extensive molecular survey has been carried out to date (Andersen et al., 2011).

Ace Lake, a meromictic saline lake (up to 4.3 % salinity) in the Antarctic, has been the target of metaproteomic and metagenomic work. Microbial diversity was found to be high in both the upper and lower layers of the lake, but low within the oxycline. Green sulfur bacteria (GSB), important for global sulfur and carbon cycles, were determined to be the dominant species at the O₂-H₂S interface. Consistent with the marine origin of the lake, the microbial composition of the surface layer was found similar to that of surface marine waters, but with lower overall species richness, implying little microbial input from foreign airborne bacteria (Ng et al., 2010; Lauro et al., 2011).

While community composition is now being described in greater detail, another by-product of cheaper sequencing costs is the accessibility of the genetic functional potential in conjunction with taxonomy of microorganisms in cryoenvironments through metagenomic analysis. Metagenomes have recently been completed for cryoenvironments such as glacial ice (Simon et al., 2009), cold saline lakes (Lauro et al., 2011), and permafrost (Yergeau et al., 2010; Mackelprang et al., 2011). In the permafrost metagenome, genes associated with the processes of methane cycling, nitrogen cycling, and carbon decomposition were identified (Yergeau et al., 2010). A glacial ice metagenome found a higher number of genes related to coping with oxidative stress and cold, and also assimilation of C1 substrates, including a higher abundance of genes involved in methane metabolism compared to other metagenomes (Simon et al., 2009). Similar analyses of the genetic functional potential of cryoenvironments have been achieved through amplification of functional genes (Bell et al., 2011; Barbier et al., 2012) or through use of functional gene microarrays (Yergeau et al., 2008; Hinsä-Leasure et al., 2010). As more deep sequencing and metagenomic datasets from cryoenvironments become available, comparative analysis should determine which functional genes and metabolisms are important for these unique cold environments.

4. Cryoenvironments, More Than Biological Freezers: Subzero Microbial Activity

The preservative properties of cold environments are well established; for example, “freezing” of bacterial isolates in glycerol at -80 °C is a routine method for storing bacteria in the laboratory. A fundamental question that remains, however, is whether the microorganisms identified in cryoenvironments are actually active or viable in situ. For example, the potential for cryopreservation of nucleic acids in cold, dry environments makes it especially difficult to differentiate between active, dormant, and dead populations based on molecular surveys (Willerslev et al., 2004; Ah Tow and Cowan, 2005). The use of treatments such as propidium monoazide (PMA) on environmental samples prior to DNA extraction allows only DNA from cells with intact cell membranes to be available for downstream enzymatic reactions, permitting identification of viable microorganisms and their functional genes (Yergeau et al., 2010). There is, however, a growing body of

evidence that indicates that cryoenvironments are more than natural microbial freezers, sustaining an actively metabolizing population of bacteria. Work on both bulk environmental samples and on microbial isolates in the lab shows measurable microbial metabolism at subzero temperatures (Tables 2 and 3). Additionally, the finding of anomalous CO₂, N₂O, and CH₄ gas concentrations and isotopic compositions in ice environments is indicative of active cryophilic life in situ.

4.1. MEASURING ACTIVITY FROM BULK ENVIRONMENTAL SAMPLES

Activity assays performed on bulk environmental samples have the advantage of targeting whole sample communities without the bias of working on specific isolated strains and by preserving, at least in some part, the community integrity of the original sample. Rivkina et al. (2000) assayed activity on permafrost samples using ¹⁴C-acetate to show respiration by native microbial populations down to -20 °C. Similar techniques have since been used to assess subzero activity on other permafrost samples, as well as cold-spring sediments, and reports of active microbial members in cryoenvironments have become numerous (Steven et al., 2006, 2007b; Lay et al., 2012). However, the use of specific substrates (e.g., ¹⁴C-acetate, ¹⁴C-glucose) to assess activity is limited to the capability of the microbial community to metabolize such compounds and also constitutes a relatively selective method. As such, failed attempts to detect measurable amounts of mineralization in permafrost-associated ice environments (i.e., ice wedges and massive ground ice) cannot rule out the possibility of active indigenous communities (Wilhelm et al., 2012).

Compared to glacial and permafrost ice environments, increased microbial activity has been observed in sea ice. Subzero activity in summer sea ice has in fact been known since the 1970s (Deming, 2010). The habitability of the more extreme wintertime Arctic sea ice, however, was only recently shown. By combining microscopy with CTC (5-cyano-2,3-ditolyl tetrazolium chloride) respiration experiments, Junge et al. (2004) exposed how sea ice bacteria and archaea not only populate highly concentrated brines in winter sea ice but are likely metabolically active in situ in liquid veins with salinities of 20 % at -20 °C. Despite many investigations of subzero activity in cryoenvironments, no clear demonstration to date of microbial activity on environmental samples has been reported below -20 °C, a temperature that has been proposed by some to constitute a practical limit for cell division and below which metabolism evidences are still scarce (Beaty et al., 2006; Bakermans, 2008).

4.2. IN SITU MEASUREMENTS OF MICROBIAL RESPIRATION

Independent of laboratory experiments, measurements of putatively microbially produced gases can serve as direct proxies for biological activity in situ without

Table 2. Microbial activity seen in environmental samples at subzero temperatures.

Environment	Temp (min)	Activity	References
Siberian permafrost	-20 °C	Incorporation of ¹⁴ C acetate into lipids	Rivkina et al. (2000)
Arctic permafrost	-15 °C	Microbial respiration of ¹⁴ C acetate	Steven et al. (2008)
Siberian permafrost	-16.5 °C	Methanogenesis	Rivkina et al. (2004)
Arctic ice wedge	-10 °C	No microbial respiration of ¹⁴ C acetate, in situ CO ₂ flux detected	Wilhelm et al. (2012)
Yukon ice wedge		Occluded CO ₂ and O ₂ , isotopic signatures	Lacelle et al. (2011)
Massive ground ice	-15 °C	No microbial respiration of ¹⁴ C acetate	Steven et al. (2008)
Basal ice, Greenland ice core	-9 °C	Methanogenesis inferred from elevated CH ₄ concentrations and isotopic signatures	Tung et al. (2006) and Miteva et al. (2009)
Arctic wintertime sea ice	-20 °C	CTC reduction	Junge et al. (2004)
Vostok ice	-40 °C	Occluded N ₂ O gases, isotopic signatures	Sowers (2001), Miteva et al. (2007)
Lost Hammer spring (~25 % salinity)	-20 °C	Mineralization of ¹⁴ C-labeled acetate	Steven et al. (2007b) and Lay et al. (2012)
Gypsum Hill springs (7.5–7.9 % salinity)	-0.5 °C	In situ methanogenesis detected	Perreault et al. (2008)

CTC 5-cyano-2,3-ditolyl tetrazolium chloride.

Table 3. Isolated microorganisms capable of subzero growth or metabolism.

Isolate (source)	Salt tolerance (% NaCl)	Temp.	Known adaptations and research interests	References
<i>Acetobacterium</i> sp. Ls1 (Lake Fryxell sediment, McMurdo Dry Valleys)	3.6 %	-2.5 °C ^c	Subzero acetogenesis	Sattley and Madigan (2007)
<i>Methanococoides burtonii</i> ^a (Ace Lake, Antarctica)		-2.5 °C ^b	EPS Oxidative stress proteins Subzero methanogenesis Genome and proteome completed	Allen et al. (2009), Franzmann et al. (1992) and Williams et al. (2011)
<i>Planococcus haloeryophilus</i> ^a (Active layer, Canadian High Arctic)	19 %	-15 °C ^b	EPS production Osmolyte uptake genes Osmolyte production (e.g., glycine betaine) Lower acidic residues Encrustation of cell at cold temperature	Mykytczuk et al. (2011), 20112
<i>Psychrobacter arcticus</i> ^a (Siberian permafrost)	10 %	-10 °C ^b	Less proline, arginine residues, and acidic amino acids Cold-shock proteins First genome sequenced From a terrestrial psychrophilic bacteria	Bakermans et al. (2006) and Ayala-del-Río et al. (2010)

<i>Psychrobacter cryohalolentis</i> ^a (Siberian permafrost)	10 %	-80 °C ^c	Increased ATP levels upon freezing	Amato and Christner (2009)
<i>Psychromonas ingrahamii</i> ^b (Arctic sea ice)	10 %	-12 °C ^b	Less hydrophobic proteins EPS production Osmotic pressure regulation Lowest temperature growth has been observed	Breezee et al. (2004) and Riley et al. (2008)
<i>Nitrosomonas cryotolerans</i> (Vostok ice core)		-32 °C ^c	Ammonia oxidation of ¹⁵ NH ₄	Miteva et al. (2007)
<i>Psychrobacter</i> sp. <i>Arthrobacter</i> sp. (Glacial ice isolates)		-15 °C ^c	Incorporation of ³ H precursors into DNA and protein	Christner (2002)
<i>Paenispinosarcina</i> sp. <i>Chryseobacterium</i> sp. (Antarctic glacial ice isolates)		-33 °C ^c	Microbial respiration of ¹⁴ C acetate, CTC reduction	Bakermans and Skidmore (2011a)

^aGenome sequenced.

^bLowest temperature growth observed.

^cLowest temperature microbial activity observed.

the caveats of additional manipulations. Several anomalous CO₂, N₂O, and CH₄ gas concentrations and isotopic signatures have been reported in cryoenvironment studies, hinting at cryophilic life metabolizing in situ. For example, the bottom of the Greenland ice sheet has been found to contain elevated concentrations of CH₄ and CO₂ (Tung et al., 2006; Miteva et al., 2009). The findings of high numbers of cells attached on glacial clay minerals at these measured depths (Table 1), in concert with *F*₄₂₀ autofluorescence imaging (as a proxy for methanogenesis), revealed that at least some of the reported cells are active in situ (Tung et al., 2006). Methane concentrations four orders of magnitude higher in silty glacial ice than in clear ice samples, alongside with CH₄ isotopic compositions, were also indicative of biogenic CH₄ production (Miteva et al., 2009). Similarly, isotopic compositions of the occluded gases O₂ and CO₂ found in ice wedges from the Yukon suggested microbial respiration by heterotrophic bacteria in situ (Lacelle et al., 2011).

To date, the report of anomalous N₂O concentrations and isotopic ratios consistent with biogenic production within Vostok glacial ice (Antarctica) perhaps represents the most extreme case of possible activity in ice at an in situ temperature of approximately -40 °C (Sowers, 2001; Miteva et al., 2007). Nitrification in ice has since been described in both pure culture experiments as low as -32 °C (Miteva et al., 2007) and implied in other glacial environments to potentially occur at -40 °C (Rohde et al., 2008). While trapped gas measurements are suggestive of in situ metabolism, they do not indicate when or on what timescale these gases may have accumulated. Measurements of CO₂ flux have been used to detect putative microbial respiration in polygon and trough surfaces in Canadian High Arctic permafrost. A net CO₂ flux was observed at significantly higher levels above atmospheric values, with ambient surface soil temperatures of -9 °C and underlying permafrost temperatures of -16 °C (Wilhelm et al., 2012).

4.3. PUSHING THE LIMITS FOR LIFE: ARTIFICIAL FREEZING OF PSYCHROPHILES

Work on isolated strains has rendered it possible to look at cryophilic life under controlled laboratory settings, allowing us to probe the biological limits to cold and gain insights on responses and adaptations to cryo-related stresses. The lowest recorded temperature for microbial division has recently been pushed down to -15 °C in *Planococcus halocryophilus* OR1, 3 °C below the previously held record of *Psychromonas ingrahamii* (see Table 3) (Breezee et al., 2004; Mykytczuk et al., 2012, 2013). Experiments on cryophilic isolates have also succeeded in expanding our view of subzero microbial activity. Recent work on ice isolates incubated in frozen M9 medium, for example, showed that viable cells were respiring down to -33 °C (Bakermans and Skidmore, 2011a). The same research group compared how a liquid brine media of comparable salinity to that of the liquid veins in frozen M9 media affected microbial growth at -5 °C. Diminished activity in ice veins as opposed to brine was observed (Bakermans and Skidmore, 2011b), a trend that

has also been observed by others at lower temperatures (Amato and Christner, 2009). These findings suggest that the osmotic stress caused by the concentration of solutes during freezing is not the sole constraint to microbial activity in ice.

Extreme subfreezing incubations, down to -80 and -196 °C (in liquid nitrogen), have also been performed on permafrost and marine isolates (Junge et al., 2006; Amato and Christner, 2009). Though true that such extremely low temperatures are generally not found on Earth, these are relevant to astrobiological research on other planetary bodies and our understanding of how life may cope with such low temperatures. Interestingly, both studies provided evidence of activity, yet only for relatively short incubation periods (less than a day), followed by virtually inactive states, reflecting probable cold-shock responses and entries into dormancy.

5. Microbial Adaptations to Cold

Whole genome sequencing, transcriptomic, and proteomic work in recent years is revealing several trends into how microorganisms cope with cold environments at the molecular level. To date, at least 47 genomes from cold-adapted bacteria have been sequenced (Bakermans et al., 2012), though only a small proportion of these isolates are capable of subzero growth. Extensive proteomic and transcriptomic work has been carried out on a few model organisms, primarily *Psychrobacter cryohalolentis*, *Psychrobacter arcticus*, and *Methanococcoides burtonii* (Formisano et al., 2004; Allen et al., 2009; Bakermans et al., 2009, 2012; Ayala-del-Río et al., 2010). Reviews on cold-adaptation mechanisms identified in microorganism have been discussed extensively elsewhere (Bakermans et al., 2009; Qiu et al., 2009; Casanueva et al., 2010). Molecular adaptations to cold environments can be grouped into adaptations which protect the cell from freezing, increase membrane fluidity, preserve enzymatic function, maintain essential cellular functions such as transcription and translation, and protect against reactive oxygen species (ROS) due to increased oxygen solubility at low temperatures. In addition, cold-adapted microorganisms have been found to decrease energy metabolism or go into a state of dormancy to resist cellular damage, adaptations which promote long-term survival in permanently cold environments (Bakermans et al., 2009; Casanueva et al., 2010).

Because the main source of liquid water in subzero environments results primarily from freezing-point depression caused by increased solute and salt concentrations, many of the microbial adaptations to cold include mechanisms to cope with osmotic stress (Chin et al., 2010). As a result, a high number of the culturable microorganisms isolated from cryoenvironments are observed to be halotolerant or halophiles. For example, 33 % of the culturable organisms isolated from an ice wedge were found to be tolerant to 5 % NaCl (Wilhelm et al., 2012). All isolates from a permafrost sample, and 32 % of the culturable isolates from the overlying active layer, were similarly halotolerant (Steven et al., 2008).

Isolated from an active layer sample from the Canadian High Arctic, *Planococcus halocryophilus* strain OR1 is capable of growth in up to 19 % NaCl and in temperatures as low as $-15\text{ }^{\circ}\text{C}$, the coldest temperature recorded yet for an isolated microorganism (Mykytczuk et al., 2011, 2012). Some of the cold-adapted traits of *P. halocryophilus* strain OR1 include a large amount of genes associated with extracellular polysaccharide substances (EPS) production and a large genetic redundancy in genes involved with osmolyte uptake and synthesis (e.g., glycine betaine) (Mykytczuk et al., 2011). The production of EPS is a commonly observed adaptation to low temperature by microorganisms (see Table 3). EPS production has been found to counteract the effects of ice-crystal formation and increase brine salinity and has been found to be a more effective cryoprotectant for cells frozen at $-80\text{ }^{\circ}\text{C}$ than glycerol when scaled to equivalent carbon (Marx et al., 2009). Increased compatible solutes uptake (e.g., glycine betaine, choline, glycerol, trehalose, mannitol), antifreeze proteins, and ice-binding proteins (IBPs) can lower the freezing point of the cytoplasm as well as prevent ice-crystal formation (Casanueva et al., 2010).

Membrane fluidity can be maintained at cold temperatures by increasing unsaturated lipids, decreasing branched lipids, shortening acyl chain length, and altering polar head groups (Bakermans et al., 2009). Protein function may be preserved in a number of ways that increase flexibility by increasing molecular entropy. This destabilization in protein structure can occur by reducing hydrogen bonds and salt bridges, decreasing acidic and hydrophobic amino acid residues, decreasing proline and arginine content, and increasing solvent-exposed hydrophobic residues (reviewed in Casanueva et al., 2010). The increased production of cold-shock proteins and chaperones, which assist in protein folding as well as maintain transcriptional and translational function, has been observed in several proteomic studies of cold-adapted bacteria (Mykytczuk et al., 2011; Piette et al., 2011).

As proteomic, whole genome, transcriptome, and metagenome sequencing efforts on cold-adapted strains and cryoenvironments increase, we may begin to answer questions about which adaptations are common to all cold-adapted microorganisms, which are common to a specific cryoenvironment, and which are shared by a particular type or family of microorganisms (Bakermans et al., 2009).

6. Astrobiology Implications of Terrestrial Cryoenvironments

The primary targets for astrobiology investigations of other solar system bodies are Mars, in the short term, as well as Jupiter's moon Europa and Saturn's moon Enceladus, in the mid to longer term. Extremely cold temperatures characterize these targets, and in this respect, polar cryoenvironments, especially briny subzero habitats, arguably offer the best terrestrial analog sites that resemble conditions known or suspected to exist on these worlds. With average surface temperatures of $\sim -190\text{ }^{\circ}\text{C}$ on Enceladus, $-160\text{ }^{\circ}\text{C}$ on Europa, and $-60\text{ }^{\circ}\text{C}$ on Mars (with lows

of $-130\text{ }^{\circ}\text{C}$ and highs of $20\text{ }^{\circ}\text{C}$), the habitability of extant or extinct life forms on these planetary bodies would be constrained by liquid water availability, similar to earthly cryoenvironments.

The discovery of plumes of salty water vapor and ice particles emerging from warm fractures (the “tiger stripes”) in the south pole region of Saturn’s small, icy, moon Enceladus has been a highlight of the ongoing Cassini Mission (Hansen et al., 2006). The plumes were recently shown to contain simple organic compounds, ammonium, and methane and most likely originate from a subsurface saltwater reservoir (Postberg et al., 2011), potentially making Enceladus the most habitable environment within the solar system after the Earth (Kerr, 2011). On Europa, there is a compelling evidence that below the $\sim 20\text{--}40\text{ km}$ ice surface, there is a salty liquid water ocean kept warm by tidal forces (Pappalardo et al., 1999; Kivelson et al., 2000); a recent study also inferred the putative presence of shallow ($\sim 3\text{ km}$) subsurface water lenses of sizes comparable to North American Great Lakes underlying Europa’s ice surface (Schmidt et al., 2011). Life could exist in these under-ice waters, perhaps subsisting in an environment similar to Earth’s deep-ocean hydrothermal vents or the subglacial lakes found under the Antarctic ice cap such as Lake Vostok.

Intriguing has been the discovery of “contemporary gully activity” on Mars which is found on numerous impact crater slopes and which forms and grows in warm seasons (late spring to early fall) and fades or vanishes in cold seasons (Malin et al., 2006; McEwen et al., 2011). Liquid brines near the surface might explain this activity as the presence of salts is found to be widespread on the surface of Mars (Davila et al., 2010; Osterloo et al., 2010), yet, the exact mechanism and source of water are not understood (McEwen et al., 2011). Geomorphological evidence indicates that Mars had liquid water in its past: thermokarst lakes (Soare et al., 2012), springlike structures (Allen and Oehler, 2008; Rossi et al., 2008), hydrated minerals (silicates and sulfates) (Gendrin et al., 2005; Mustard et al., 2008), and deltas and alluvial fans preserved on the surface of Mars are signs that water once flowed on the Martian landscape (Kraal et al., 2008) and, thus, could have been a potential abode for past microbial life.

The Phoenix Lander also surprisingly detected perchlorate ($\sim 1\%$) in Martian permafrost soils (Hecht et al., 2009). Perchlorates (ClO_4^-) are highly soluble salts with low eutectic temperatures which can act as freezing-point depressants, creating subzero salty liquid habitats within frozen permafrost. As an example, a saturated solution of $\text{Mg}(\text{ClO}_4)_2$ has a freezing point of $-67\text{ }^{\circ}\text{C}$, within the range of the diurnal temperature cycle of the Phoenix landing site in the summer (Rennó et al., 2009; Catling et al., 2010; Stoker et al., 2010). Perchlorates can also act as electron acceptors, allowing anaerobic microbial respiration to occur where perchlorate replaces oxygen as the terminal electron acceptor (Coates and Achenbach, 2004). Approximately 30 bacterial strains capable of growth in up to 12% $\text{Mg}(\text{ClO}_4)_2$ have been isolated from the Canadian High Arctic, although anaerobic growth at cold temperatures using perchlorates as terminal acceptor has yet to be shown (Whyte, unpublished data, 2013).

While the cold and dry Martian surface environment is considered inhospitable to microbial life, subsurface permafrost environments are extensive on Mars and are considered to be primary astrobiology targets where life could have survived. The discovery of polygonal terrain on Mars underlain by ice, confirmed at the Phoenix landing site, heightens interest in the possibility that this water-bearing habitat may be, or may have been, a suitable habitat for extant life (Smith et al., 2009). This possibility is supported by the detection of active microbial communities in subsurface permafrost environments, such as ice wedges found beneath tundra polygon features on Earth (Wilhelm et al., 2012). Interestingly, approximately 20 % of microorganisms that we have isolated from high Arctic permafrost environments are capable of subzero growth and all of these organisms are salt tolerant (Whyte, unpublished data, 2013).

The discovery of Mumma et al. (2009), that the 10 ppb methane reported in the Martian atmosphere (Formisano et al., 2004) may originate from localized “hotspots” or “plumes” of methane arising from the frozen terrestrial Martian surface bears important astrobiological significance but is still under extensive debate (Lefevre and Forget, 2009; Zahnle et al., 2011). The origin of the Martian methane could be attributable to either abiotic (Keppler et al., 2012) or biological sources, the latter including methanogenesis by microbial communities inhabiting the Martian subsurface. On Earth, ~90–95 % of methane is biological in origin with ~65 % directly produced through the activity of methanogenic archaea that can generate methane under anaerobic conditions (Conrad, 2009). The methanogen detected in diverse cold and saline environments illustrate that this group of archaea may be well suited to survival in extreme environments and could also be a source of methane on Mars by halophilic methanogens (Potter et al., 2009; Ulrich et al., 2012). A unique terrestrial analog for potential methane ecosystems in cryoenvironments can be found at the high Arctic spring Lost Hammer, which provides a model of how a methane seep can form in a hypersaline, cryoenvironment characterized by thick permafrost (Niederberger et al., 2010). Although isotopic analyses of the gas (~50 % methane) emitted from the Lost Hammer spring were consistent with a thermogenic, rather than biogenic, origin of methane, the methane itself can act as an energy and carbon source for sustaining anaerobic-methane-oxidation metabolism (Niederberger et al., 2010). The presence of anaerobic-methane oxidizers reported in the Lost Hammer outlet sediments illustrates the possibility that methane emanating from the frozen subsurface can support viable microbial metabolism within these extreme environmental constraints.

7. Conclusion

Once thought dead or hostile to life, Earth cryoenvironments are now recognized as active microbial habitats most likely supporting in situ metabolism. Evidence also supports a strong correlation between cryophilic life and salt- or osmotolerance,

given that freezing-point depression by the concentration of solutes constitutes the main process that maintains liquid water at subzero temperatures and that most, if not all, known cryophilic microorganisms characterized to date are salt tolerant. Overall, these findings further underline how microbial life could inhabit subzero briny extraterrestrial environments existing on Mars, Europa, and Enceladus. The microorganisms that could survive and potentially remain viable under such growth conditions would most likely be halophilic cryophiles, highlighting both the importance of understanding microbial life in cryophilic salty environments on Earth and the significance of identifying, characterizing, and targeting salty features on these solar system bodies for life detection.

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MICROBIAL DIVERSITY AND ENZYMES IN IKAITE COLUMNS: A COLD AND ALKALINE ENVIRONMENT IN GREENLAND

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1. Introduction

A very unusual alkaline and cold natural environment is found in the southwestern part of Greenland (61°11'N; 48°01'W), the ikaite columns of the Ikka Fjord (Fig. 1). The columns constitute a cold (4 °C), alkaline (pH 10.4), and low-salinity (0.9 %) environment, and they harbor a microbial community adapted to this polyextreme environment.

There are more than 600 columns of various sizes with a maximum age of ~10,000 years, although most of them are probably much younger (Seaman and Buchardt, 2006). Being visible from the water surface, the ikaite columns were known to the early settlers in Greenland and have been mentioned as far back as in old Inuit legends (Krogh, 1982; Rink, 1866). This fascinating environment was declared an officially protected area by the Government of Greenland in 2000 (Seaman and Buchardt, 2006).

Geological investigations of the columns were initiated in 1962 (Pauly, 1963) and resumed in 1995, whereas scientific analyses of the microbial community living in this extreme environment have been carried out since 2002. Focus has been on diversity studies and description of novel bacterial species as well as on the biotechnological potential of enzymes adapted to this environment (Schmidt and Stougaard, 2010; Schmidt et al., 2006a, b, 2007, 2010, 2012; Stougaard et al., 2002).

In this chapter, research on the microbial diversity in this polyextreme environment will be reviewed.

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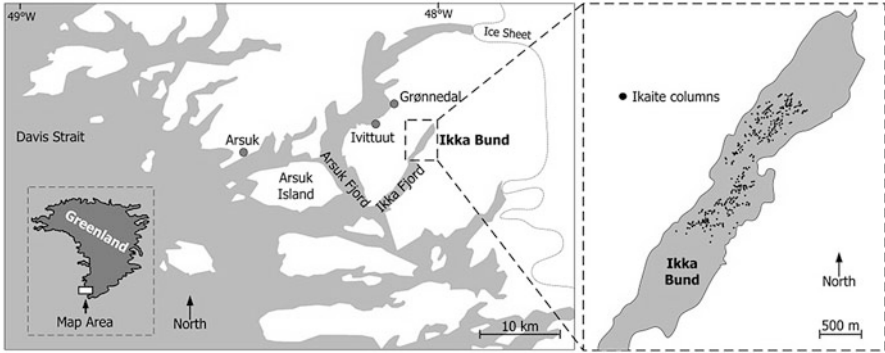


Figure 1. Location of the Ikka Fjord in southwest Greenland and the ikaite columns in Ikka Bund (Redrawn and modified from Seaman and Buchardt, 2006).

2. Geochemical Characteristics

2.1. THE IKKA FJORD

The Ikka Fjord was formed around 8,000 B.C. by transgression of seawater and deglaciation (Seaman and Buchardt, 2006; Kelly, 1977). It is a glacial valley surrounded by steep, 500 m high mountains dominated by lower Proterozoic (Ketilidian) gneisses. The inner fjord is cut in a NW-SE trending belt approximately 3 km wide by alkaline magma intrusions belonging to the Grønnedal-Ika igneous complex (Emeaus, 1964; Allaart, 1976). The Ikka Fjord is 13 km long, 170 m deep, up to 1.6 km wide, and characterized by two distinct parts: a deep outer fjord and a shallow inner fjord area called Ikka Bund. Between the two areas is a shallow sill called “Snævringen” (The Narrowing) that prevents most of the larger icebergs from the Davis Strait and the outer Ikka Fjord from entering Ikka Bund. The ikaite columns are located in Ikka Bund, and the area has been described as Ikka Column Garden (Fig. 1) (Buchardt et al., 1997, 2001; Seaman and Buchardt, 2006).

The Ikka Bund is 30 m deep and the uppermost 1–2 m is composed of freshwater runoff. Below the freshwater layer, the water is marine with a salinity of 33‰ and a temperature permanently below 6 °C. In contrast, the freshwater above the halocline can reach a temperature of 12 °C (Buchardt et al., 2001). From November to May, frozen freshwater covers the Ikka Fjord (Seaman and Buchardt, 2006).

2.2 THE IKAITE COLUMNS

In an area of 0.5 × 2.5 km in the Ikka Bund, more than 600 individual ikaite columns can be found in different shapes and sizes. Column heights vary from a few cm to more than 18 m and cross sections of up to 15 m have been reported

Table 1. Characteristics of seep water from the ikaite columns and sea water of the Ikka Fjord.

Component	Seep water	Sea water
Conductivity (mS cm ⁻¹)	18.2	42.5
Salinity (‰)	9.3	31.1
Temperature (°C)	4.0	3.6
pH	10.4	8.1
Na ⁺ (mmol L ⁻¹)	198	413
K ⁺ (mmol L ⁻¹)	1.9	9.5
Ca ²⁺ (mmol L ⁻¹)	0.17	8.9
Mg ²⁺ (mmol L ⁻¹)	1.7	45.7
Cl ⁻ (mmol L ⁻¹)	21.2	506
SO ₄ ²⁻ (mmol L ⁻¹)	2.8	28.6
PO ₄ ³⁻ (mmol L ⁻¹)	0.26	Below detection
Alkalinity (mmol L ⁻¹)	153	<0.5

Adapted from Buchardt et al. (1997).

(Buchardt et al., 1997, 2001; Seaman and Buchardt, 2006). Some columns appear as great towers only limited by the halocline, whereas others seem almost needle-like and very fragile. The term “unique” has been used to describe the columns, largely due to the geological factors that allow the columns to exist (Seaman and Buchardt, 2006).

The major mineral in the columns was named ikaite by the discoverer Hans Pauly in 1963 (Pauly, 1963). Ikaite is calcium carbonate hexahydrate (CaCO₃·6H₂O) a metastable cold-water mineral that forms when alkaline groundwater rich in carbonate ions mixes with calcium-rich seawater in the Ikka Bund (Table 1). Formation of ikaite is closely related to the Grønnedal-Ika complex as the alkalinity of the groundwater is proposed to originate from dissolution of secondary sodium carbonate minerals in the intrusion. This theory was supported by ¹⁴C analyses of newly precipitated ikaite that showed high contribution of inorganic carbon from the Precambrian intrusive carbonatitic rocks (Buchardt et al., 2001; Seaman and Buchardt, 2006). Phosphate from the carbonatite also dissolves in the precipitate that runs from the top of the intrusion to the ground beneath the Ikka Bund. High hydraulic head forces the water out under the Ikka Fjord, resulting in submarine springs penetrating an impermeable glaciomarine clay layer under the fjord bottom. Ikaite has a solubility of one to two orders of magnitude larger than that of calcite and aragonite, which are more common carbonate minerals. However, ikaite growth is favored by the low temperature in the fjord (2–6 °C) and the high phosphate concentration in the springwater, which inhibits the nucleation of calcite and aragonite (Bischoff et al., 1993; Brooks et al., 1950). Ikaite will decompose into calcite and water within hours when kept at room temperature (Seaman and Buchardt, 2006; Pauly, 1963).

The columns have been observed to grow directly from the mud that covers the flat areas of the fjord bed with upward growth at the tip of the columns (Buchardt et al., 2001; Seaman and Buchardt, 2006). Vertical growth is facilitated by buoyancy of the springwater due to the lower density compared to seawater in Ikka Bund, which has a salinity of up to 33‰ (Seaman and Buchardt, 2006). Furthermore, growth is promoted by the permeable framework of the monoclinic ikaite crystals, which ensure that the columns act as conduits for the springwater (Buchardt et al., 1997; Seaman and Buchardt, 2006). Buchardt and coworkers (2001) investigated the growth rate of the columns and found the increase in height to be 25–50 cm year⁻¹ for a column with 15 cm diameter and estimated porosity of 50 %. Column mergers have been observed when columns grow adjacent to each other and damage to the columns due to boring organisms or mechanical influence initiates ikaite growth at the point of damage resulting in a large diversity of column shapes (Buchardt et al., 2001).

3. Biological Diversity Covering the Ikaite Columns and in the Ikka Fjord

The ikaite columns have a rich fauna of marine eukaryotic organisms living on the outside, which give the columns an appearance resembling coral reefs found in warmer waters (Fig. 2). This fauna is dominant on older columns, whereas newly formed ikaite columns as well as active growth zones show no or little attachment of biota (Dahl and Buchardt, 2006). The eukaryotic organisms have been studied and described in detail by Thorbjorn and Petersen (2003), and a few examples will be highlighted in this section.

The organisms found on the ikaite columns are similar to organisms found on offshore fishing banks, wave beaten shores, and narrow channels, and the



Figure 2. Sea urchins, sea anemones, coralline red algae, ascidians, mussels, and several other species inhabit the outside of the ikaite columns (Photos: Richard Martin and Jesper Kikkenborg).

environment is sheltered and without any high-energy water movement. In general, diversity increases when moving inwards in the fjord and with increasing depth (Thorbjorn and Petersen, 2003). Several of the organisms found on the columns and in the Ikka Fjord have been reported as new to this part of Greenland, including polychaetes, ascidians, and a copepod parasite (Thorbjorn and Petersen, 2003). Two of the most abundant species found encrusting the ikaite columns are the coralline red algae *Clathromorphum* and *Lithothamnion*. Both can be found on the columns all the way down to the bottom, due to the good light penetration in the Ikka Fjord. Buchardt et al. (1997) suggested that they help stabilize the columns (Fig. 2).

3.1. EUKARYOTES AND ARCHAEA INSIDE THE COLUMNS

So far, only a small number of eukaryotic organisms have been identified in the interior of the ikaite columns. Kristiansen and Kristiansen (1999) described a new species of *Chroomonas*, *C. ikaitensis*, and Sørensen and Kristensen (2000) reported a new species of rotifer, *Notholca ikaitophila*, in the ikaite columns. However, DNA sequencing of 18S rRNA genes from DNA extracted from one column indicated the presence of numerous other, previously unidentified, eukaryotic species (Stougaard et al., 2002). The results suggested the presence of ascomycetes, annelid worms, diatoms, green algae, ciliates, dinoflagellates, and *Mesomycetozoa*, although all with low similarity to known sequences in databases (<80–96 %). All attempts to amplify archaeal 16S rRNA gene sequences have failed, suggesting a limited presence of Archaea in the ikaite columns. These analyses were conducted on new material from the top of columns, thereby likely only identifying a fraction of the total diversity (Stougaard et al., 2002).

4. Microbial Diversity Associated with the Ikaite Columns

The microbial community inhabiting the cold and alkaline environment of the ikaite columns has been studied for more than a decade. So far three new bacterial species and genera have been described (Schmidt et al., 2006b, 2007, 2012). Both cultivation-dependent and cultivation-independent methods have been applied to establish information on the bacteria adapted to this rare polyextreme environment.

4.1. TOTAL BACTERIAL DIVERSITY

Phylogenetic analyses based on the 16S rRNA gene were carried out on DNA isolated directly from the ikaite columns. These analyses showed that ~50 % of the phylotypes are similar (90–99 %) to known meso- or thermophilic alkaliphiles,

Table 2. Bacterial phyla present inside ikaite columns analyzed by pyrosequencing of the 16S rRNA gene.

Phylum	Reads	%
Proteobacteria	4,067	57
Cyanobacteria	899	13
Firmicutes	599	8
Actinobacteria	391	5
Bacteroidetes	368	5
Others	36	1
Unclassified bacteria	768	11
Total	7,128	100

whereas the remaining ~50 % showed less than 90 % identity when compared to available 16S rRNA gene sequences (Stougaard et al., 2002). Furthermore, Schmidt et al. (2006a) established a 16S rRNA gene library where 33 % of the clones showed less than 97 % identity to known sequences, suggesting that these may represent new species. It is therefore reasonable to assume that the alkaline, cold, and low-saline environment of the ikaite columns harbor a microbial community rich in novel species.

Recently, a metagenomic analysis of the total bacterial diversity on a pool of six different ikaite columns was conducted by pyrosequencing of the V3 and V4 hypervariable regions of the 16S rRNA gene. The RDP database was used to assign phylogenetic relationships (<http://rdp.cme.msu.edu>), and the analysis revealed that the most dominant phyla were Proteobacteria (57 %), Cyanobacteria (13 %), Firmicutes (8 %), Actinobacteria (5 %), and Bacteroidetes (5 %) (Table 2) (unpublished data).

Among the Proteobacteria, Beta- (44 %) and Alphaproteobacteria (31 %) were the dominant groups followed by Gammaproteobacteria (12 %). The pyrosequencing results, which show a dominance of Proteobacteria, are in agreement with earlier investigations of cultured isolates and 16S rRNA gene libraries (Stougaard et al., 2002; Schmidt et al., 2006a).

Terminal-restriction fragment length polymorphism (T-RFLP) analysis on three different ikaite columns indicated that each column harbors a distinct microbial community (Schmidt et al., 2006a). The community structure within a column also differs at varying depths, a difference not caused by the presence of other bacteria, but by a varying local abundance of individual species. These results were obtained on material collected in 2002 (Schmidt et al., 2006a) and confirmed on material collected in 2006 (Pedersen, 2007).

Alkaliphilic bacteria from the ikaite columns have been shown to be unique to the columns and not present in the surrounding sea water (Aarup, 2006; Schmidt et al., 2006a; Pedersen, 2007).

4.2. CULTURED BACTERIA

The first attempts to culture bacteria from ikaite columns were performed by Stougaard et al. (2002). Working on material that had been stored at $-18\text{ }^{\circ}\text{C}$ for several years, they were able to cultivate ten different isolates with three phylogenetic affiliations: Firmicutes (6), Proteobacteria (3), and Bacteroidetes (1). All isolates were able to grow at $5\text{ }^{\circ}\text{C}$ with an optimal growth around $13\text{ }^{\circ}\text{C}$, and three of the isolates had optimal growth at high pH (9–10).

Using ikaite material that had been preserved at $5\text{ }^{\circ}\text{C}$ or at $-20\text{ }^{\circ}\text{C}$ in 20 % glycerol, Schmidt et al. (2006a) successfully obtained more than 200 isolates, which were further characterized with respect to pH and temperature tolerance. Five of the cultured isolates were true psychrophiles, whereas the remaining were psychrotolerant. Nine of the isolates were only able to grow at pH 10 and 15 isolates only at pH 9 and pH 10, while the majority of the isolates were able to grow at pH 8–10.

Phylogenetic analysis on 67 of the cultured isolates showed that 54 were affiliated with Proteobacteria: Gamma- (27), Alpha- (26), and Betaproteobacteria (1). Sixteen of the cultured Alphaproteobacteria isolates showed 98 % sequence identity to *Loktanella vestfoldensis*, a psychrotolerant bacterium isolated in Vestfold Hills, Antarctica (Van et al., 2004), and nine isolates showed 92–99 % sequence identity to *Rhodobaca bogoriensis*, an alkaliphilic bacterium isolated from a sodalake in Africa (Milford et al., 2000). Most of the Gammaproteobacteria belonged to the *Pseudomonadaceae* family, eight of which showed 99 % sequence identity to *Pseudomonas antarctica*, a psychrophilic bacteria from Antarctica (Reddy et al., 2004). Four of the isolates analyzed were related to the Gram-positive *Bacilli* in the phylum Firmicutes, one being 97 % identical to *Bacillus alkaliphilus*.

T-RFLP analysis demonstrated that when bacteria from ikaite material are cultivated, the diversity drops and a community of Gamma- and Alphaproteobacteria, *Bacilli*, and *Clostridium* is established. Attempts to develop cultivation media resembling ikaite conditions showed no effect in terms of increased diversity (Aarup, 2006).

As in the metagenomic analysis presented in the previous section, Proteobacteria also dominate the cultivable part of the bacteria found in the cold and alkaline ikaite columns.

4.2.1. New Species from the Ikaite Columns

Phylogenetic analyses of cultured isolates and 16S rRNA gene libraries indicated that approximately one third of the isolates may represent new species. So far, three novel species have been described.

Rhodonellum psychrophilum is a red-pigmented bacterium of the phylum Bacteroidetes and family *Flexibacteraceae* (Schmidt et al., 2006b). It is a strictly aerobic psychrophile with optimal growth at $5\text{--}10\text{ }^{\circ}\text{C}$ and a growth range from 0 to $22\text{ }^{\circ}\text{C}$. *R. psychrophilum* has a pH range from 7.5 to 10.7 and a growth optimum at 0.6 % NaCl (w/v). Based on 16S rRNA gene sequence analysis and

DNA-DNA hybridization, the closest relative is *Belliella baltica*, a pink-colored bacterium with a growth optimum at 25 °C isolated from surface water in the Baltic Sea (Brettar et al., 2004). The cell wall of *R. psychrophilum* has a very high amount of branched and unsaturated fatty acids (97 %), which increases membrane fluidity and thereby cellular activity and transport processes, a characteristic adaption for bacteria living at low temperatures and high pH (Schmidt et al., 2006b). Weak biofilm production was observed at 5 °C.

Arsukibacterium ikkense is a nonpigmented, psychrotolerant Gammaproteobacterium (Schmidt et al., 2007). The growth range is from 0 to 30 °C with an optimum at 15 °C. The pH growth range was reported from 7.5 to 10.0 with optimum at 9.2–10.0 and a salinity optimum at 3 %. The closest related species is *Rheinheimera baltica*, a blue-colored bacterium isolated from the Baltic Sea with a growth optimum at 20–25 °C (Brettar et al., 2002). Only 60 % of the total fatty acids in *A. ikkense* are branched and unsaturated, which is low compared to *R. psychrophilum*. This shows that not all bacteria present in the ikaite columns exhibit the typical features of cold adaption (Schmidt et al., 2007). *A. ikkense* show biofilm formation and alkali-stable and cold-active amylase and protease activity (Sect. 5).

Alkalilactibacillus ikkensis is a nonpigmented psychrotolerant bacterium belonging to the phylum Firmicutes (Schmidt et al., 2012). It is able to grow from 0 to 28 °C and at pH from 8.5 to at least 11.5. Based on 16S rRNA gene sequencing, fatty acid composition, and DNA-DNA hybridization analyses, the closest related species is *Halolactibacillus xiariensis*, a halophilic and moderately alkaliphilic bacterium isolated from a soda lake in Inner Mongolia (Cao et al., 2008). *A. ikkensis* was isolated based on its β -galactosidase activity (Schmidt and Stougaard, 2010; Schmidt et al., 2012), but it also shows α -amylase, β -glucuronidase, α -galactosidase, and β -1,3-glucanase activity at low temperatures (Sect. 5) (Schmidt et al., 2012).

4.3. BACTERIA FROM SIMILAR ENVIRONMENTS

Natural alkaline environments are relatively rare, and the most intensively studied are *soda lakes*. Like ikaite columns, they are very alkaline (pH 8 to >12), but unlike ikaite columns, they are highly saline and often found in temperate or subtropical regions with temperatures in the range from 20 to 44 °C (Jones et al., 1998; Duckworth et al., 1996; Grant and Heaphy, 2010). Analyses of microbial diversity in known soda lakes show that Gammaproteobacteria dominate the Gram negative, while Gram-positive bacteria are often related to *Bacillus*. Archaea are also common and are related to the genera *Natronococcus* and *Natronobacterium* (Jones et al., 1998; Duckworth et al., 1996).

More similar to ikaite columns is the Lost City Hydrothermal Field, which is alkaline (pH 9–11) and has low salinity. However, the temperature ranges from 40 to 93 °C (Schrenk et al., 2004). Analyses by Schrenk and coworkers (2004)

Table 3. Characteristics of natural environments with properties similar to ikaite columns.

Characteristics	Ikaite columns	Cabeço de Vide	The Lost City	Soda Lakes
pH	10.4	11.4	9–11	8 to >12
Temperature (°C)	2–6	20.5	40–93	20–44
Salinity	Low	Low	Low	High
Dominant bacteria	Beta-/ Alphaproteobacteria	Actinobacteridae	Gamma-/ Epsilonproteobacteria	Gammaproteo- bacteria
Archaea	N.D.	–	Yes	Yes

N.D. not detected, not investigated.

showed that the microorganisms inhabiting the carbonate columns in Lost City often are associated with dense biofilm. Total cell counts were relatively high (2.0×10^6 – 3.1×10^8 cells gdw^{-1}), but diversity was low and dominated by Archaea of the order *Methanosarcinales* (Schrenk et al., 2004). This is in contrast to ikaite columns, where Archaea have not been identified despite several attempts (Stougaard et al., 2002). Bacteria related to Proteobacteria and Firmicutes were also identified in the Lost City Hydrothermal Field (Schrenk et al., 2004).

The ground water of the Portuguese City Cabeço de Vide constitutes a natural environment with a pH of 11.4, a low salinity, and a temperature of 20.5 °C (Tiago et al., 2004). An analysis of the bacterial diversity showed that total bacterial counts were low (3.4 – 7.2×10^3 cells L^{-1}), as was the diversity of the cultivable community. The majority were associated to Actinobacteria and some to Firmicutes. In contrast to ikaite columns, where Proteobacteria are abundant, only very few Proteobacteria were observed in the groundwater of Cabeço de Vide (Tiago et al., 2004).

The results from environments with some of the characteristics of ikaite columns show that the microbial community found in ikaite columns is unique and very different from previously described environments (Table 3).

5. Enzymes

The springwater seeping through the columns is characterized by a very low content of nutrients (Bucharadt et al., 2001). Therefore, it has been hypothesized that the bacteria living in the columns grow on organic matter derived from degradation of animals, algae, and bacteria that either inhabit the columns or have been trapped during the process of ikaite column formation (Schmidt et al., 2006a, b). This theory is supported by the metagenomic analyses conducted on ikaite material, where heterotrophic bacteria are dominant.

Extracellular enzymes produced by the bacteria are the key factors in nutrient degradation outside the cell and they are, like the bacteria, well adapted to the alkaline and cold environment. Intracellular enzymes of alkaliphilic bacteria are

Table 4. Distribution of enzyme activities among 450 enzyme-producing bacterial isolates.

Enzymes	Isolates	%
β -Glucosidase or β -glucuronidase	237	53
Phosphatase	137	30
β -Galactosidase	120	27
α -Amylase	112	25
Protease	104	23
Mannanase	48	11
Cellulase	42	9
Xylanase	8	2

often adapted to the neutral or slightly alkaline pH in the cytosol (pH 8.0–9.3) (Yumoto, 2002). Cold-active and alkaline-stable enzymes have considerable application potentials in industrial and biotechnological processes, particularly in the detergent industry, and enzymes that are cold active at neutral pH may be applied to numerous processes where low operating temperatures are preferred, e.g., in food processing, for medical purposes, the chemical industry, waste treatment, and biotechnology. The large number of new bacterial species isolated from the ikaite columns implies that there is a high possibility of finding enzymes with novel properties in this environment. So far, several reports have described ikaite bacteria that produce cold-active and/or alkaline-stable extracellular enzyme activities and two cold-active enzymes, a β -galactosidase and a lipase, have been characterized in detail (Schmidt and Stougaard, 2010; Schmidt et al., 2006b, 2007, 2010, 2012).

5.1. ENZYME DIVERSITY

A collection of 672 bacterial isolates derived from ikaite material plated on R2A medium (pH 10) was screened for enzyme activity (Pedersen, 2007). Nine different enzyme activities were examined by screening on substrates linked to azurine (AZCL linked) or to 5-bromo-4-chloro-indolyl (X linked). Intracellular enzymes included β -galactosidase, β -glucosidase, β -glucuronidase, and phosphatase, and extracellular enzymes were protease, α -amylase, cellulase, xylanase, and mannanase. The results demonstrated that out of the 672 isolates, 450 (67 %) exhibited enzymatic activity on one or more substrates and up to five different enzyme activities could be produced by the same isolate. In total, 53 % of the positive isolates produced β -glucosidase or β -glucuronidase, 30 % phosphatase, 27 % β -galactosidase, 25 % α -amylase, 23 % protease, 11 % mannanase, 9 % cellulase, and 2 % produced xylanase (Table 4).

α -Amylase and cellulase producing isolates were further analyzed at different pH values ranging from pH 7 to 10. Enzyme activity associated with growth was observed: all isolates showed enzymatic activity at pH 9 and 10, whereas only a few showed activities at pH 8 and no activity was observed at pH 7.

Temperature and pH analysis on extracts containing extracellular α -amylases from four isolates showed that the enzymes were active at pH 5–10 and at temperatures ranging from 5 to 50 °C (Pedersen, 2007).

5.1.1. Enzymes of Novel Species

A total of four enzyme-producing bacterial isolates from ikaite columns have so far been described.

Schmidt et al. (2006b) characterized the psychrophilic *Rhodonellum psychrophilum* which produces a substantial range of enzymes including alkaline phosphatases, esterases, proteases, and galactosidases.

Protease, amylase, and intracellular phosphatase activity was described for the novel Gammaproteobacterium *Arsukibacterium ikkense* (Schmidt et al., 2007). Preliminary bioinformatic analyses of the genome sequence have revealed a high number of extracellular proteases and α -amylases (unpublished data).

The isolate *Alkalilactibacillus ikkensis* produces α -amylase, α -galactosidase, β -galactosidase, and β -glucuronidase (Schmidt et al., 2012). The intracellular β -galactosidase showed 57 % identity to the closest related β -galactosidase from *Bacillus megaterium* (Schmidt and Stougaard, 2010), and the amino acid composition of the protein showed features of cold-active enzymes. The number of Arg and Pro residues and the Arg/(Arg+Lys) ratio were decreased compared to *Escherichia coli* β -galactosidase, indicating fewer hydrogen and ionic bonds. The *A. ikkensis* β -galactosidase was successfully expressed in *E. coli*. The enzyme showed maximum activity at pH 8 and at 20–30 °C, with 90 % activity remaining at pH 9 and ~60 % at pH 7. The enzyme retained 60 % of the maximum activity at 0 °C, which is the highest activity reported for a recombinantly produced cold-active β -galactosidase (Schmidt and Stougaard, 2010). Furthermore, when compared to a commercially available β -galactosidase, the enzyme showed a twofold higher conversion rate at temperatures between 0 and 20 °C (Schmidt and Stougaard, 2010). The β -galactosidase of *A. ikkensis* could be irreversibly inactivated by heating to 50 °C for 5 min or 40 °C for 10 min.

An uncharacterized Gammaproteobacterium with close relationship to an uncharacterized *Nitrincola* sp. E-048 (96 % 16S rRNA gene identity) was isolated due its ability to produce lipase (Schmidt et al., 2010). A novel triacylglycerol lipase with similarity to a lipase from *Rhodoferrax ferrireducens* (51 % identity at the amino acid level) was identified. The lipase showed enzymatic activity from 5 to 80 °C. Maximum activity was observed at 55 °C, which is not typical for cold-adapted proteins. The ability to remain active at high temperatures was reflected in the amino acid composition of the protein. The lipase contained similar amounts of the polar hydrogen-binding residues (Ser, Thr, Asn, and Glu) as a thermophilic lipase produced by *Bacillus stearothermophilus* P1. However, in silico

analysis of the secondary and tertiary structure of the lipase indicated larger loop sizes, which is often associated with cold-adapted enzymes (Schmidt et al., 2010; Gianese et al., 2001; Roy and Sengupta, 2007). The pH optimum was observed at pH 8 and 70 % of the maximum activity remained at pH 9 and 40 % at pH 7. No activity was found at pH 6 or below. It was not possible to measure activity at pH 10 due to instability of the screening substrate.

6. Biofilm

The ikaite columns are rich in biofilm, which has been visualized by cryo-SEM when analyzing the ikaite crystals (Bucharadt et al., 2001). The biofilm is often associated with sedentary diatoms, known to secrete large amounts of biofilm, but bacterial production is also likely to occur, as several of the bacteria isolated from ikaite columns are confirmed producers of biofilm, including *R. psychrophilum* (Schmidt et al., 2006b) and *A. ikkense* (Schmidt et al., 2007). The biofilm has been proposed to protect the ikaite crystals and stabilize the columns (Seaman and Bucharadt, 2006) and may be involved in establishing a protective environment for the bacteria by nutrient sequestering, protection against grazers, and attachment to the columns (Jefferson, 2004; Nichols et al., 2005). The natural substrate for the β -galactosidase activity found in *A. ikkensis* could be polymers from, e.g., algal cell walls, but it is also possible that the β -galactosidase is able to degrade complex galactose-containing sugars in the biofilm, in which case the biofilm acts as a nutrient in itself (Schmidt and Stougaard, 2010).

In a preliminary study, 250 strains isolated from ikaite columns were analyzed for biofilm production (Aarup, 2006). Under the experimental conditions, 19 of the strains were categorized as excellent (11) or good (8) producers of biofilm, while the remaining showed little (96) or no (135) biofilm production. The best producers of biofilm were all closely related to *A. ikkense* as determined by 16S rRNA gene sequencing, except one, which was a Firmicutes related to the *Clostridium* family.

7. Concluding Remarks

The unique environment of the Ikka Fjord was first described in 1962, but further investigations were not initiated until 1995. Here we have reviewed the research conducted on the nature of the ikaite columns and the bacteria surviving in this polyextreme environment.

The geochemical data suggest that the Ikka columns are a unique phenomenon and phylogenetic and metagenomic analyses indicate that the bacterial diversity differs from other known alkaline environments. Three novel species have been described, and two novel enzymes with industrial potential have been

characterized. Additionally, screenings of cultured bacteria have shown a wide range of enzymatic activities. The data derived so far describes a unique biological environment with a good prospect of finding novel species and enzymes for industrial applications. Presently, more thorough metagenomic and genomic analyses are being conducted in order to establish more information on the microbial population in the ikaite columns.

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MICROBIAL COMMUNITIES THRIVING IN VARIOUS ICE ECOSYSTEMS

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1. Introduction

By definition the cryosphere is the portion of the Earth where water exists in the solid phase as snow or ice. It collectively includes vast areas of sea ice, lake and river ice, glaciers, ice sheets and caps, as well as the snow cover and frozen ground including permafrost (Fig. 1).

The Earth's biosphere is cold with 14 % being polar and 90 % (by volume) consist of cold oceans. Nearly three quarters of the Earth's freshwater occurs as ice, and a large fraction of the soil ecosystems is present as permafrost. The majority of the world's ice volume is found in Antarctica. In terms of areal extent, however, the winter snow and ice extent of the Northern Hemisphere comprises the largest area, amounting to an average of 23 % hemisphere surface in January.

All various components of the cryosphere can be considered as ecosystems which are settled by mainly microorganisms. Freshwater systems harbor cells mainly either in alternate layers of ice and slush (if there is precipitation which enables the formation of the layered structure) or in clusters of sediments which are entombed in ice. The multiple stress by desiccation, low nutrients, and cold temperatures can be compensated by solar radiation which creates a microfilm of liquid water to enable metabolism as seen in polar lakes without precipitation. The same effect can be seen in supraglacial habitats where microbial communities and also metazoa can inhabit the surface of glaciers. Sea ice is probably the best investigated ecosystem and is characterized by internal brine channels with relatively high salt concentrations where not just microbes but also metazoan life can thrive. Snow covers the Earth in large areas; also here we can observe mainly microbial communities between the boundary layers of snow crystals.

Within the cryosphere there are important linkages and coupling mechanisms generated through its influence on surface energy and moisture fluxes, precipitation, clouds, hydrology, and oceanic circulation. Through these feedback processes, the cryosphere plays a significant and integral role in climate and in the response of climate models to global change. All parts of the cryosphere contribute to short-term climate changes, with permafrost, ice shelves, and ice sheets also contributing to longer-term changes including the ice age cycles. Most of these

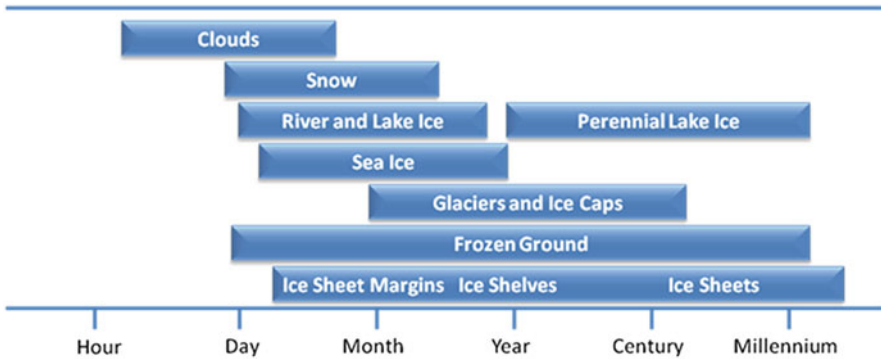


Figure 1. Various ice ecosystems of the cryosphere and their longevity (Modified after IPCC-Report ARA 4 (2007)).

components are overlapping with the hydrosphere and show different residence times ranging from hours to thousands of years. Snowfall may remain for several hours or longer, and ice covers can seal a water body for one recurrent winter season or stays perennially, given that the summer temperatures do not exceed freezing point. One water droplet, however, can remain for millions of years in the deep ice of the East Antarctic ice sheet. Hence, the longevity of these cold environments is a crucial factor also for the establishment of living communities (Fig. 1). The impacts the cryosphere exerts on the biosphere are factors like the light climate, albedo, soil humidity, hydrology, and air and water temperatures. Therefore, it has been addressed as an exclusively physical component. Moreover, due to hostile conditions such as low temperatures, strong wind, low-nutrient concentrations, and UV radiation, areas around freezing point have been seen as devoid of life. However, the general picture of being a repository for wind-transported and ice-trapped microorganisms has changed dramatically with the improvement of methods and accessibility to extreme environments. With increasing awareness that the cold regions of our planet harbor living communities, studies regarding the cryosphere are not solely restricted to physical aspects anymore but became substantially enriched by, e.g., ecological disciplines, and later on even biotechnological applications of, e.g., cold-shock proteins were of great interest.

2. Rethinking from Void of Life to a Living Cryosphere

Mankind's interest in the cryosphere has shown many facets that have largely changed with technical progress. In the early days, high-latitude and high-altitude regions, respectively, have been connected to adventurism, armchair romantics for the general public by observing alpine and polar explorers struggle with the hard conditions, or as hardship for residents in high-mountainous or high-latitude regions. Throughout history however, we find rare observations of encounters between humans and microorganisms on ice and snowy surfaces such that of

Aristotle, the Greek. The observation of Bauer (1819) about “dark snow” attracted further scientific investigations. They have been succeeded by Nansen’s (1897) observations of microbes colonizing sea ice, and a later study by Lean (1919) discussed the presence of a variety of microbes associated with brine channels within sea ice. These early studies did actually consider large areas of snow and ice to be possible viable ecosystems for life.

Early observations of ecosystems in the cold have been very patchy and this was for a good reason: the lack of investigations during the cold season or subzero temperatures was mainly caused by logistic difficulties which have been expressed by troublesome accessibility to the study sites and the nontrivial character of working in the cold. Additionally, the prior understanding of life in ice was simply a paradoxon; hence, the ecological relevance of these extreme environments has never been taken into account. For long time, limnologists have considered the cold winter season where the lakes are covered by a thick and long-lasting ice sheet and cut off from any atmospheric exchange as poorly relevant for ecological processes. The overall opinion was that processes in the cold are in slow motion and metabolic rates are negligible or even inhibited by freezing temperatures. What at the first glance appeared to be a contradiction in terms, being frozen and leading an active life at the same time (Psenner and Sattler, 1998), turned out to be an exciting example of the adaptation of microorganisms to environmental extremes.

Moreover, with the improvement of standard methods to investigate even ultra-oligotrophic environments and increased interest in the cold biosphere, finally most diverse niches for microbial life have been acknowledged as components of the cryosphere interacting with each other in complex ways.

Another aspect of survival at subzero temperatures has been a crucial issue in daily life not even hundred years ago, when food hygiene posed substantial questions regarding cold-adapted organisms. In the industrial age, the survival of microorganisms in ice focused on public health aspects. It has occupied the attention of bacteriologists due to the popular custom of mingling ice with food or drinks to either extend or improve the quality (Jensen 1943). In the early twentieth century, typhoid fever has been a big issue where impure ice has never been suspected to be the origin of infections – survival of pathogens in ice has simply been seen as impossible (Jordan, 1911). The reason for this was the belief that when contaminated water freezes the great majority of typhoid bacteria will be immediately destroyed (Jordan, 1938).

The recognition of (mainly) microbial survival at freezing point is questioning adaptation mechanisms which enter both the ecological and the biotechnological realm. The general understanding of extending the limits for life increased, and simultaneously organic compounds such as freeze-shock proteins were applied in food and medical industry. To grow successfully in cold habitats, cold-adapted microorganisms have evolved a complex range of adaptations of all their cellular constituents, including membranes, proteins, energy-generating systems, components responsible for nutrient uptake, and the synthesis of compounds conferring cryotolerance to avoid the destructive effect of intracellular ice formation (Cavicchioli et al., 2002). These strategies offer multiple biotechnological applications

of cold-adapted organisms and/or their products in various fields. The characteristic features of cold-active enzymes (high catalytic efficiency at low and moderate temperatures; thermolability) offer a number of advantages for biotechnology processes, such as the shortening of process times, saving of energy costs, prevention of the loss of volatile compounds, performance of reactions that involve thermosensitive compounds, and reduced risk of contamination (Margesin et al., 2007).

With increasing knowledge about ice ecosystems on our planet and how organisms adapt to survive or even thrive under these harsh conditions, many researchers have begun to look at icy exoplanets like the moons of Jupiter (Europa, Io, and Ganymede) or on Mars. Cold and extreme environments in general and the therein living adapted microbial communities are a substantial component in the field of astrobiology. The McMurdo Dry Valleys in Antarctica are often described as possible analogues for life on Mars. The cold and xeric Dry Valley soils formed under environmental conditions resemble those of past and present Mars (Javaux, 2006). According to McKay (1997) the field-based investigation of parts of the Antarctic yields valuable information about soils and microbial life that may bear significantly on future manned and unmanned missions to Mars, especially since the Martian surface archives an active and varied geologic history similar in many ways to that of Antarctic terrains. Warm temperatures on Mars may not have persisted beyond the end of the heavy bombardment 3.8 billion years ago. Hence, Mars would have quickly had conditions where ice-covered water bodies were the main habitat for life which resembles the conditions in the Antarctic Dry Valleys where precipitation is less than a few centimeters of snow per year and the mean annual temperatures are below -20°C . The dry cold conditions make the valleys virtually lifeless. However, microbial ecosystems in the Dry Valleys found beneath the ice of the perennially ice-covered lakes (Parker et al., 1982), in the ice itself (Priscu et al., 1998; Priscu, 2009), or within the porous subsurface of sandstone rocks (Friedmann, 1982) prove that microbial communities are thriving even under the most extreme conditions. Mars is similar to Earth in various ways, having many of the same “systems” that characterize our planet, such as an atmosphere, a hydrosphere, a cryosphere, and a lithosphere. In other words, Mars has (thin) air, ice, and rocks that all interact to produce an environment which might have been able to sustain life about a billion years after its formation and – in certain areas – maybe still as of today (NRC, 2006).

Spread across the Arctic, Antarctic, and alpine regions of our world, the cryosphere of our planet is most likely an ancient and vital key for the persistence of life on Earth. Strong evidence continues to accumulate indicating that the Earth has been completely ice covered for ten million years or more on at least two occasions in a process now known as “snowball Earth” (Kirschvink et al., 2000).

During these periods of massive global glaciation, icy microbial ecosystems would have served as the central reservoir for life, including the photosynthetic primary producers such as the cold-tolerant cyanobacteria (Vincent and Howard-Williams, 2000; Priscu and Christner, 2004). To survive in one of the most hostile, extreme environments on Earth, to deal with high ultraviolet radiation, freeze-thaw

cycles, and organic resources dependent over the long term on photosynthetic primary production, these communities would contain the elite specimens of terrestrial evolution (Tranter et al., 2004).

So the prior view from a purely physically driven sterile cryosphere has turned to an active ecosystem with teeming life even under the most extreme conditions. But when considering the living cryosphere, we should distinguish between different capabilities and conditions of organisms: is the cryosphere (1) just a repository (a deep freezer) for life, e.g., in ancient ice (Castello and Rogers, 2005); (2) does it harbor life on the edge, i.e., species that barely sustain life functions; or is it even (3) an oasis where organisms can thrive under harsh but stable conditions? However, the main driver for activity within cold habitats is the prerequisite of life which is liquid water.

Ice and snow covers on solid ground can – if they are not too thick and solar radiation is strong enough – initiate the formation of liquid water layers. Solar radiation is essential in two ways, i.e., for producing heat to melt the ice and to provide radiation for photosynthesis. Hence, active metabolism in solid ice depends either on direct sunlight and/or exclusion of ions during the freezing process to create veins inside the matrix filled with brine. The formation of brine channels is the prerequisite for the numerously reported metabolic rates within sea ice where organisms can thrive during the entire ice-covered season. Especially in the course of discussions about possible life forms in Lake Vostok (a subglacial lake under 4 km of ice in the eastern Antarctic), Buford Price has been one of the pioneers to establish solid ice, formed by the transition from snow to glacial ice, as another potential microbial habitat (2000).

“I propose a habitat consisting of interconnected liquid veins along three-grain boundaries in ice in which psychrophilic bacteria can move and obtain energy and carbon from ions in solution,” he stated in 2000, when he described the possibility of microbial life in the brine veins between ice crystals. Compared to the brine channels in sea ice, these veins are relatively small with a diameter of a few μm . Hence, microbial abundances and metabolic rates will not reach those high values as observed in the ice cover of the polar ocean. Polar lake ice, for instance, is showing these boundary layers between ice crystals as well; however, the metabolic “hot spots” are located in sediment clusters which sunk into the ice due to the absorption of solar energy (Priscu et al., 1998, 2002). The sinking distance is limited by the solar heat and hence a function thereof and the radius of the sediment particle. If there is no other source of organic matter, chemical energy, or heat, the thickness of the ice layer is a limiting factor for life. However, we must consider the possibility of the existence of ice ecosystems without solar radiation. Deformation of thicker ice layers can exert heating by friction at their base, but also geothermal heat can create liquid water under the ice – which is a good insulator.

Solar radiation also provokes the existence of so-called cryoconite holes which are unique freshwater environments and are found on glacial ablation zones (Fig. 2). These microcaverns can cover 0.1–10 % of the ablation zone of



Figure 2. (a–c) Various forms of glacial cryoconite holes (c) with frozen lid.

a glacier and occur globally in glaciated environments. They are predominantly water filled and frozen throughout the winter or even during a day-night cycle. The living conditions in this extreme habitat are characterized by a high light intensity (UV radiation), low water conductivity ($1.9\text{--}6.0\ \mu\text{S cm}^{-1}$), low temperatures ($0.1\text{--}0.2\ ^\circ\text{C}$), little dissolved oxygen, and lack of nutrients. The communities are complex microbial consortia of bacteria, cyanobacteria, microalgae, viruses, and protozoa. Also Metazoa such as tardigrades, rotifers, and nematodes can be found. Both the microflora and fauna are probably decomposed by fungi and bacteria. *Hypsibius klebelsbergi* Mihelčič, 1959, a tardigrade, has the ability to occupy a particular ecological niche on the glaciers of the Alps. This species only occurs in cryoconite holes in the Alps. However, very little information is available about the role of metazoans since most of the studies to date have focused on microbial processes. During the short summer period when the water in cryoconite holes is mostly liquid and active metabolism is possible, the abundance of Metazoa is very high. Up to date, the biological activity within a cryoconite hole was mostly addressed to microbial communities since the role of Metazoa has been rather underestimated. It is probably necessary to reconsider the carbon budgets within cryoconite holes after investigation of abundances and biomass as well as the composition of the food web of alpine supraglacial environments. The importance of tardigrades and their food preference within the food web of cryoconite holes are still unknown.

On most alpine glaciers (which are temperate throughout – with the exception of a handful of cold spots), summer temperatures lead to surface melting which transports airborne organic matter (leaf debris, soil particles, pollen, insect remains, etc.), gases, and living microorganisms to the glacier bed thus providing nutrients and organic matter for heterotrophs. Glacier beds have stable temperatures, and when they reach the pressure melting point, water is present in the subglacial sediments or it may form larger water pockets. Sharp et al. (1999) tried for the first time to use the frequency of dividing cells as an indication of in situ bacterial activity at the base of two temperate glaciers in the Swiss Alps. They suggested that bacterial activity at the glacier bed is important to provide elevated concentrations of CO_2 , thus leading to enhanced weathering. An inoculum of debris-rich samples from the basal ice increased pyrite oxidation significantly.

These experiments, however, were performed in the laboratory at 4 °C, so evidence for in situ activity is still missing.

In Antarctica, a different situation exists, characterized by the existence of more than 140 deep subglacial lakes identified recently under the Antarctic ice sheet (Siegert et al., 2005). The largest of these lakes is Lake Vostok (ca. 14,000 km²; ca. 1,000 m deep), which is thought to lie in a deep rift valley. The water column of Lake Vostok remains liquid under nearly 4 km of ice as the result of the insulating properties of the overlying ice sheet, natural heating from the Earth's surface, and pressure-induced freezing-point depression caused by the weight of the ice sheet. The lake has been isolated from the atmosphere since the ice sheet formed (about 15 million years before present). Owing to systematical change of ice thickness by about 400 m over the length of the lake, melting occurs at the north end, where the ice is thicker, and refreezes (accretes) at the southern end where the ice is thinner. These differences in ice thickness and melting temperatures are the cause for a sustained mixing of the lake water (Wüest and Carmack, 2000). Ice cores taken from the accreted lake ice and the overlying meteoric ice have all shown the presence of microbes which can metabolize when exposed to liquid water (Priscu et al., 1999; Karl et al., 1999). This contention implies that the biological seed for the Lake Vostok organisms is atmospheric deposition followed by about 500,000 years of transport to the bottom of the ice sheet where they are released, along with nutrients and gases. Actual conditions within Lake Vostok will be revealed when finally the first reliable liquid sample will be retrieved from the water body. Despite findings of bacteria in accreted ice, however, there are skeptic voices stating that oxygen concentrations which are 50 times higher than organisms could possibly survive lead to a toxic oversaturation (Bulat et al., 2009). With that new perspective the dispute about the principal possibility of life in Lake Vostok has begun.

Another example for ice melting without solar radiation was the surge of Vatnajökull in Iceland caused by an under-ice volcanic eruption, a phenomenon called Joekulhlaups (Björnsson, 2002), or the most recent example from the under-ice eruption of the volcano Eyjafjalla in Iceland in April 2010. This dramatic eruption caused one third of the glacial ice mass to melt. Volcanic activity under a glacier can provide both heat to produce liquid water, suitable chemical compounds and redox couples to allow microbial growth. However, water bodies without solar radiation can only function as an ecosystem if a redox gradient exists and is permanently sustained. As long as suitable organic matter is present, fermentation or respiration with electron acceptors such as oxygen, nitrate, or sulfate may occur, and some bacteria can use chemical energy and CO₂ to grow. Lithoautotrophy in the subsurface has been suggested several times, and also the mechanisms to generate available energy for bacteria have been discussed. Data on in situ activity are not yet available. Nonetheless, chemolithoautotrophic microorganisms have been detected in very deep and isolated rock layers or cave systems, and it would be interesting to compare such warm or hot subsurface habitats with ice ecosystems. However, if all redox pairs (organic and inorganic)

are exhausted and there exists no mechanism to reestablish a disequilibrium between oxidized and reduced forms of matter, life as we know it is not possible. In the case of Lake Vostok and other water masses covered by thick layers of ice, one could in principle envisage a redox cycle driven by dissolution/precipitation of (iron, manganese, and other) minerals, caused by geothermal heat or hydrothermal vents as suggested by Bulat et al. (2004).

More generally – and more theoretically – even in closed ice-water systems where there is no replenishment of substances (e.g., stable ice caves), also other gradients may allow certain forms of life, for instance, a pH, an electrical, an ion concentration, or a temperature gradient at the ice-water interface. Freezing comprises a change in the free energy of the water, a rise in the concentration of salts due to exclusion of ions during the freezing process and a shift in the ionic ratio of the surrounding water because some ions are incorporated more easily into the growing ice crystal than others. Also the concentration and the ratio of dissolved gases will change if ice melts. Thus, freezing/thawing cycles could principally be used by organisms to support vital functions, for instance, to drive ATP production by using an H^+ gradient at a mineral-water or water-ice interface. This, however, could happen only at very low rates and by life forms which are actually unknown.

Therefore, we can hardly suggest that life may exist without a redox gradient, but when exploring new ice systems on Earth or on other planets, one should at least consider the possibility of alternative energy gradients suitable to sustain life (Psenner and Sattler, 1998).

3. The Atmosphere: Living Space or Transit Corridor for Microbes?

Surprisingly, there is no reference that mentions the atmosphere as an extension of the cryosphere's ecological niches. So far, the atmosphere has always been addressed as a driver of global climate interacting with the other components of the climate system and as a conveyor of aerosols and inactive spores. Recent investigations, however, have changed this picture substantially, but general acceptance of the atmosphere as a – although short lived – habitat is still lacking.

Life is floating in the clouds. Not simply as inactive spores but a considerable portion thereof is metabolizing and reproducing in the atmosphere which has been thought for long time to be too hostile for active life. Clouds have traditionally been beyond the purview of biologists – too hostile and too short lived to be a habitable environment. This picture has changed with the notion that one can find microbial communities consisting of viruses, bacteria, algae, spores, cysts, fungi, and even protists from the well-mixed boundary layer to the higher atmosphere.

The establishment of microbial communities of isolated environments actually begins in the air. Antarctica is isolated from the other continents of the world by the Southern Ocean and Antarctic circumpolar current (Smith, 1991; Wynn-Williams, 1991). However, it has been known for more than six decades that

bacteria, algae, and fungi are easily transported over significant distances by the wind (Gislén, 1948). In fact, hundreds of millions of tons of dust that includes viable microorganisms, trace metals, and organic material are transported between continents each year (Choi et al., 1997; Garrison et al., 2003). Bacteria have even been shown to divide on airborne particles (Dimmick et al., 1979a, b), and approximately 20 % of the total atmospheric aerosol mass is carbonaceous material (Bauer et al., 2002). Cloud water can contain high concentrations of organic acids such as formate and acetate that bacteria can utilize as energy sources (Herlihy and Mills, 1986; Herlihy et al., 1987), and it is now clear that microorganisms can grow and metabolize even in icy supercooled cloud droplets. Whatever its origin may be, biological material present in Antarctic air represents a diverse consortium of life forms including moss spores, pollen, fungal spores, bacteria (including cyanobacteria), algal propagules, viruses, lichen propagules, tardigrade cysts, nematodes, and arthropod fragments (Burckle et al., 1988; Wynn-Williams, 1991; Marshall, 1996a, b).

For fungi, probably the most important dispersal route is air transport as well. However, airborne distribution of fungi in the Antarctic is constrained by the low levels of airborne particles in Antarctic air. Counts of total colony forming units (bacteria and fungi) in low-altitude air systems are in the order of $0.5\text{--}3\text{ m}^{-3}$ (Cameron et al., 1977; Wynn-Williams, 1991; Marshall, 1996a). Meteorological conditions that would allow for such transport occur regularly, although not necessarily frequently. Nevertheless, more than 900 fungal species have been identified in the Antarctic, and the presence of fungi pathogenic to plants and invertebrates not found in Antarctica suggests dispersion from other regions (Onofri et al., 2000; Tosi et al., 2002). In addition, fungi normally associated with particular hosts have been found in Antarctic ecosystems where their hosts are absent (Göttlich et al., 2003).

Molecular biological studies of airborne bacterial diversity and transport in Antarctica are not as extensive as the work on fungi. However, it does appear that prokaryotic life in the air of Antarctica may be of distant, local, or regional origin. PCR-based investigations of microbial diversity in air samples collected at Rothera Point on Adelaide Island in the Antarctic Peninsula identified a wide variety of cyanobacteria, actinomycetes, and diatom plasmids (Hughes et al., 2004). The closest 16S rRNA gene matches for many of the sequences were to organisms already identified in Antarctic or other cryosphere environments. While the majority of matches were to clones of local origin, wind trajectory calculations indicated that the air had recently traversed the Antarctic Peninsula raising the possibility that a significant portion of the microbiota identified may have been of widely distributed origin. Regardless of origin, the link between airborne microbial life and the ice of glaciers and Antarctic lakes depends on the fundamental physics of light, heat, and the adhesive characteristics of organic molecules.

The challenging question connected with life in extreme environments such as the atmosphere is still if the sheer presence of such microorganisms actually

implies activity, or even indeed a functional role. To date, studies of life in remote icy environments have generated a huge range of estimates of microbial numbers, and estimates of activity and impact will become available in the coming years. The implication of airborne transport for future studies of icy environments is that it is essential to know whether the microorganisms identified are (1) merely transient organisms, (2) established but nonviable, (3) established and viable, or (4) established, viable, and reproducing.

4. Altitude Limitations for Extreme Microbial Life

The challenges faced by microorganisms in aerial environments could be considered a continuum, whereby the severity of the environment increases with altitude. Viable microorganisms have been obtained from the upper atmosphere, but have also been launched into Earth orbit in an attempt to study their survival outside the atmosphere (Wainwright et al., 2003, 2004). Although isolates from the stratosphere have been recognized as common bacteria from the Earth (i.e., *Staphylococcus*, *Bacillus*) (Narlikar et al., 2003), an even tiny survival rate of freeze-dried bacteria in space is all that would be needed to permit the movement of cosmic microbial life around the galaxy (Wickramasinghe, 2004). The experiments of Horneck and Rettberg (2002) have shown that bacteria could survive space travel, including lift off from the place of origin and reentry into a planetary atmosphere. It has been shown that the Gram-positive, spore-forming organism, *Bacillus subtilis*, can survive for 6 years in space with some of the original population remaining viable after their recovery (Horneck et al., 1984; Horneck, 1993), and they can survive an atmospheric entry velocity of 1.2 km s^{-1} in a basalt rock attached to a sounding rocket (Fajardo-Cavaros et al., 2005). Despite the general opinion that small particles get burnt by frictional heating during entry into the stratosphere, there might be ways to permit intact injection, as suggested by Wainwright et al. (2003), where microbial life could be transported in connection with comet dust. Hence, the potential for transfer into and out of the atmosphere has been studied. Cockell et al. (2007) launched a cryptoendolithic habitat, made of a gneissic impactite inoculated with *Chroococciopsis* sp., into Earth orbit. After orbiting the Earth for 16 days, the rock entered the Earth's atmosphere and was recovered in Kazakhstan. The heat of entry ablated and heated the original rock to a temperature well above the upper temperature limit for life and also to below the depth at which light levels are insufficient for photosynthetic organisms (~5 mm), thus killing all of its photosynthetic inhabitants. This experiment showed that atmospheric transit acts as a strong biogeographical dispersal filter to the interplanetary transfer of photosynthesis. However, studying adaptations for the aerial transport of microorganisms might help in the search for microbial life on other planets.

5. The Cryosphere and the Global Carbon Cycle

By attributing a value to the cryosphere – and doubtlessly organic carbon can be seen as a “strong currency” in ecology – there are good reasons to consider the cold biosphere as a substantial contributor of organic carbon. Priscu and Christner (2004) made an attempt to estimate the total number of bacterial cells in the ice sheets of Antarctica and Greenland which resulted in 9.61×10^{25} cells corresponding to a carbon content of 2.65 Tg. These values approach those reported for the Earth’s freshwater lakes and rivers (1.3×10^{26} cells; 2.99 Tg), suggesting that ice sheets contain nontrivial amounts of cells and carbon. These estimates of the number of prokaryotes and organic carbon associated with Antarctic subglacial lakes and glacial ice are clearly tentative and result from a handful of studies and will be refined once additional data become available. However, they do imply that icy habitats contain an organic carbon reservoir that should be considered when addressing issues concerning global total carbon storage reservoirs and dynamics and the potential connectivity between ice ecosystems.

Assessing the carbon pool of single ice ecosystems is a tricky task and even more on a global scale. As mentioned before, some ice ecosystems persist for thousands of years, others for a winter season or – for instance, in the case of cloud droplets – for just 1 day. Organic carbon produced in an ice ecosystem by photosynthesis will eventually be released to the next adjacent system. This is the case for organic carbon production on glacial surfaces such as cryoconite holes and superficial habitats. Some cryoconite holes can be literally flushed from DOC by heavy meltwater which results in a relocation of organic carbon and biomass into lower areas such as the glacier forefield.

The same mechanism is valid for annual sea ice and alpine lake ice. Once the ice cover is melting, unusually high concentrations of nutrients and cells are part of a sudden release into the water column. This deposition is mostly going along an inoculation and fertilization effect of the pelagic communities. However, these effects are vital for higher levels of the food web such as feeding krill in Antarctica.

Assessing the contribution of the cryosphere to the global production of organic carbon is based on very patchy observations so far and is clearly demanding more detailed investigations. It seems to be clear, however, that cold environments have generally been underestimated in their potential to fix and produce carbon. Sea ice with ca. 1.3 Gt C a^{-1} (Longhurst et al., 1995) is certainly the major player in carbon production, compared to the global phytoplankton production of ca. $45\text{--}50 \text{ Gt C a}^{-1}$. All land plants are contributing with $45\text{--}68 \text{ Gt C a}^{-1}$ (Longhurst et al., 1995), while glaciers, for instance, produce ca. 64 Gg C a^{-1} .

These numbers need to be handled with extreme care since measurements are patchy. Up to now there is no single consecutive measurement regarding carbon fixation and production throughout a whole season. Moreover, with the concept of connectivity, there is a substantial support of neighboring systems with a considerable reservoir for bacterial diversity and carbon. Observing the

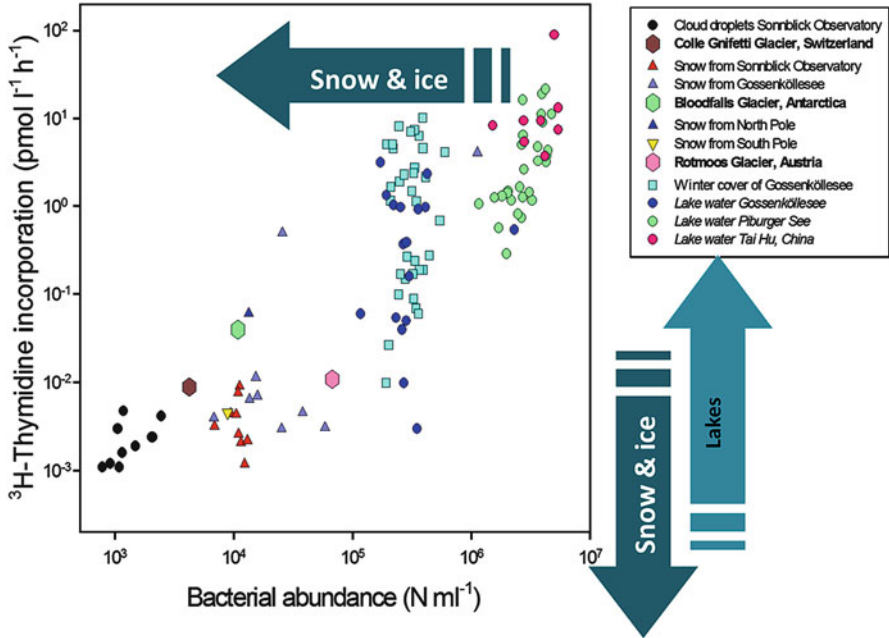


Figure 3. Bacterial cell numbers versus bacterial activity as measured via ³H-thymidine incorporation of various ice ecosystems in comparison with hypereutrophic habitats (e.g., Lake Tai Hu in China) (Modified by Roland Psenner after Sattler et al., 2001).

succession of primary and secondary production in cold environments along a temporal scale will show a light- and temperature-dependent shift between autotrophy and heterotrophy. From where we stand now, we can simply use the recognition of ice ecosystems as significant carbon contributors to stress the need for more detailed investigations to finally include cold environments into the global carbon budget. An example to show bacterial activity in various ice ecosystems compared to higher trophic levels is shown in Fig. 3.

6. Longevity of Ice Ecosystems?

The previously described, mostly microbial, ecosystems which are collectively covering massive areas of the globe are now facing a new set of challenges. Few places on Earth have been affected by climate change more than the Antarctic Peninsula with an increase in annual average temperatures of almost 3 °C during the past 50 years (Hansen et al., 1999). The impact of this change is complex. For instance, increased temperature is accompanied by an increase in humidity and availability of liquid water producing a significantly increased growing season. But increases in humidity can alter the ultraviolet-induced inactivation of airborne bacteria

(Peccia et al., 2001). Radiation can inactivate airborne bacteria at both moderate (50–60 %) and high (85–95 %) levels of relative humidity, but inactivation rates are greatest at moderate levels (Paez-Rubio and Peccia, 2005). In addition, the elevated awareness in the scientific community of the importance of these extreme ecosystems has led to significant increases in human contact with these fragile microbial consortia inhabiting ice ecosystems. The price for that contact will be an increase in the risk of forward contamination of the cryosphere with human microbiota and the possibility of chemical contamination, e.g., with the hydrocarbons inherent in human transportation and survival, e.g., in Antarctica.

Although we are still at the beginning of the exploration of microbial communities in snow and ice, those factors which are crucial for the formation, duration, and physicochemical conditions of ice habitats, e.g., global warming, atmospheric deposition of acids and dust, and UV radiation, are changing rapidly. If we include supercooled cloud droplets, it is not clear whether the area and the number of ice ecosystems will increase or decrease by global warming. While some temperate glaciers will certainly retreat and consequently some ice ecosystems will literally disappear, climate warming is expected to change cold glaciers (i.e., those frozen to the bed rock) to temperate ones (i.e., with liquid water at least during summer season), and also large permafrost regions will transform from repositories of frozen organisms into a habitat for living ones. On the one hand, perennially ice-covered lakes would show a decrease in ice-cover thickness; on the other hand, the increased availability of liquid water is enhancing metabolism. Increase in the water vapor content of the atmosphere may lead to enhanced precipitations which tend to improve the conditions for ice organisms at high altitudes and high latitudes. Hence, climate change has severe effects on the expansion and shrinkage, respectively, of these sensitive environments. With increase of global temperature, the prerequisite for life, which is water, will increase substantially as well which will result first in a release of entombed cells and, consequently, in a revitalization of a portion of those cells.

7. Oasis or Repository: Stable or Temporal?

Water as the clear driver of the fate of microbial communities can dramatically change the character of the matrix of both an oasis and a repository. The deep cores of the Greenland ice sheet show that microbial cells can be entombed in the ice for hundreds to thousands of years. The same is known for Lake Vostok ice cores or the vast Antarctic ice sheets. These environments will clearly stay repositories due to their insulating thickness. However, there are habitats which can be transformed from a deposit to an oasis, as can be seen in ice caves, for instance. Ice caves are characterized by a relatively stable temperature regime throughout the season. Sometimes the temperature rise does not necessarily need to be high to change the cave's climate substantially to enhance the metabolism of previously entrapped cells at the ice surface.

Another point of discussion is the longevity of the atmospheric habitat. Microbial cells hitchhiking on aerosols, suspended in cloud droplets, and floating through the air: this picture is clearly limited by a time frame characteristic for the residence time of clouds. Clouds can live and die within a day (e.g., cumulonimbus clouds). Clouds with the longest life span are cirrus clouds which occur in higher altitudes and can remain for several weeks at the maximum. But if microbial cells are spun into the atmosphere and happen to be deposited to the ground on the same day, is it still acceptable to talk about a habitat, not to say an ecosystem? We personally consider it still an ecosystem since cells metabolize and reproduce therein.

For extreme environments, we most likely need to leave the well-known path of ecosystems with clear boundaries or ecotones like alpine lake ice. We may even be careful with definitions such as “habitat” since – depending on factors such as temperature, water, and radiation – they may eventually switch their habitability: an oasis may thus become a repository or vice versa.

8. The Role of Cold-Loving Bacteria in the Tree of Life

For decades, scientists have used a comprehensive tree of life showing heat-loving bacteria as the Earth’s earliest life forms. However, it is still debatable whether life has originated in the hot or in the cold. Even a deep subterranean igneous rock environment, probably hot with a high pressure, has been suggested as the cradle of life. There are some good arguments for a hot origin of life, for instance, the phylogenetic relationship of organisms based on small subunit ribosomal RNA sequences. According to Levy and Miller (1998), the stability of RNA is not given under these circumstances. However, the validity of this relationship is sharply denied by researchers who assume that phylogenetic relationships are strongly biased by the use of just one single gene for constructing the tree of life. If lateral gene transfer is common among all (prokaryotic) organisms, then a hierarchical universal classification is difficult or impossible, and the history of life can be represented more properly as a web rather than a tree. Interestingly, a similar change of views or paradigms has also occurred – although much earlier – in ecology where the concept of a linear food chain has transformed into the now broadly accepted vision of a food web. New results coming from phylogenetic trees based on genes that do not code for ribosomal RNA but also chemical experiments with alternative structures for the nucleic acid backbone considerations about the thermal stability of basic molecules found in all organisms (amino acids, nucleic acids, lipids) and statistical analyses of the GC content of DNA do not emphasize a hot origin of life. On the contrary, life in hot environments seems to be a late adaptation (Brochier and Philippe, 2002). Also the low luminosity of the early sun hints at generally lower temperatures at the time when the first living cell appeared on earth. As long as we do not have stronger insight in the general phylogeny, however, it is premature to conclude a cold origin of life. Thus, before raising another origin-of-life hypothesis, we suggest that evidence must come from different

sources, i.e., a consistent phylogenetic tree with known evolution rates of single genes, knowledge about the chemical stability of biomolecules under different constraints, and a clear description of the physicochemical environment which is assumed to have led to the first living cell. In a way we apply a similar reasoning as for the definition of an ice ecosystem.

9. Outlook

Beside all speculations about other icy worlds, studies on ice ecosystems have clearly shown that temperatures at or below 0 °C do not inhibit the proliferation of microbial communities. Rather, active and diverse assemblages of organisms develop in ice and snow covers of distinct geographical regions of the world. Even supercooled cloud droplets seem to be a suitable site for bacterial growth, which leads us to the suggestion that life can be expected everywhere in the cold where liquid water exists. The existence of ice ecosystems adds to global biodiversity in at least two ways: first, the area with active life is much bigger (by several million km²) than assumed hitherto; second, ice ecosystems show peculiar ways of interactions between organisms that are otherwise strictly separated, creating – sometimes short lived – ecotones between atmosphere, hydrosphere, and pedosphere. Our present knowledge of ice ecosystems is biased by the selection of specific examples and the relatively short time passed since their first exploration. In addition, we lack also methodologies to measure activities and growth rates of organisms under in situ conditions, for instance, at the ice-water or the liquid-gas interface and at subzero temperatures.

These facts draw the bow from terrestrial extreme environments to the exciting field of astrobiology. If mankind ever is to touch the surface of Mars, then very sophisticated and robust methods are required to exclude terrestrial contamination. What is valid throughout the methodology in oligotrophic environments must be applicable for investigation of extraterrestrial samples, either rock, soils, or even water ice. As long as we are not capable to avoid all possible contamination vectors (back or forth), we are not ready for these demanding studies. The best example for this delicate requirement is subglacial Lake Vostok, the eighth largest lake on Earth and covered by 4 km of solid ice. As the glacier has traversed the lake, lake water has frozen to the bottom of the glacier forming a 200 m accretion ice layer. The density of bacteria of the retrieved ice cores is among the lowest concentrations of microbes recorded on this planet. However, to drill through the lake to finally gain lake water, which has been separated from the atmosphere for approximately at least 15 million years, is another task requiring absolutely sterile performance. Lake Vostok might be the most prominent test area for sampling microorganisms outside of planet Earth. Tedious decontamination protocols are recently ready to be applied for samples with low cell concentrations. The investigation of ice ecosystems are in definite need thereof.

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SNOW ALGAE: ADAPTATION STRATEGIES TO SURVIVE ON SNOW AND ICE

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1. Introduction

1.1. A SHORT HISTORY

Snow algae are a group of freshwater microalgae that have encountered the extreme habitats of persistent snow and glacier fields in the polar and high-alpine regions of our earth. In suitable locations they can build up massive blooms resulting in a macroscopically visible pigmentation of the snow (Fig. 1). The dominating species belong to the green algae (Chlorophyta), and depending on the life cycle stages and dominating pigments observed, this results in green and different shades of orange, pink, or red snow – for the latter three hereafter I will use the term “red snow” only. Green snow is caused by the trophic, actively dividing sexual or asexual cells stages, whereas red snow is the result of their carotenoid-rich resting stages, such as hypnospores or hypnozygotes. Other snow tints have been described referring to other taxonomic groups, e.g., purple-grey ice caused by the Zygnematophyceae *Mesotaelium berggrenii* or *Ancylonema nordenskiöldii*.

One of the first field samples of red snow was collected during the Northern Expedition under Captain Ross in August 1818 to Baffin’s Bay between northern Greenland and Canada (Bauer, 1819) and brought back to England to be investigated. Numerous scientists have studied the samples containing “red and colorless globules,” and following early misinterpretations as a fungus, Wille (1903) identified the organism as an alga. Numerous taxonomic changes followed and finally the taxon *Chlamydomonas nivalis* (Bauer) Wille (1903) was erected. Kol (1968) was the first one to write an extensive monograph about these unusual organisms, clearly stating that the taxon *Ca. nivalis* has to be regarded as a collective name, as she realized that not only one species was responsible for the color. This view still is valid today, nevertheless, often neglected.

In the second half of the twentieth century, most work on snow algae concentrated on the identification of different taxa found on snow (Duval et al., 1999; Hoham, 1974a; Hoham and Mullet, 1978; Ling, 1996; Ling and Seppelt, 1998), the clarification of their life cycles (Hoham, 1974b, 1975a; Hoham et al., 1979, 1983), and aspects of their ecology (Hoham, 1975b; Hoham et al., 2000; Kawecka and



Figure 1. (a) Red snow on glacier Doktorbreen 25 km inland in Nathorst Land (Spitsbergen, Svalbard), (b) intensely tinted snow from carotenoid-rich resting stages of *Chloromonas* and *Chlamydomonas* spp., (c) field sample of resting stages covered with sediment particles, (d) green snow in coastal Bjørnhamna in Albert I Land (Spitsbergen, Svalbard), (e) sporangia of the psychrophilic *Chloromonas nivalis*. All scales = 10 μm .

Eloranta, 1986; Müller et al., 1998). Later, molecular phylogeny became a valuable method to understand snow algal diversity (Buchheim et al., 1997; Hoham et al., 2002; Pröschold et al., 2001). A first overview on snow algal ecology was given by Hoham and Duval (2001), and since the turn of the millennium, also the scientific community working with snow algae became more diverse publishing studies on different topics (Hoham et al., 2006, 2007; Leya, 2004; Müller et al., 2001; Nedbalová and Sklenár, 2008; Novis et al., 2008; Pocock et al., 2004; Remias et al., 2010; Stibal and Elster, 2005; Takeuchi and Koshima, 2004). For a more complete list of literature on snow algae, see above examples and references therein.

Realizing how diverse the group of snow algae are and also as a result of numerous new descriptions, the taxonomy became more and more confusing, often also as a result of numerous descriptions of “new” species based on field material only. For a long time virtually no snow algal strains were available from public algal collections, only some very few academic collections existed, e.g., in the laboratories of Ron Hoham (Colgate University, NY, USA) and Hau U. Ling (Australian Antarctic Division, Australia), two of the pioneers of recent snow algal research. With molecular phylogeny being established as a valuable tool, field samples as well as laboratory strains could be analyzed in more detail, and it showed that often strains were misidentified as *Ca. nivalis* or misinterpreted as different species. This was also due to the fact that even until today no clear description of the leading taxon of *Ca. nivalis* exists, especially no type material one can refer to. These days the situation regarding the availability of snow algae is different. The UTEX (the Culture Collection of Algae at the University of Texas) hosts many algal strains from Hoham’s work including some type material in their snow collection, however, not of *Ca. nivalis*. The CCCryo (Culture Collection of Cryophilic Algae) is specialized on algae strains from polar and alpine environments and serves as a valuable bioresource. Currently there is also the neotyping of the taxon *Ca. nivalis* in progress. Apart from basic research, cryophilic algae recently also came into focus of biotechnological applications due to their ability to grow at lower temperatures and their special metabolites.

1.2. WHAT MAKES AN ALGA FROM SNOW A TRUE SNOW ALGA?

Some definitions have to be observed when working with snow algae. One obvious character, nevertheless for a long time rather neglected, is their adaptation to low temperatures. Psychrophilic algae (= true snow algae) are obligatorily adapted to snow temperatures, i.e., their preferred temperature for growth is near 0 °C. Indeed only strains belonging to the genera *Chlamydomonas* or *Chloromonas* plus some closely related taxa like some *Desmotetra* spp. or from the recently revised genus of *Microglena* (Demchenko et al., 2012) within the Chlorophyceae (Chlorophyta) show such a strong adaptation to low temperatures with an optimum for their growth below 15 °C, and they will not survive much more than 20 °C. Some strains from the CCCryo have proved to have optimum temperatures for growth below

5 °C and would not even tolerate an increase to 10 °C (Leya, 2004). The other typical group of snow algae among the green algae belongs to the Trebouxiophyceae (Chlorophyta), and though regarded as soil algae, some species are regularly found on snow, such as *Raphidonema nivale* and closely related species, and make up green snow. This species shows a temperature optimum around 12 °C though also higher values have been reported for other species from this genus isolated from snow (Stibal and Elster, 2005). Using the temperature threshold of 15 °C for optimal growth, one can separate the psychrophilic algae from the psychrotrophic ones. The latter are regarded as non-obligate cryophiles as they survive well-elevated temperatures and have an optimum for growth above 15 °C. This classification goes well in accordance with Morita (1975) who works with psychrophilic bacteria. Hoham and Duval (2001) discuss this issue intensely. In fact often algae strains were isolated from snow and regarded as true snow algae until taxonomic studies revealed their identity as closely related psychrotrophic or mesophilic taxa such as *Chlamydomonas augustae* or *Chloromonas reticulata*. Many of the really interesting adaptations, like, e.g., the synthesis of ice-structuring proteins (ISPs, see Sect. 3.4.2 below), can only be found in truly psychrophilic strains. These basics have to be observed when doing snow algal research. The group of algae neighboring psychrophilic snow algae in nature is permafrost algae. These are psychrotrophic soil algae occasionally also thriving on snow, but rather due to passively being dispersed from the permafrost soil onto adjoining snow and glacier fields.

Apart from freshwater snow algae, also marine psychrophilic algae can be found, e.g., on the bottom of ice flows; however, these are often diatoms and rather termed ice algae to separate them from their freshwater counterparts. This group is not part of this book chapter. However, as nature is known to hardly ever provide clear boundaries, some phylogenetic clades of originally psychrophilic freshwater Chlamydomonadaceae can be characterized with a clear preference for brackish or marine culture media. A revision is under way and those taxa might be transferred to the green algal genus *Microglena* (Demchenko et al., 2012).

The following sections will deal with interesting adaptation strategies of psychrophilic snow algae from the Chlamydomonadaceae mainly.

2. Stressors and Adaptation Strategies

Snow algae are exposed to a range of abiotic and biotic stressors which determine whether they find suitable conditions to establish stable populations and mass developments as green and red snow in their extreme habitat.

2.1. TEMPERATURE

When working with snow algae, temperature clearly is the key factor deciding about metabolism activity. It decides about water availability in the habitat and influences most of the other stressors. In general a more or less constant temperature

of around 0 °C can be measured on snow fields during the short vegetational period in summer. During freeze incidents water becomes locked, hindering transport of nutrients over the snow field and hence limiting nutrient availability to the algae. In return, rising temperatures by a few degrees will release liquid water and disperse nutrients. Thus, a temperature of just above 0 °C with the result of liquid water and nutrient availability is a prerequisite for actively dividing (trophic) algal cells. When temperature falls just below 0 °C, the algae slow their metabolism and either stay in their trophic state preventing cellular freezing by accumulation of sugars and/or antifreeze substances (see Sect. 3.4.2 below) or, if able, transform into thick-walled resting stages to withstand the unfavorable conditions. So, water availability is the key for cellular processes and cell division and in turn is directly dependent on temperature. Sung et al. (2003) give a good overview and depict a model of temperature-dependent molecular signal transductions.

Snow algae in general can be regarded as being stenothermic, tolerating only a small range of temperature.

2.2. NUTRIENTS

Snow algae have to cope with varying levels of nutrients. This can range from almost pure water after snow fall or rain under melting conditions to locally high concentrations under freeze conditions, as only the water is locked and dissolved ions become highly concentrated in the intercrystalline spaces where the snow algae live. Biotic sources of nutrients can be the surrounding vegetation like mosses and lichens or fecal pellets from animals like nearby penguin colonies, colonies of migrating birds, or passing by polar foxes, polar bears, or reindeers. Also inorganic nutrients from mineral sources, e.g., the surrounding rock substrates, can be made available or locked under the same conditions.

Snow algae can be regarded as eurytrophic, in the sense that they tolerate a broad range of nutrient concentrations.

2.3. OSMOTIC AND DESICCATION STRESS

Low and freezing temperatures are the prime stressors directly provoking osmotic and desiccation stress. In their trophic (green) stage, snow algae are primarily planktonic as flagellated unicells and can move freely within the intercrystalline space of snow and meltwater to areas best suitable for them. When water freezes, the algae are opposing osmotic stress, as, though low in concentration initially, salt and mineral ions are being concentrated under such freezing conditions (Kirst, 1990). The high ion concentrations have a strong desiccation effect on the trophic algal cells in this space. To prevent cell death thick-walled spore stages are formed reducing water loss; additional extracellular mucilage layers have a similar effect, most probably also serving as a mechanical protection and keeping predators

away from the cell. In life culture collections many of the Chlamydomonadaceae from snow, though for this group flagellated cells are typical, often can be observed easily shedding their flagella or even being unflagellated. Thus, living on the top layer of snow, this algal group can be counted among the cryoseston (Kol, 1968) rather than cryoplankton. An interesting morphological adaptation recently has been described for the marine diatom *Chaetoceros dictyota* living in sea ice with significantly shortened and inside bent setae compared to the free-living planktonic form with long and outward bent setae (Ligowski et al., 2012).

2.4. LIGHT

Light obviously also is a strong stimulus, primarily providing energy for photosynthesis, but high levels in combination with decreasing levels of nutrients will induce synthesis of secondary carotenoids like, e.g., astaxanthin which acts as a strong antioxidant. This pigment gives red snow its dominating dark red color. In combination with low temperatures and especially in snow algal species with no secondary carotenoid metabolism, excessive energy is directed towards the xanthophyll cycle and dissipated as heat through non-photochemical quenching (Szyszka et al., 2007).

In the run of the annual season, and especially in high polar regions, light is extremely variable. On Spitsbergen (Svalbard, Norway) at 75–81°N, snow algae have approx. 3 months in summer with continuous light for 24 h with high light conditions during midday to build up their massive populations. After this vegetational period snow fall usually covers the snow algae during autumn, leaving the cells in a day/night cycle of twilight and darkness for another 3 months, followed by 3 months of total darkness in winter. During the next 3 months of spring, days become longer again and with the melting snow cover light starts penetrating this insulating layer. By some not yet fully understood mechanism germination of cysts that have formed during the previous summer is induced, and cell proliferation starts to form green snow for a very short time until again cysts are formed, and secondary carotenoids are accumulated to prepare for winter, making up the red snow (for theories about annual life cycles of snow algae, see Remias (2012) and Müller et al. (2001)).

Snow algae tolerate an extremely broad range of light intensities and qualities.

3. Adaptation Strategies in Snow Algae: Some Examples

So far, little has been published about the adaptation strategies of psychrophilic snow algae, mainly due to the fact that suitable strains have not been available from culture collections until now. This chapter will attempt to give a first and short overview about current results.

3.1. PIGMENTS/ANTIOXIDANTS AGAINST HIGH-LIGHT STRESS

High light intensities in combination with increased irradiation of UV light have destructive effects on different parts of the cell. Induced free radicals and singlet oxygen species built up and accumulating, especially in the resting stages, can lead to damages in DNA (Buma et al., 1995), proteins (Chauhan et al., 1998), and also (photosynthetic) pigments (Rajagopal et al., 2000). The combination of high-irradiation and low-nutrient availability at low habitat temperatures seems to be especially stressful (Bidigare et al., 1993). In general snow algae can cope very well with light conditions varying in quantity and quality. Different taxonomic groups have evolved diverse sets of pigments with either absorbing capacities or simply as shielding pigments to protect the photosynthetic apparatus and other light-sensitive cell components. Secondary carotenoids with many double bonds, but also other UV-absorbing pigments, play a vital role in snow algal physiology.

For the glacier inhabiting desmid *Mesotaenium berggrenii*, an unusual phenolic compound, a purpurogallin carboxylic acid-6-*O*- β -*D*-glucopyranoside, is described with absorption in the far UV-B at 275 nm and especially the UV-A range at 338 nm (Remias et al., 2012). This pigment is responsible for the dark violet-brown pigmentation of the cells. The dark red color observed in snow algae forming the red snow is mainly accounted to the keto-carotenoid astaxanthin and its esters which have absorption maxima in the UV-A range at 371 and at 470 nm (Müller et al., 1998; Remias and Lütz, 2007). Apart from direct absorption of harmful radiation and serving as a valuable pool of antioxidants for scavenging free radicals (Duval et al., 2000), these pigments together with a whole range of other carotenoids and xanthophylls (Leya et al., 2009) can shade the chloroplast from excessive radiation and, thus, light inhibition. The antioxidant activity seems especially important for the red resting stages, though these are metabolically not completely inactive (Remias et al., 2005; Stibal et al., 2007), the alga cannot repair, e.g., DNA damages caused by UV irradiation during cell division by molecular repair mechanisms but instead has to use chemical reactions between the carotenoids' double bonds and the free radicals for inactivation. The fat-soluble carotenoids are stored in lipid globules outside the chloroplast, with the lipids themselves simultaneously serving as valuable longtime energy storage products. Some of those red snow species, when in their vegetational, green phase, and, thus, without a big pool of secondary carotenoids available, still have a chemical option to inactivate free radicals with the help of an active pool of xanthophylls, as, e.g., in *Chloromonas nivalis* (strain CCCryo 005–99) (Remias et al., 2010).

Those algal species completely lacking a secondary carotenoid metabolism, like, e.g., *Raphidonema* spp., have high capacities for the production of α -tocopherol (vitamin E) and again of the xanthophyll cycle pigments (Leya et al., 2009), presumably representing an alternative way of adaptation to cope with high-light stress. This might be an explanation why these species, usually found on the permafrost soils adjoining snow fields also, are able to build up

considerable blooms as green snow. Why they are not occurring regularly together with species typical for red snow is still under debate, but might be a question of snow chemistry which seems to be influenced by the underlying rock substrate. Kol (1968) observed that green snow often is typical for calcitrophic substrates opposed to red snow usually observed on silicotrophic locations.

Snow algae obviously have found a variety of ways to cope with the different light conditions encountered on snow, thus helping them to live in this extreme habitat.

3.2. LIPIDS/FATTY ACIDS

While the primary and short-time energy storage product in snow algae, especially in the green stages, is starch, high amounts of lipids are built up in resting stages, in which they serve as highly energetic secondary storage products and as depots for the fat-soluble carotenoids (see above) during overwintering conditions. In many species large lipid globules can also be observed in the green cell stages.

The lipids occur as fatty acids in globules throughout the cell, in the form of phospholipids as part of membrane bilayers, and as glycolipids, e.g., in the outer membrane layer (Arts et al., 2009). Low temperatures, nutrient depletion, and also solar radiation are the key factors influencing the fatty acid composition in the lipids (Piorreck et al., 1984; Roessler, 1990), of which temperature has the dominating influence (Poerschmann et al., 2004). But also the nutritional regime changes the FA composition of algae considerably (Spijkerman and Wacker, 2011). For snow algal communities in Antarctica, Bidigare et al. (1993) describe a higher content of unsaturated FAs in red snow (89 %) than in green snow (28 %) with the monounsaturated oleic acid (C18:1n-9) accounting for nearly 60 % in the red snow and for 11 % of all FA in green cell samples. In red snow from different locations on Spitsbergen as well as in some clonal laboratory culture experiments of snow algae isolated from Spitsbergen under nitrogen limitation, this FA was also the dominant one with amounts of 33–45 mg g C⁻¹ (20–38 % of the total FA contents) in red snow samples and up to 71 mg g C⁻¹ (= 31 % of total FA) in green cell cultures of strain CCCryo 010–99 of *Chloromonas rostrifinskii* under nitrogen depletion (Spijkerman et al., 2012). When analyzing green snow predominantly consisting of *Cr. brevispina* in its green flagellated cell stages, Řezanka et al. (2008) found the oleic acid accounting for approx. 7 % of all FAs. This relatively low value might be explained by the fact that the green cell population was not nutrient limited, as Spijkerman et al. (2012) also determined a proportion of 11 % only in the unstressed culture of above *Cr. rostrifinskii*. Tazaki et al. (1994) found a proportion of 5.6 % of oleic acid in red snow samples from northern Canada. The dominance of oleic acid in nutrient depleted cultures and in resting stages of snow algae reflects its importance as a precursor in the synthesis pathway of polyunsaturated FAs. From this depot the algae can comfortably scoop to metabolize longer and especially polyunsaturated FAs (PUFAs) which are of importance to

oppose cold and light stress. PUFAs typically found in major amounts in red snow samples are linoleic acid (C18:2n-6) with 8–16 %, α -linolenic acid (C18:3n-3) with 13–18 %, and stearidonic acid (C18:4n-3) with 4–6 % of all FAs (Spijkerman et al., 2012). Similar values were determined from nutrient-limited green laboratory cultures of above strain of *Cr. rostrifinskii* for linoleic acid (C18:2n-6) with 4 %, α -linolenic acid (C18:3n-3) with 23 %, and stearidonic acid (C18:4n-3) with 6 % of all FAs. Řezanka et al. (2008) found similar values studying above *Cr. brevispina* for linoleic and stearidonic acid, but considerably lower values (6 %) for α -linoleic acid. They also found relatively high contents of PUFAs with chains shorter than C16 in their field material, which they relate to the extreme habitat. Spijkerman et al. (2012) observed considerable differences not in the type but in the proportions of FAs between different snow algae species. This might simply be an effect of when samples were taken from the stressed cultures for analyses. Due to different growth rates, different species reach a different grade of “ripeness” after a certain time, i.e., the amount of secondary carotenoids and FAs can vary.

Interestingly Řezanka et al. (2008) found only one type of saturated FA in their sample, the palmitic acid (C16:0). This is unusual, as Bidigare et al. (1993) list many more (C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C24:0) for their green sample, some of them amounting to 27–35 % of the total FAs. Also Spijkerman et al. (2012) could detect FAs of types C16:0, C18:0, C20:0, C21:0, and C22:0 with the palmitic acid dominating with up to 25 % of total FAs in the unstressed culture of strain CCCryo 005–99 of *Cr. nivalis*. Teoh et al. (2004) also investigated some Antarctic microalgae of which only the *Chlamydomonas* strain UMACC 229 seems to be psychrophilic. They state that in all other algae studied, the saturated FAs were dominant, except for the PUFA hexadecatrienoic acid (C16:3) which was dominating the *Chlamydomonas* strain with almost 50 % of the total FAs.

3.3. COLD-INDUCED TRANSCRIPTS AND COLD-ADAPTED PROTEINS

Very little to date is known about cold-induced or cold-active enzymes and other proteins from psychrophilic algae. The following section will cite some reviewed articles but also some yet published work to give an insight in possible adaptation mechanisms from this field.

To prove that many proteins are newly or increasingly synthesized under cold conditions, Pawella (2008) performed differential display studies on 2D-SDS gels of soluble and membrane-bound proteins in psychrophilic snow algae strains from the CCCryo. She compared protein extracts from algal cultures maintained at 2 °C with such from 8 °C. For strain CCCryo 050–99 of *Chlamydomonas cribrum*, she found more of the large subunit of RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) being produced at 2 °C than at 8 °C. Also for *Raphidonema*

sempervirens (strain CCCryo 011–99), this could be observed. Comparing the RuBisCO of two psychrophilic *Chloromonas* spp. (strains ANT1 and ANT3) with that of the mesophilic *Ca. reinhardtii*, Devos et al. (1998) found that the carboxylase activity of the psychrophilic RuBisCO was lower at low temperatures than that of *Ca. reinhardtii* which is contrary to what is usually observed in psychrophilic enzymes. Also the optimal temperature for both enzyme types was similar; however, the psychrophilic RuBisCO was more thermolabile. Interestingly Devos et al. also observed that the relative amount of RuBisCO subunits in the psychrophilic isolates was twice as high as in *Ca. reinhardtii*. The authors state that this might balance the low catalytic efficiency of the psychrophilic RuBisCO at low temperatures. This strategy is contrary to what since has been observed in psychrophilic bacteria whose enzymes show a higher specific activity at lower temperatures compared to their mesophilic homologues (Aghajari et al., 1998; Choo et al., 1998). It may appear as if this mechanism might have been lost in eukaryotic organisms and that in fact the strategy simply to synthesize more of a specific enzyme to achieve the same turnover of the substrate at low temperatures would be the only adaptation mechanism in psychrophilic algae, but the following examples suggest that the prokaryotic strategy also is used in eukaryotes.

As studies on cold-induced proteins in snow algae are extremely scarce, some more results from unpublished student theses are worth mentioning. In her proteome studies on the psychrophilic strain CCCryo 020–99 of *Chloromonas* cf. *platystigma*, Pawella (2008) could show that cultures from 2 °C compared to 8 °C strongly increased the production of homologues of the leghemoglobin C2, the α -subunit of the H⁺-transporting 2-sector adenosine triphosphatase and a precursor of the chlorophyll-*a/b*-binding protein. Bley (2006) performed transcript studies on the same strain and found a strong upregulation of a membrane-bound ABC-transporter protein after 2 h of cold shock at 0 °C compared to RNA material from a culture maintained at 8 °C. Proteins from this family are important for the transmembrane transport of inorganic or organic molecules. This might be important for an efficient nutrient uptake or the excretion of ISPs (see Sect. 3.4.2. below).

When comparing the nitrate reductase (NR) of the psychrophilic *Koliella antarctica* with that of the closely related thermophilic *Chlorella sorokiniana*, Di Martino Rigano et al. (2006) found that the optimal temperature for the specific activity of the psychrophilic NR and other thermal features were all shifted by about 10 K towards lower temperatures compared with the mesophilic NR. When studying the NR and the argininosuccinate lyase of above-mentioned strain ANT1, Loppes et al. (1996) found that for both enzymes the temperature for the apparent optimal activity was about 20 K lower in the ANT1 strain than in *Ca. reinhardtii*. They also observed that both enzymes from the psychrophilic strains were more heat sensitive than the mesophilic ones. Zacke (2007) compared the malate dehydrogenase (MDH) of two psychrophilic snow algae with that of *Ca. reinhardtii* (Fig. 2). He observed that the optimal temperature for the apparent activities of the MDHs from the two psychrophilic strains CCCryo 020–99 of *Cr.*

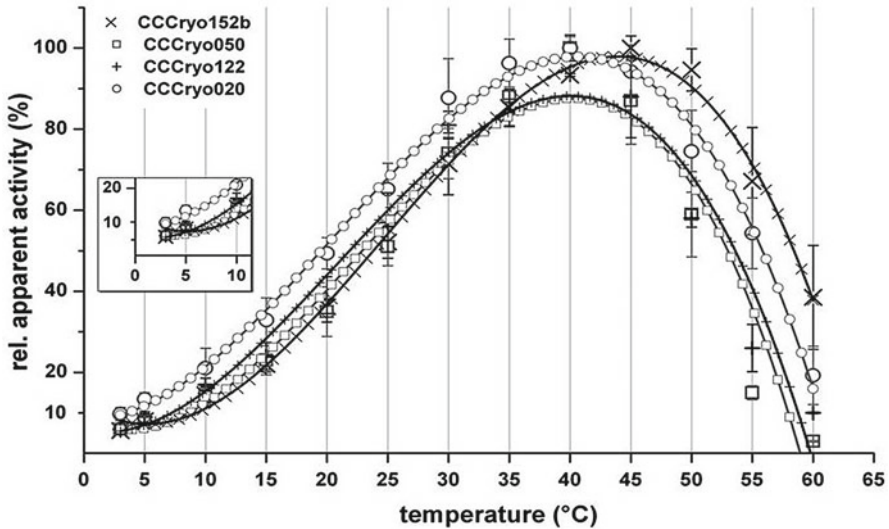


Figure 2. Relative apparent activities of the malate dehydrogenase (*MDH*) of two psychrophilic (CCCryo 050–00 and 020–99) and one psychrotrophic (CCCryo 122–00) strain compared to the *MDH* of the mesophilic *Chlamydomonas reinhardtii* (CCCryo 152–01).

cf. platystigma and CCCryo 050–99 of *Ca. cribrum* was shifted by approx. 5 K towards lower temperatures. Looking at 3 °C, the relative apparent activity of these strains' *MDH*s was nearly double compared to the *MDH* of *Ca. reinhardtii*. The apparent activation energy E_a laid at 47 kJ mol⁻¹ for CCCryo 020–99 compared to 62 kJ mol⁻¹ for the two other strains from snow (CCCryo 050–99 and 122–00) and 64 kJ mol⁻¹ for the *Ca. reinhardtii* strain CCCryo 152b-01 (= SAG 11-32b; UTEX 90; CCAP 11/32A).

Interestingly also the *MDH* from the psychrotrophic strain CCCryo 122–00 (= SAG 26.86; UTEX LB 1969; CCAP 11/51B) of *Cr. typhlos* (also known as *Cr. reticulata*, *C. augustae*, *C. nivalis*), one of the first strains incorrectly described as *C. nivalis*, showed similar activities as the *MDH* from the psychrophilic strains.

3.4. HOW TO PREVENT DESTRUCTIVE ICE CRYSTAL FORMATION

Living on persistent snow fields or ice with ambient temperatures around 0 °C during the vegetational season when air temperatures are well above, or at freezing temperatures during the rest of the year when air temperatures decrease below that encountered under the snow cover, snow algal cells are constantly in danger of freezing. Intracellular and extracellular ice crystals would be damaging to membrane and cell structures and eventually fatal to the whole cell. Strategies are different in different snow algal taxa. One also has to differentiate between

intracellular and extracellular freezing. In winter, when air temperatures, e.g., in northwestern Spitsbergen in 1999 cooled down to a mean of $-15\text{ }^{\circ}\text{C}$ with extremes as low as $-25\text{ }^{\circ}\text{C}$ (Leya et al., 2004), the snow algae had concentrated, due to thawing processes towards the end of the summer, in a well-insulated layer under a thick snow cover with temperatures stabilizing at $-7\text{ }^{\circ}\text{C}$ from the beginning of January until end of April. Thus, snow algae do not really have to stand the much lower air temperatures.

3.4.1. Intracellular Freeze Protection

To prevent intracellular freezing and also the hyperosmotic stress, the algae synthesize considerable amounts of different sugars and sugar alcohols. In different CCCryo strains from the Chlamydomonadaceae (Chlorophyceae), trehalose could be measured with very variable concentrations between 14 and $814\text{ }\mu\text{mol g}^{-1}$ lyophilized dry mass (lyo-dm) (2008, unpublished; measurements performed by L. Gustavs). Trehalose only has a very weak osmoprotective effect, but rather is discussed as a mechanical cryoprotectant as during desiccation it can bind to membranes by replacing water and thus maintains their basic structure as it was described for yeast, higher plants, insects, etc. (Yancey, 2005 and references therein). When Gustavs et al. (2010) studied various green microalgae (mainly Trebouxiophyceae) from different aeroterrestrial habitats in temperate regions, they found that trehalose was more abundant under matric stress, while glycerol was rather accumulated under osmotic stress. Gustavs also found much lower trehalose concentrations in aeroterrestrial Trebouxiophyceae ($<10\text{ }\mu\text{mol g}^{-1}$ lyo-dm, personal communication) than in our snow algae from the Chlamydomonadaceae (see above). Interestingly also in the psychrotrophic *Raphidonema nivale* (Trebouxiophyceae) strain CCCryo 112-00, apart from living on snow also a typical inhabitant of cold-soil substrates, she determined trehalose concentrations at $6\text{ }\mu\text{mol g}^{-1}$ lyo-dm only. Probably the ability of trehalose as to act a mechanical stabilizer under desiccation/osmotic stress is less important in the aeroterrestrial Trebouxiophyceae than in the primarily planktonic Chlamydomonadaceae. Trehalose is also reported from Antarctic lichens (Roser et al., 1992). The same authors also investigated the glacier inhabiting *Mesotaenium berggrenii* (Streptophyta) and report that this was the only organism among the lichens and snow algae investigated that produced glycerol. From the brief screening of L. Gustavs in 2008, we know that also one psychrophilic *Chloromonas* sp. (strain CCCryo 020-99) and also *R. nivale* produces this compound. So, in contrast to trehalose which was produced by all strain screened, the production of glycerol seems to be limited to some species only. In their studies on aeroterrestrial algae, Gustavs et al. (2010) found the accumulation of glycerol under osmotic stress. Saccharose, as the primary product from photosynthesis, is a typical sugar found in green algae. It is often found at enhanced levels, but only has a low osmoprotective effect (Kirst, 1990). In the screening in 2008, Gustavs found remarkable amounts of saccharose in *R. nivale* (CCCryo 112-00) with $116\text{ }\mu\text{mol g}^{-1}$ lyo-dm and much less in *Chloromonas* sp. (CCCryo 020-99)

with $5 \mu\text{mol g}^{-1}$ lyo-dm and in *Chloromonas nivalis* (CCCYo 005–99) with only $2 \mu\text{mol g}^{-1}$ lyo-dm. Higher amounts ($25 \mu\text{mol g}^{-1}$ lyo-dm) were detected in *Sphaerocystis* sp. (CCCYo 101–99), but presumably this has to be accounted to the massive formation of sucrose containing mucilage layers surrounding the cell packages, a strategy to oppose desiccation stress often observed in permafrost algae, but also in some cell stages of snow algae species. Not much to date can be said about other sugars or sugar alcohols possibly synthesized by snow algae to fight intracellular freezing, as data on this are still scarce. A detailed overview on different low molecular weight carbohydrates in red algae in relation to osmotic acclimation, however, is given by Eggert and Karsten (2010).

3.4.2. Extracellular Freeze Protection

Though when thinking of freeze protection, we usually think of intracellular adaptations the other side of the outer cell membrane must not be forgotten. During freeze events the cells dwell in a hyperosmotic channel system between numerous ice crystals which might be fatal to the cells when uncontrolled in growth. Ice-structuring proteins (ISPs), formerly also termed antifreeze proteins [AFP; (Clarke et al., 2002); in this chapter I will use the term ISP only], have the ability to bind to ice crystal surfaces and modify their shape by inhibiting growth and recrystallization processes (Knight and Duman, 1986). ISPs are widely spread among very different cold-acclimated and cold-adapted organisms, e.g., cold-water fish (DeVries, 1983; Fletcher et al., 2001; Sicheri and Yang, 1995); Alaskan insects and spiders (Duman et al., 2004) and other terrestrial arthropods (Duman, 2001); higher plants (Atıcı and Nalbantoğlu, 2003) such as winter rye (Griffith et al., 1997) or the grass *Lolium perenne* (Pudney et al., 2003); enoki and shiitake mushrooms (Raymond and Janech, 2009); an Arctic psychrophilic yeast (Lee et al., 2010); Antarctic moss, cyanobacteria, and the green alga *Prasiola* (Raymond and Fritsen, 2001); and marine sea ice diatoms (Bayer-Giraldi et al., 2010, 2011; Raymond, 2000; Raymond and Knight, 2003), and only recently have been described from psychrophilic green algae from marine environments (Raymond et al., 2009), and psychrophilic freshwater snow algae from the CCCryo collection (Connor, 2011; Dolbinow, 2010; Petasch, 2008; Speth, 2010). Interestingly, though the ISPs are similar in function, hardly or virtually no homologies can be found on nucleotide or amino acid level which makes their molecular characterization difficult. Also from an evolutionary point of view, ISPs are quite interesting. Based on different molecular studies and assays on antifreeze proteins from winter rye, Hon et al. (1995) suggest an evolution from pathogenesis-related proteins (endochitinases, endo- β -1,3-glucanases, and thaumatin-like proteins). Studying the antifreeze glycoproteins (AFGPs) in Antarctic fish, Chen et al. (1997) propose that these evolved from ancestral trypsinogen genes and date this evolutionary process at 5–14 million years ago.

In all organisms producing ISP or proteins with similar function, these are excreted out of the cells into either the hemolymph, the blood, or in multicellular plants into the apoplastic space. In unicellular algae, such as the snow algae, the

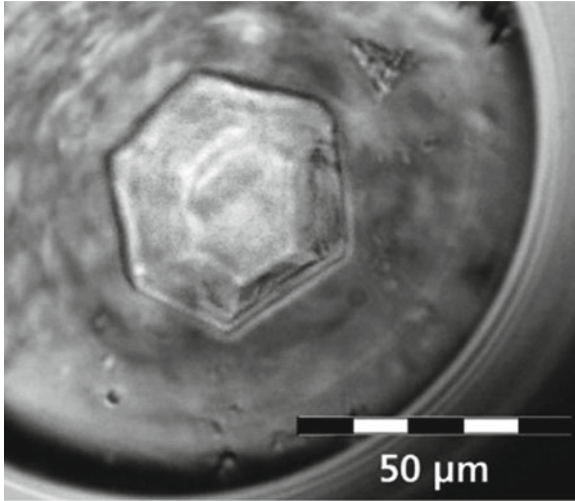


Figure 3. Ice crystal shaped by ISP from the psychrophilic strain CCCryo 050–99 of *Chlamydomonas cribrum* (Modified from Petasch, 2008).

ISPs are excreted into the cell near environment and accumulate in the channels between the ice crystals. To date it can only be assumed that this is the location where they bind to the surrounding ice crystals and modify these in a way so that the algal cells are not harmed by uncontrolled and destructive growth. We screened various snow algae strains from the CCCryo and found that only psychrophilic species from the Chlamydomonadaceae were able to produce ISPs. Neither cold-tolerant (psychrotrophic) Chlamydomonadaceae isolated from snow nor any *Raphidonema* spp. (Trebouxiophyceae) from snow had this ability. In the laboratory psychrophilic snow algae can be cultured at 0–3 °C, and the ISPs which are excreted into the culture medium can easily be harvested and purified by the cool finger method described by Kuiper et al. (2003). Purification, however, is not necessary to test the activity of ISPs. The filtered culture supernatant can be used, and depending on the concentration of ISPs, these hinder small ice crystals to grow over time (recrystallization) and also force them into a specific shape, namely, hexagonal bipyramids by binding to specific planes of the ice crystal surface (Jia and Davies, 2002; Wathen et al., 2003).

Figure 3 shows an ice crystal shaped by the ISP from the psychrophilic strain CCCryo 050–99 of *Chlamydomonas cribrum*. Clearly visible is the hexagonal basal plane and one side of the blunt pyramid protruding to the front. Depending on the overall activity of ISP and the specific concentration, ice crystals structured by them can be rather blunt or spiky (see Wathen et al., 2003). For testing the recrystallization activity (RI-activity) of ISPs, the so-called RI-assay (Regand and Goff, 2006) can be used. Over time the initially small and numerous crystals will either recrystallize, i.e., larger ones will grow at the expense of smaller ones,

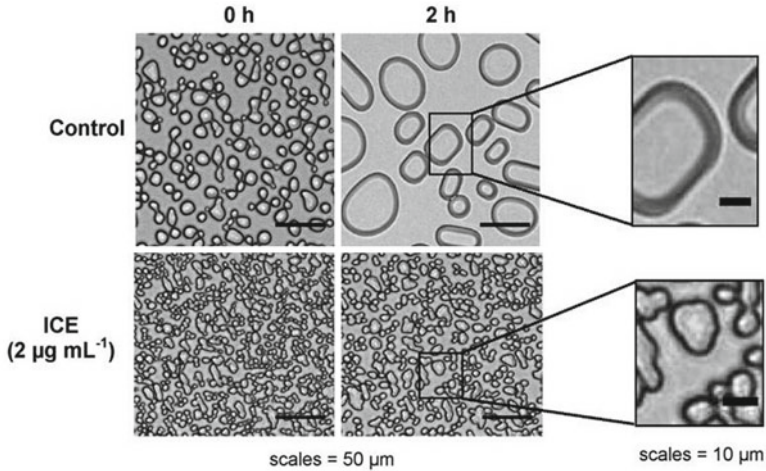


Figure 4. Recrystallization assay with $2 \mu\text{g mL}^{-1}$ purified ISP (ICE) from the psychrophilic strain CCCryo 050-99 of *Chlamydomonas cribrum* in 23 % sucrose solution at $-5 \text{ }^{\circ}\text{C}$. The control contained no ISP.

when no ISPs are present, or if ISPs are active, the initially small crystals will hardly grow or only little in size while changing towards a somewhat angular shape (Fig. 4).

Regarding active concentrations of ISP, it is hardly possible to give one single answer as this depends much on the type of ISP and its specific activity. In contrast, e.g., to sodium chloride which lowers the freezing point of a solution in a colligative way, i.e., the freezing point depression is directly dependent on the concentration of sodium chloride, ISPs act in a non-colligative way. The molecular and biochemical structure determines its function and ability to bind to ice surfaces, and therefore ISPs are rarely lowering the freezing point of a solution, but they structure ice crystals. Nevertheless, some proteins are described having thermal hysteresis activity (Barrett, 2001; Knight and Duman, 1986; Urrutia et al., 1992), and these often are termed thermal hysteresis proteins (THP).

For several of the snow algal ISPs, we have studied from the CCCryo strains, full RI-activity (no significant growth in crystal size at $-5 \text{ }^{\circ}\text{C}$ over 1 h) was observed at a total protein concentration of about $20 \mu\text{g mL}^{-1}$ in the natural culture supernatant (this fraction naturally contained all other, non-ISP, excreted proteins plus the ISP from the algal culture, as the ISP concentration cannot be measured specifically). With the purified fraction containing predominantly ISPs, the same effect was obtained at about $2 \mu\text{g mL}^{-1}$. On the other hand, many ISPs from other psychrophilic snow algae strains required higher concentrations to maintain full activity. This reflects the variability of ISP, even within this small taxonomic algal group.

First, protein and molecular studies support this high diversity. Each single snow algal species has evolved a unique type or a set of ISPs of different sizes ranging between approx. 14 and 28 kDa. For strains CCCryo 257–06 (*Desmotetra* sp.) and 273–06 (*Chloromonas* sp.), full length sequences could be determined (Connor, 2011). These ISPs are similar to those characterized by Raymond from the *Chlamydomonas* strain CCMP 681 from the high intertidal in the Antarctic (Raymond et al., 2009), which is also reflected by their close phylogenetic relation. As more information on ISPs from freshwater algae does not exist, the anti-freeze protein from the marine ice diatom *Fragilariopsis cylindrus* described by Bayer-Giraldi et al. (2011) is worth being mentioned. From an ecological point of view, they suggest that the production and excretion of the diatoms' AFP into the environment might change the microstructure of the nearby ice surface and thereby act in favor of the diatoms' need for attachment surface.

Regarding snow algal ecology this theory could also be applied to snow and glacier fields. The snow algae, though basically being planktonic, have to remain in their cold habitat providing them with suitable conditions, as low temperature and light. Being rinsed away by meltwater, possibly even out on the surrounding permafrost soils where considerably higher temperatures prevail, would be fatal for psychrophilic snow algae. Thus, by altering the microstructure of nearby ice crystals in the intercrystalline channels might facilitate this intended immobility. Interestingly the active concentration of ISPs as stated above ($20 \mu\text{g ml}^{-1}$ in the culture supernatant) equals the protein concentration we measured in an algal culture in its mid-exponential phase ready to harvest the ISPs. Possibly this concentration is also reached in the natural habitat in the ice crystal channel system where the algae dwell. Bayer-Giraldi et al. (2011) state that due to the hydrodynamics in a sea ice brine channel system, a sufficient accumulation of ISPs does not sound plausible. This might be different in a more static snow field, especially under freezing conditions, when meltwater mobility comes to a rest. Liquid water in the intercrystalline channels and around the algal cells will still be existent, and a concentration of ISPs sufficient to be actively influencing ice crystal habit sounds realistic. Being trapped in this channel system, the algal cells possibly can keep crystals small and, due to their pyramidal shape, less harmful as if the crystals would be growing uncontrolled without ISPs.

To date we know far too little about the role of these ISPs in snow algal ecology, but they seem to be of major importance for the survival of psychrophilic algae. It also remains open why these proteins with such a common mode of action – binding to the surface of ice crystals – have evolved into such an extremely high not to say species-specific diversity. Or may their ice-related function just be a positive by-product? Possibly their putative evolution from pathogenesis-related proteins could help understanding the role of ISPs in future snow algal research and also help finding industrial applications for this interesting and unusual group of proteins, obviously playing an important role in snow algal adaptation to their natural habitat.

4. Conclusions

Psychrophilic snow algae, naturally exposed to light, nutrient, temperature, osmotic, and desiccation stress, are adapted to their extreme environment in many ways, and different taxa have evolved different mechanisms. High-light and UV stress is opposed by the production of absorbing or shading pigments as well as by the accumulation of effective antioxidants. Low-nutrient availabilities can be balanced, e.g., by an effective and cold-active nitrate reductase or the use of ammonia as a nitrogen source; on the other hand, the algae thrive under increased nutrient levels and build up massive blooms resulting in red or green snow. Temperature is a parameter having a direct slowdown effect on the cells' metabolism which is opposed by modifications in enzymes or increased levels of these, but it also has some indirect effects, as under freezing conditions cells become exposed to osmotic and desiccation stress. These in return are fought by changes in cell morphology, such as the formation of thick-walled cysts or the excretion of mucilage layers. When snow and ice crystals threaten the snow algae, they produce ice-structuring proteins to stop the crystals from growing and force them in less harmful shapes.

Though young on an evolutionary scale, snow algae have evolved numerous remarkable adaptations and are worth to be studied further and in more detail.

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ADAPTATION OF ANTARCTIC FRESHWATER GREEN ALGAE TO EXTREME ENVIRONMENTS

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1. Introduction

Antarctica is the coldest habitat on earth with an average temperature varying from -10 to -30 °C in winter and of around 0 °C in summer (Wiencke and Dieck, 1990). In addition, Antarctica presents various kinds of stresses besides severe year-round coldness, such as nutrient limitation, nearly 5-month total darkness in winter, high salinity, low humidity, freeze-thaw cycles, and long periods of light and ultraviolet radiation (UVR) (Becker, 1982; Hawes et al., 1999).

Despite of the extreme conditions, Antarctica possesses a rich diversity of algal flora, which forms the base of the food webs as producers in the Antarctic ecosystem. Antarctic freshwater green algae as the important constituents dwell in lakes, soil, on rock, snow and ice surface, and so on (Hawes, 1990). To grow and survive in the unfavorable environment, these algae which colonized in Antarctica have developed a number of mechanisms related to the alteration of cell morphology, adaptations of cellular structures with a higher proportion of unsaturated or short-chain fatty acids, and greater flexibility of proteins or enzymes as well as gene expression (Nagashima et al., 1995; Loppes et al., 1996; Teoh et al., 2004; Hu et al., 2008; Nagao et al., 2008; Li et al., 2009; Lu et al., 2010).

Green algae residing in lakes, snow, soil, and dry land may evolve significantly different strategies for the adaptation of the ambient environment (Morgan-Kiss et al., 2006). The Antarctic biota living in lakes may have a relatively high diversity of species that could well adapt to the narrow temperature range and very low-light conditions, while terrestrial Antarctic green algae exposed to terrestrial habitats have to face a 50 °C temperature range and changes in temperature, seasonally and diurnally. Besides, these species also endure desiccation, osmotic stress, and large fluctuations of solar irradiation and freeze-thaw cycles, which contributes to the alteration of distribution patterns, species diversity, polymorphism, and abundance (Huiskes, 2007). Studies of freshwater green algae inhabiting in

Antarctica mainly focused on *Chlamydomonas*, *Stichococcus*, *Chlorella* and *Scenedesmus* (Morgan-Kiss et al., 2006; Hu et al., 2008; De Wever et al., 2009; Chen et al., 2012a). *Chlamydomonas raudensis* UWO 241 (hereafter referred to as UWO 241), a psychrophilic phytoplankton organism from the permanent ice cover over Lake Bonney, is the most researched model species to date (Morgan-Kiss et al., 2006). *Chlorella vulgaris* NJ-7 (henceforth referred to as NJ-7), a psychrotolerant microalgal strain from the surface of wet rocks in Antarctica, has retained a high versatility to encounter rapid environmental change and also serves as a model species for the study of the adaptation to extreme environments (Hu et al., 2008; Lu et al., 2009). However, studies on the adaptation of Antarctic green alga to low-temperature environments are still in their infancy.

Additionally, it would be interesting to verify whether the green algae residing in Antarctica have the same origin. Some species seemed to have been introduced to Antarctica for they were identified to be closely related to strains from temperate latitudes (Hu et al., 2008). However, a recent study revealed that Antarctic microchlorophyte strains isolated from lacustrine habitats exhibited a wide diversity of apparently endemic Antarctic lineages at different taxonomic levels based on phylogenetic analysis. It was indicated that rare colonization events occurred and long-term survival took place in glacial refugia in Antarctica (De Wever et al., 2009). Therefore, it is likely that ancient patterns of isolation and long-range evolution characterized Antarctic freshwater green algae, which show a remarkable divergence from taxa originating from other continents.

The purpose of this review is to summarize the research on the Antarctic freshwater green algal adaptation to the extremely cold environment in recent years, with special emphasis on the two well-characterized green algae strains UWO 241 and NJ-7.

2. Adaptation to the Permanently Cold Environment

Temperature is one of the most important determining factors that limit the distribution and species of microalgae. Cold-adapted microalgae can be divided into two classes, namely, psychrophile and psychrotolerant. The former with their optimal temperature for growth at or below 15 °C are unable to survive above 20 °C, while the latter with an optimal growth temperature above 18 °C exhibit a wider temperature range for growth (Morgan-Kiss et al., 2006). Many concerns have been concentrated on the Antarctic psychrophiles and few reports on the psychrotolerants can be found. To our knowledge, Antarctic green algae from lakes or transitory ponds mainly belong to the psychrophiles, while most of the terrestrial ones isolated from soil and rock surface are psychrotolerants (Morgan-Kiss et al., 2006, 2008; Hu et al., 2008; Chen et al., 2012a).

Antarctic terrestrial and limnetic green algae face different low-temperature environments and may evolve adaptive strategies of their own to survive, including the alteration of cell morphology, ultrastructure, photosynthetic apparatus, and

gene expression, together with the evolution of cold-adapted enzymes and antifreeze proteins, and the development of more fluid biological membranes through the accumulation of polyunsaturated fatty acyl chains.

2.1. CELL MORPHOLOGY

Cold acclimation is related to changes in cell structure in temperate algae (Nagao et al., 2008). Algae inhabiting Antarctica are prone to aggregate and form cell packages embedded in mucilage (Ling, 2001), and cell walls were found to be very thick or surrounded by an envelope or the colloid in Antarctic green algae (Pocock et al., 2004; Hu et al., 2008; Morgan-Kiss et al., 2008; Chen et al., 2012a). Moreover, Antarctic green algae exhibit convergent evolution towards reduced morphology (e.g., coccoid forms) (De Wever et al., 2009). For example, *Scenedesmus* sp. NJ-1, a psychrotolerant strain isolated from the surface of wet rocks in Antarctica, is spherical, which is quite different from the majority of this genus (Chen et al., 2012b). In addition, a number of starch grains and lipid droplets were often observed in Antarctic green algae, which might be associated with the adaptation to the extreme coldness (Pocock et al., 2004; Hu et al., 2008; Morgan-Kiss et al., 2008; Chen et al., 2012a, b).

2.2. PHOTOSYNTHESIS

Long-term exposure to suboptimal temperature may alter the structure and function of the photosynthetic apparatus. UWO 241 has been discovered to be unable to utilize red light and unable to undergo state transitions (Morgan-Kiss et al., 2002b). This enigmatic green alga possesses varieties of unusual features in the organization and composition of the photosynthetic apparatus. In comparison with the mesophilic species *C. reinhardtii*, UWO 241 exhibits comparable levels of D1, which plays a central role in the reaction center protein of photosystem II (PS II), but reduced levels of PsaA/B in PSI as well as the absence or reduction of all light-harvesting I proteins. Moreover, this alga exhibits significantly higher levels of the oligomeric form of light-harvesting II complexes in the photosynthetic apparatus (Morgan et al., 1998; Morgan-Kiss et al., 2002b, 2005). Thus, the organization of the photosynthetic apparatus in UWO 241 results in an unusually high PSII-to-PSI ratio, which might be an adaptive strategy under constant low-light environment for chlorophyll *a* and *b* in PSII absorb light mainly in the blue region (Melis, 1998).

Cytochrome *f* in UWO 241 was identified to be about 7 kDa smaller than the 41 kDa cytochrome *f* in *C. reinhardtii* (Morgan-Kiss et al., 2002b), while the amino acid sequence of cytochrome *f* from UWO 241 showed 79 % identity with that of *C. reinhardtii*. Besides, the heme in cytochrome *f* from UWO 241 is significantly less stable to high temperature than that of *C. reinhardtii*, which indicates an adaptation to a cryophilic environment at the protein sequence level.

UWO 241 also exhibited higher levels of subunits of CF1 in chloroplast ATP synthase as well as higher adenylate pools compared with *C. reinhardtii*, which may be an adaptive strategy to provide adequate energy for ATP-dependent biochemical reactions at low temperatures (Morgan et al., 1998). Meanwhile, this psychrophilic alga possesses a structurally and functionally downregulated PSI, suggesting higher rates of PSI-driven cyclic electron transport (Morgan et al., 1998). The temperature at maximum F_0 fluorescence in UWO 241 was 10 °C lower relative to that in the *C. reinhardtii* (Morgan-Kiss et al., 2002a), which indicates a higher thermal lability of the photosynthetic apparatus in the psychrophilic alga. Different from UWO 241, *Chlorella* sp. BI, an Antarctic psychrophilic isolated from the Ross Ice Shelf near Bratina Island, was able to adjust light energy distribution between PS II and photosystem I (PS I) in a short time (Morgan-Kiss et al., 2008).

In contrast to the temperate *Stichococcus bacillaris* FACHB753, NJ-10 and NJ-17, two Antarctic psychrotolerant *Stichococcus* strains shared a high photosynthetic capacity of growth at low temperatures (<10 °C) (Chen et al., 2012a). Likewise, *Chlorella* sp. 82A isolated from wet soil of the shore of Lake Miers and *C. vulgaris* KG-5C from King George Island exhibit higher photosynthetic activity at low temperatures than *C. pyrenoidosa* C-28, a temperate strain (Nagashima et al., 1993). After freezing at -20 °C, these Antarctic green algae retained their photosynthetic activity, while temperate strains lost their photosynthetic activity (Nagashima et al., 1993; Hu et al., 2008; Chen et al., 2012a). Since growth rate and photosynthetic rate appeared highly correlated in phytoplankton (Coles and Jones, 2000), the high photosynthetic activity at low temperatures and freeze-tolerant properties of psychrotolerants might be conducive to their adaptation to Antarctica.

2.3. COLD-ADAPTED ENZYMES AND COLD-INDUCED PROTEINS

The molecular adaptation of enzymes in cold-adapted microorganisms is of vital importance to catalyze the chemical reaction rates at low temperatures. The optimal activity of nitrate reductase, glutathione reductase, and argininosuccinate lyase all shift to lower temperatures compared to mesophiles, and these enzymes exhibit a greatly reduced thermal stability (Loppes et al., 1996; Vona et al., 2004; Di Martino Rigano et al., 2006; Ding et al., 2007; Chen et al., 2012a). In addition, these cold-adapted enzymes usually show a higher level of specific activity than those of the mesophiles (Chen et al., 2012a).

Group 3 late embryogenesis abundant (LEA) proteins, which can protect enzymes against dehydration and inactivation by freezing (Reyes et al., 2005) and prevent varieties of proteins from aggregation (Chakrabortee et al., 2007), are cold inducible in *C. vulgaris*, and they were expressed at significantly higher levels in NJ-7 than in UTEX 259, a temperate strain isolated from the Netherlands (Hu et al., 2008; Li et al., 2009; Liu et al., 2011). So far, four LEA proteins, namely, HIC6, HIC12, Ccor1, and Ccor2, have been identified in *C. vulgaris*.

2.4. FATTY ACID PROFILE

The fluidity of the cell membrane can be increased with the increase of branched fatty acid content or the degree of fatty acid unsaturation (Feller, 2007). These fatty acids will lower the temperature of gel-liquid crystalline-phase transition of the lipid and therefore have the membrane maintaining proper fluidity at low temperatures. Antarctic green algae may depend on the content of polyunsaturated fatty acids (PUFA) to adapt to the harsh environment (Morgan-Kiss et al., 2006).

UWO 241 exhibited a significantly higher content of unsaturated fatty acyl bonds and higher levels of polyunsaturated fatty acids in chloroplast galactolipids compared to *C. reinhardtii*, suggesting that low-temperature adaptation of UWO 241 was specifically associated with the photosynthetic membranes. In addition, it possessed some novel polyunsaturated fatty acids with positional shifts in the unsaturated bond closer to the lipid head group, which may enhance the fluidity of lipid membrane (Morgan-Kiss et al., 2006).

In contrast, cold adaptation of *Chlorella* BI was not linked to the presence of short-chain fatty acids or high levels of membrane-associated PUFA (Morgan-Kiss et al., 2008). *Chlorella* BI may utilize other mechanisms to maintain a low lipid transition temperature, such as altering the membrane protein/lipid ratio or adjusting lipid head group class distribution. Alternatively, the moderate levels of unsaturated FAMES could be sufficient for low-temperature adaptation in this Antarctic chlorophyte.

PUFAs were increased in NJ-7 during cold hardening and may be involved in the development of freeze tolerance. An extremely high amount of Δ^{12} unsaturated fatty acids (UFA) was identified, which indicated that Δ^{12} fatty acid desaturase may be involved in the coldness adaptation of NJ-7 (Lu et al., 2009). The amount of the Δ^{12} UFAs in NJ-7 accounts for 58.1 % of total fatty acids, which is 2.1- and 1.9-fold compared with UWO241 and *Chlorella* BI, respectively. However, the adaptation to cold conditions of *Chlorella* sp. NJ-18, a psychrotolerant organism from the surface of wet rocks in Antarctica, and two Antarctic *Stichococcus* strains NJ-10 and NJ-17 was not entirely dependent on the content of unsaturated fatty acids (Hu et al., 2008; Chen et al., 2012a).

2.5. GENE REGULATION

Cold adaptation is dominated by a series of genes which may exert cumulative effects on freeze tolerance. Some cold-induced genes, such as *hiC6*, *hiC12*, *rpl10a*, *hsp70*, *Ccor1*, and *Ccor2*, have been identified in *C. vulgaris* NJ-7. Although very few variations of deduced amino acid sequences were found in these genes, the transcription of *hiC6*, *hiC12*, *rpl10a*, *Ccor1*, and *Ccor2* was greatly intensified in NJ-7 compared to that in UTEX 259, which is correlated to the significantly enhanced freeze tolerance of the Antarctic isolate (Hu et al., 2008; Li et al., 2009; Liu et al., 2011). There is a tandem array of five *hiC6* genes (*NJ7hiC6*-1, -2, -3, -4 and -5) in NJ-7 and a tandem array of four *hiC6* genes (*259hiC6*-1, -2, -3 and -4)

in UTEX 259. *NJ7hiC6-2* and *259hiC6-2* were not expressed or expressed at low levels, whereas *259hiC6-1* and *NJ7hiC6-3/4* exhibited the highest *hiC6* transcript levels in the respective strains. Unlike *hiC6*, *hiC12* is present as a single gene in the two *Chlorella* strains (Wang et al., 2011). *Ccor1* and *Ccor2* are co-organized in the same gene cluster *Ccor1-Ccor2-Ccor1-Ccor2* in the two *Chlorella* species. Interestingly, the expression of *hiC6* genes in NJ-7 can reach a relatively high level even without cold hardening, which suggests that the genes are less dependent on temperature. *Ccor1* and *Ccor2* also showed much higher expression in NJ-7 than in UTEX 259 not only at 4 °C but also at 20 °C. Thus, some of cold-induced genes in NJ-7 evolved towards constitutive expression to adapt to permanently cold environments (Wang et al., 2011).

In addition, several studies demonstrated that the expression of genes associated with photosystems increases at low temperature in the polar microalgae (Mock and Hoch, 2005). For instance, the PSII-CP47 gene was over-expressed in *Chlorella* sp. UMACC 243 grown at 4 °C, an Antarctic psychrotolerant alga from Casey (Chong et al., 2011), and the over-expression may contribute to the maintenance of photosynthetic activity.

3. Adaptation to Low-Light Conditions and Total Darkness

3.1. LOW-LIGHT CONDITIONS

UWO 241 adapted to a stable light environment of low intensity behaves differently in response to light of specific qualities. Although the alga could grow under white or blue light, cells failed to grow under red light. UWO 241 has acquired a suite of strategies to adapt to a constant low-light condition with narrow spectral quality in its habitat, such as the unusually low ratios of chlorophyll *a* to *b* as well as the low levels of antheraxanthin and zeaxanthin (Pocock et al., 2004), functional and structural augmentation of LHCII-PSII, and structurally and functionally downregulation of PSI and associated LHCI. It has been proposed that UWO 241 relies entirely on LHCII for light harvesting, and excitation of PSI occurs mainly via an energy spillover mechanism from LHCII-PSII centers (Morgan-Kiss et al., 2006).

3.2. TOTAL DARKNESS

Microalgae overwinter in a species-specific manner. *Chlamydomonas subcaudata* can form akinetes in winter, whereas *Chlorella* sp. accumulates a large amount of starch, lipids, and carotenoids within the cells (McKnight et al., 2000). Heterotrophic uptake of organic substrates by algae under total darkness may be an overwintering strategy. Another strategy for algae overwintering is the formation of cells in resting stages.

4. Adaptation to Nutrient Limitation

Nutrients are one of the most important factors that affect the physiology and biochemistry of microalgae. Thanks to their capacity to acquire the necessary resources for growth and the high affinity for nitrogen and phosphorus under severe nutrient limitation, Antarctic algae can grow under a wide range of natural conditions. Moreover, some species can grow under a variety of trophic modes, such as heterotrophic growth in the dark (Morgan-Kiss et al., 2008).

5. Adaptation to UV Radiation

The most extensive destruction of the ozone layer is present in the Antarctic continent. Over 50 % of ozone depletion occurs each spring since the past two decades (Frederick et al., 1998). Due to ozone depletion, enhanced solar ultraviolet radiation (UVR) has become one of the major stresses for many phototrophic organisms in Antarctica ecosystems. The effects of UVR stress on Antarctic freshwater microalgae include reduction of photochemical yield and photosystem II activity (Lesser et al., 2002; Hughes, 2006). Higher doses of ultraviolet-B (UVB) exert harmful effects on DNA replication, transcription, and translation (Buma et al., 1997) and thereby lead to increasing mortality. The percentage of polyunsaturated fatty acids in an Antarctic *Chlamydomonas* sp. decreased in response to high doses of UVB (Wong et al., 2007). Antarctic microalgae cope with the adverse environment by synthesis of UV-absorbing pigments, resorting to DNA repair system and other strategies in response to enhanced UVB radiation (Singh et al., 2011).

6. Adaptation to Desiccation and Freeze-Thaw Cycles

Desiccation and freeze-thaw cycles have a deleterious effect and result in very extensive mortality of algae. Nevertheless, Antarctic green algae could maintain photosynthetic capacity and intracellular solutes under repeated freeze-thaw cycles between 5 and -4 °C (Hawes, 1990). The thick walls, mucilaginous layers, and formation of cell aggregates of Antarctic microalgae probably provide protection against freeze-thaw cycles and desiccation (Morgan-Kiss et al., 2006).

7. Adaptation to High Salinity

Cold tolerant green algae in permanently cold environments may also show salinity tolerance due to cross adaptation. A higher degree of unsaturated fatty acids in the membrane lipids of Antarctic microalgae might be an adaptive advantage to the high-salinity stress. UWO 241 exhibits a wider range of salinity for growth

and is able to withstand high salinities (exceeding that of seawater) (Pocock et al., 2011). Genes encoding CvFAD2 and CvFAD6 have been identified to be involved in high-salinity acclimation in NJ-7 (Lu et al., 2009; Lu et al., 2010).

8. Conclusions

Antarctic freshwater green algae not only survive but thrive in their extreme habitat. Comparative studies on the responses to temperature, salinity, and desiccation of Antarctic, tropical, and temperate algae have revealed some of the adaptive strategies of Antarctic freshwater green algae to the harsh environments. To be specific, comparative studies of two *C. vulgaris* (NJ-7 and UTEX 259) and two *C. raudensis* (UWO 241 and SAG 49.72) facilitate our understanding of the intraspecies microevolution and may unveil the adaptive molecular mechanisms in response to Antarctic extreme conditions.

However, studies on Antarctic freshwater chlorophytes are still superficial. Since no genome sequences of psychrophilic or psychrotolerant green algae are available yet, studies on the genetic basis and molecular mechanisms of cold-adapted Antarctic green algae are mainly focused on specific genes and in particular on cold-adapted enzymes. Excitingly, the whole genome sequencing along with transcriptome analysis of Antarctic *Chlorella* strain NJ-7 and temperate *Chlorella* strain UTEX 259 is in progress, which may help to fully interpret the cold adaptation mechanism of microalgae to the extreme coldness (Wang et al., 2011).

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**PART V:
PRESSURE**

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DEEP SUBSURFACE OIL RESERVOIRS AS POLY-EXTREME HABITATS FOR MICROBIAL LIFE. A CURRENT REVIEW

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1. Introduction

Microbial life has proven to be adapted to various extreme conditions on earth, including extremely cold and hot, acidic and alkaline, as well as high salt (Allen and Banfield, 2005; Podar and Reysenbach, 2006). Oil reservoirs located deep within the earth crust are providing not only very high temperatures but in addition often high pressure and high salt, heavy metal, and organic solvent concentrations (Youssef et al., 2009). Consequently, microorganisms tolerating and propagating under such conditions are truly poly-extremophiles, being both (hyper)thermophilic, piezotolerant, halophilic, and solventophilic (Kotlar, 2012). Oil reservoir microbial communities are interesting research objects, also due to their potential impact on oil production and their relevance for industrial bioprocess applications including approaches of Biologically activated Enhanced Oil Recovery (Bio-EOR) and bioprospecting for thermostable biocatalysts applicable in industrial bioprocesses. Due to the enormous commercial values associated with oil production, bio-probing for the purpose of reservoir monitoring and the development of complementary methods in search for new oil prospects also represents fields with potentially high impacts.

To date, numerous oil reservoirs worldwide have been studied with respect to their content of microorganisms (Fig. 1). These studies included (1) the description of new genera, species, and strains of both *Bacteria* and *Archaea*; (2) the specific enrichment and characterization of subpopulations like methanogens and sulfate-reducing bacteria (SRB), the latter being discussed to be related to reservoir and oil production problems like souring and corrosion; (3) cultivation-dependent and cultivation-independent studies aiming at characterizing the

complexity of individual microbial communities, nowadays promoted by flourishing new cultivation-independent technologies like the meta'omics (metagenomics, metatranscriptomics, etc.); and (4) comparative studies covering multiple, distantly located reservoir sites. For such approaches, the access to representative, uncontaminated sample material is important, though often difficult to achieve and thus representing a major obstacle. Metagenome analysis and upcoming new technologies can be expected to revolutionize the view on oil reservoir microbial communities in the near future.

In this chapter, we follow the traditions of previous, excellent reviews, for example, by Magot and coworkers (Magot et al., 2000), focusing on microbiology in oil reservoirs with in situ temperatures of 50 °C or higher, including (hyper) thermophilic strain isolations and descriptions from such locations.

2. Microorganisms and Microbial Consortia of High-Temperature Oil Reservoirs

During the past three decades, many high-temperature oil reservoirs (≥ 50 °C in situ temperature) in Europe, North America, South America, Africa, Asia, and Australia have been subject to microbiological characterizations (see Fig. 1 for study types and references). The longest tradition of such studies exists in Europe and North America starting in the late 1980s, while Asian sites (particularly Chinese) have gained an increasing attention during the last few years. South American high-temperature reservoirs have only rarely been investigated so far, and a number of studies including some of the early consortia characterizations cover multiple sampling sites on different continents. The best surveyed region in this respect is the North Sea and the European part of the North Atlantic Ocean, both concerning consortium studies (mainly cultivation dependent) and related to the description of new species and strains. After 2006, obviously responding to the revolutionary technological developments in high throughput sequencing, culture-independent approaches have increased in number, mainly using samples from Asia but also from South America and Europe. Metagenomic (Kotlar et al., 2011) and other not yet applied meta'omic (e.g., metatranscriptomic, metaproteomic) approaches to study entire microbial communities, including the metabolically active fractions, can be expected to be applied in increasing frequency in the near future. Such studies will likely provide unprecedented insight into microbial in situ processes both in pristine reservoirs and reservoirs subjected to methods of enhanced oil recovery. Up to now, sampling has most frequently originated from production water or wellheads, and a large fraction of the reservoirs have also been flooded with production-associated water prior to sampling (see Fig. 1). This means that many of the strains or consortia described in the literature are likely to be contaminants relative to the original, untouched reservoir. Therefore, the significance of future microbial community studies of oil reservoirs will to a large degree depend on the quality of the sample material (closer discussed in Sect. 3), and some of the latest studies have already applied dedicated sampling devices that account for this type of challenge (Yamane et al., 2008; Kotlar et al., 2011).

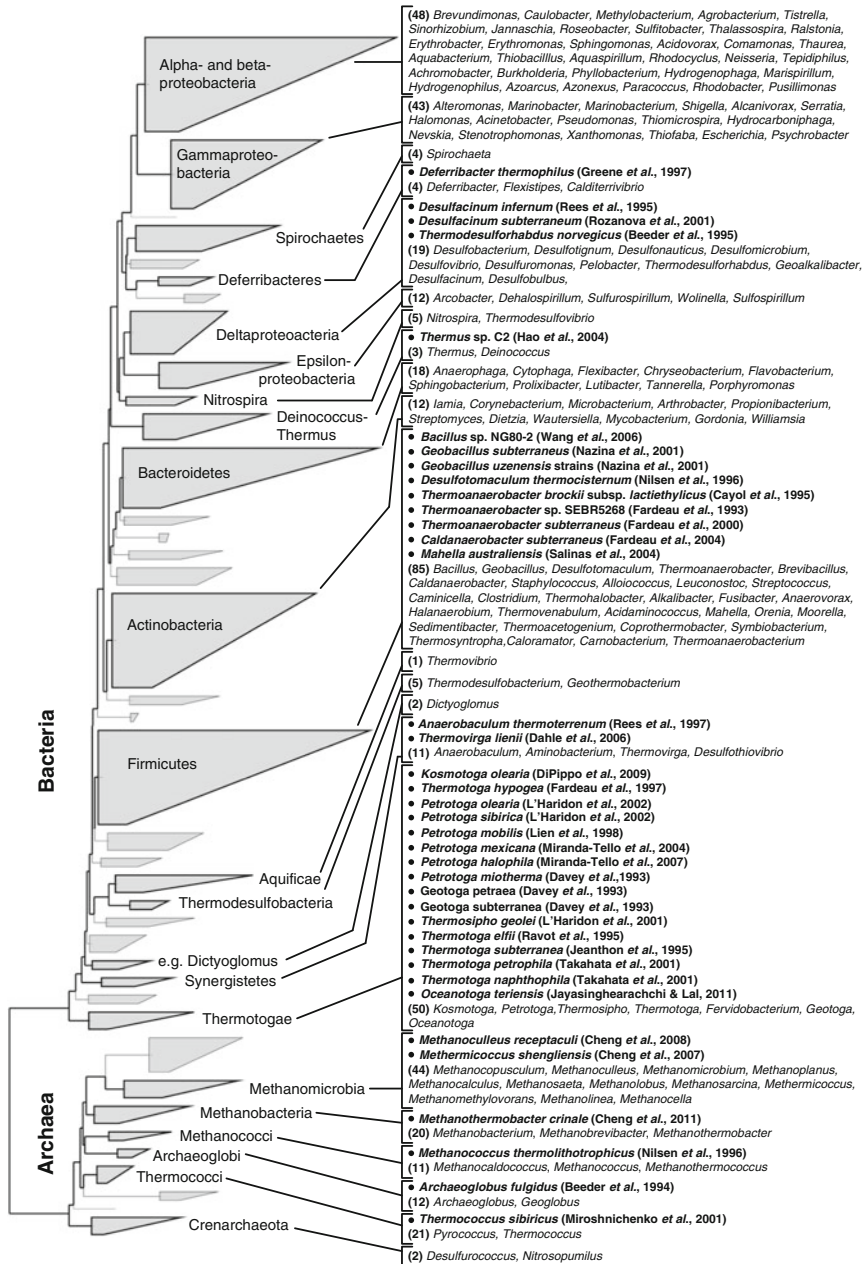
2.1. NEW MICROBIAL ISOLATES

A large number of both bacterial and archaeal species have for the first time been described as isolates from high-temperature oil reservoirs (see Fig. 2 for species names and references). Regularly, enrichment cultures have been grown, resulting in isolation of pure strain cultures, which subsequently have been characterized in detail to define their taxonomic placement in the microbial tree of life (Fig. 2). The Thermotogae, represented by the genera *Petrotoga* (6 species), *Thermotoga* (5 sp.), *Geotoga* (2 sp.), *Kosmotoga*, *Oceanotoga*, and *Thermosiphon*, account for the highest number of new strain isolates from high-temperature oil reservoirs, followed by the Firmicutes with the genera *Thermoanaerobacter* (3 sp.), *Geobacillus* (2 sp.), *Bacillus*, *Desulfotomaculum*, *Caldanaerobacter*, and *Mahella*. Isolates from other bacterial groups represent the genera *Deferribacter* (Deferribacteres), *Thermus* (Deinococcus-Thermus), *Anaerobaculum*, and *Thermovirga* (both Synergistetes). Archaeal isolates from high-temperature oil reservoirs belong to the genera *Methanoculleus* and *Methermicoccus* (both Methanomicrobia), *Methanothermobacter* (Methanobacteria), *Methanococcus* (Methanococci), *Archaeoglobus* (Archaeoglobi), and *Thermococcus* (Thermococci).

It needs to be mentioned that it is widely accepted that only a very small fraction of microorganisms can be readily cultivated using established methods (Rappé and Giovannoni, 2003). In that sense, the listing of strain isolates in Fig. 2 (bold/bullet points) includes the strong bias of cultivability and therefore does not represent a valid picture of predominant species present in oil reservoirs. In addition, it cannot be ruled out that some of these strains are not indigenous to the sampled reservoir, but rather represent contaminations from oil production processes or sampling.

2.2. CULTIVATION-DEPENDENT CONSORTIAL STUDIES

The majority of studies based on high-temperature oil reservoir derived sample material, including most of the strain isolations and descriptions mentioned above (Sect. 2.1), include enrichment culture prior to strain isolation or 16S rRNA gene amplification, library cloning, and sequence analysis (see Fig. 1 for references). Such enrichment steps are bound to introduce the potentially strong bias of cultivability in the analysis of a community composition within an oil reservoir (see comment at the end of Sect. 2.1), often on top of putative contaminations and biases originating from sampling and the way the respective original reservoir content has been altered by production processes. In spite of all these concerns, we found it interesting to relate all the reported organisms to the microbial tree of life, to see if some major trends could be observed (Fig. 2). The figure also includes the species names of the isolated strains (Sect. 2.1, bold/bullet points) and different genera detected in cultivation-independent studies (Sect. 2.3). It is striking from this figure that Proteobacteria are very frequently reported as being present based



on consortium studies, while they seem to never have been identified as single novel isolates from oil reservoir samples growing at 50 °C or above. The reason for this discrepancy is not clear but could potentially be related, for example, to the conditions commonly used for strain purification, which may be in disfavor of this group of organisms. Another possible explanation might lie in contaminating DNA, leading to misinterpretations of obtained 16S rRNA gene sequence data. For the remaining parts of the tree, the correlation between individual isolates and community compositions is more consistent, and the Firmicutes and Thermotogae are heavily represented. The same accounts for various *Archaea* (like Methanomicrobia, Methanobacteria, and Thermococci). This indicates that members of these taxonomic groups indeed are typical indigenous inhabitants of hot oil reservoirs, a conclusion that also appears reasonable based on their biological properties.

2.3. CULTIVATION-INDEPENDENT STUDIES

Many cultivation-dependent studies performed so far and discussed in Sect. 2.2 include approaches of amplification of ribosomal 16S rRNA genes followed by cloning and sequence determinations. Some of these studies follow experimental set-ups that in parts do not involve enrichment steps prior to 16S rRNA gene amplification (Orphan et al., 2000; Bonch-Osmolovskaya et al., 2003; Sette et al., 2007; Brakstad et al., 2008; Dahle et al., 2008; Korenblum et al., 2012). In addition, increasing numbers of studies in the most recent years, predominantly based on Asian reservoir samples, do not include enrichment steps at all, rendering them completely cultivation independent (see Fig. 1 for references). Such procedures should implicate a less biased picture of the microbial population in a given sample, particularly if contamination sources have also been minimized. 16S rRNA-based studies are limited with respect to the information that can be obtained, in the sense that the entire genetic make-up of the corresponding organisms remains unknown. To overcome these limitations, our group recently carried out a nontargeted metagenomic approach to sequence metagenomic DNA from an oil reservoir on the Norwegian Continental Shelf (Kotlar et al., 2011). In this case, we applied a pressurized sample methodology to a reservoir that had not been contaminated by sea water breakthrough. This study confirmed the presence of typical thermophilic bacteria (e.g., Thermotogales taxa) and Archaea (e.g., methanogens), but an apparently diverse group of Proteobacteria was also predicted from this study (Sect. 2.2). This may indicate that such bacteria are actually abundant in hot oil reservoirs but that they for some reason have not been cultivated as individual strains.

2.4. STUDIES SPECIFICALLY ADDRESSING RESERVOIR PROBLEMS

Some groups of microorganisms are seemingly related to specific reservoir conditions and problems like reservoir souring and corrosion which are discussed to be a consequence of microbial processes taking place during oil production (see Sect. 4.6),

especially in oil reservoirs exploited by application of secondary recovery methods like the injection of sea or fresh water. As a consequence, a number of studies aim at enriching and describing the microbial species possibly involved in such reservoir problems, like for the souring case, sulfate-reducing bacteria (SRB), which in several studies have been enriched and analyzed with respect to taxonomy and/or metabolism (Rosnes et al., 1991; Leu et al., 1998, 1999; Rozanova et al., 2001a; Kaster et al., 2007). Though specific markers for SRB exist (e.g., the *dsrAB* and the *aps* genes (Teske et al., 2003)), they have obviously never been applied in the context of high-temperature oil reservoir studies. Other examples of enrichment and analysis of a specific subfraction of an oil reservoir microbial community are the analysis of methanogens by enrichment and sequencing of the *mcrA* and *assA* genes (Mbadinga et al., 2012) and organisms capable of alkane degradation/hydrocarbon oxidation using *alkB* gene enrichment (Shestakova et al., 2011).

3. Oil Reservoir Sample Recovery and Processing

Deeply buried oil reservoirs are particularly challenging to sample, at least if it is considered important to keep the level of contamination at a minimum. Localization, logistics, knowledge about sampling methodology, knowledge about reservoir history, as well as the dependence on extensive oil company collaboration are relevant factors influencing the sampling. Most of the studies reported in the literature are therefore not meeting the ideal requirements for understanding the composition and metabolic performance of the indigenous microbial communities. Since we believe that these problems are very important for the future progress in the field, we have chosen to elaborate more on these problems in the Sects. 3.1, 3.2, and 3.3 below.

3.1. SAMPLING METHODOLOGY

Oil reservoir samples for microbial studies can be collected in various ways, and the samples themselves can be very different, for example, originating from the oil phase (Pineda-Flores et al., 2004), from the water phase (Nilsen et al., 1996b), or from drilling cores (Spark et al., 2000). They can also be sampled from different parts of the technical infrastructure, like wellheads (l'Haridon et al., 2002; Bonch-Osmolovskaya et al., 2003), first separators (Leu et al., 1999; Brakstad et al., 2008), or tanks (Takahata et al., 2001). In addition, some of the reported samples are “mixed” (e.g., Rozanova et al., 2001a), containing material from more than one oil well, whereas others originate from single wells only (e.g., Li et al., 2006; Kotlar et al., 2011). Even if it can be seen as an oversimplification, one may probably assume that the more distant the sampling point is from the actual oil reservoir, the higher in general is the risk for contamination (discussed below).

In addition to differences in sampling sites, the methods used for sample withdrawal can also be very different. The overall impression from the relevant literature is that sampling methodology is often poorly described. Some studies use samples collected by tapping a pipeline at atmospheric pressure (e.g., Magot et al., 2004; Dahle et al., 2008; DiPippo et al., 2009), whereas other collect pressurized samples (e.g., Kotlar et al., 2011; Yamane et al., 2011) to avoid cell lysis (Sect. 3.2.2). Even if, to our knowledge, only applied in one study so far, there are technical possibilities for more advanced sample collection, where a sample is collected in situ in an oil reservoir well, enclosed, and transported up to the platform with a maintained high pressure (Yamane et al., 2011). Such a procedure further minimizes the loss of representativeness due to cell lysis and potential contamination of the sample from pipelines and other infrastructures, however, being extremely costly due to the advanced equipment used and a longer interruption of oil production from the well.

3.2. SPECIAL CHALLENGES IN SAMPLE COLLECTION

Sample collection from deeply buried oil reservoirs is virtually impossible without a close collaboration with the relevant oil company. Due to safety regulations for oil platforms and limited access to these areas, people with very different educational background are commonly involved at the different stages of the sample collection procedure, and samples are often collected by personnel lacking microbiological background, increasing the risk of contamination (see Sect. 3.2.2). Different challenges apply, dependent on the type of sample to be collected and the sampling methodology. Some of the main and particular pronounced aspects are described below.

3.2.1. *Access to Reservoir Samples*

Oil reservoirs are often remotely located, resulting in logistical challenges concerning sampling equipment and sampled material, leading to longer transportation times than preferred from a microbiological point of view. Due to these obstacles, oil reservoir samples are in practice restricted to selected research groups only, i.e., those that have managed to establish a collaboration agreement with an oil company. The process of sample collection from producing oil wells is often in conflict with a desirable continuous oil production and the associated enormous value generation. A stop in oil production for the purpose of sampling involves beside the direct economic loss often also substantial risks for the oil company (e.g., clogging of production pipes), rendering establishment of such collaboration a difficult task.

3.2.2. *Representativeness of Sample Material*

The question of whether or not microbes isolated/described/identified using oil reservoir samples are indigenous or not, is a constantly debated issue in the literature (e.g., Magot et al., 2000; Youssef et al., 2009). The first issue is the origin

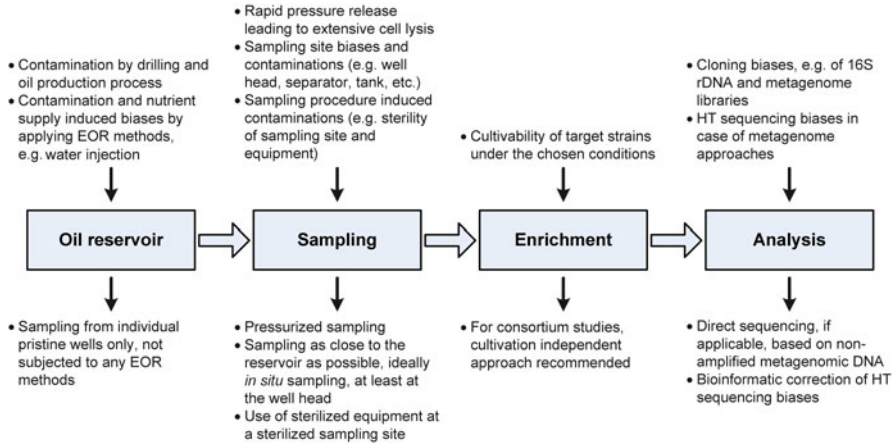


Figure 3. Sources of contamination and experimental biases as well as suggestions for their minimization for representative consortium studies of oil reservoirs.

of the microbes analyzed, whether or not they originate from the actual original reservoir or if they have been introduced to the oil reservoir by processes like drilling or well treatment (Fig. 3). In addition, the exact origin of the sample material might be difficult to elucidate, since sediment particles and fluids might, apart from the actual reservoir site, originate from pipelines and platform infrastructures. Samples used for reported studies have also been found to be collected at different structures, and depending on the sampling site, non-indigenous microbes within the sample are often likely to occur. However, isolation of new strains and description of microbes from oil reservoir samples, indigenous or not, may still be valuable for certain types of studies, for example, within bioprospecting.

Most studies using oil reservoir samples are performed on samples subjected to a rapid pressure reduction (transportation of the oil sample to the platform followed by the release of sample pressure at the production liner), potentially resulting in substantial cell lysis within the sample (similar to the effect of a French pressure cell press). A few studies (Kotlar et al., 2011; Yamane et al., 2011) perform sampling into pressure flasks to maintain high pressure upon sampling. From these flasks, upon arrival in the laboratory, pressure is subsequently released by a procedure that is slow enough to presumably avoid extensive cell lysis. This approach does most likely result in the collection of a more representative sample with respect to the *in situ* microbial community. Therefore, such samples are preferred for total oil reservoir microbial community descriptions (Fig. 3).

The analysis and downstream handling of the reservoir samples might also influence whether or not indigenous microbes are analyzed (Fig. 3). Cultivation in general might be selective for nonindigenous microbes, since the *in situ* reservoir conditions might be difficult to mimic in the lab (e.g., Whitman et al., 1998; Reeder and Knight, 2009). Analysis of isolated DNA would therefore be a more accurate

approach for the characterization of microbial communities (e.g., Amann et al., 1990; Reeder and Knight, 2009); however, contamination of the sample might still pose a problem, and DNA-based methods might also be biased towards more well-known microorganisms (e.g. primer design for 16S rRNA gene analysis). To date, a metagenomic approach (analyzing the total DNA content, i.e., the metagenome, of the sample) is believed to be the most accurate approach to environmental microbial community characterization (Cowan et al., 2005; Quince et al., 2008; Sleator et al., 2008).

3.3. LIMITATIONS AND RESTRICTIONS ON SAMPLE INFORMATION AND DISSEMINATION OF ANALYSIS RESULTS

Characterization of oil reservoir microbial communities usually requires (as described above) the collaboration with an oil company, which often raises the issues of confidentiality and Intellectual Property Rights (IPR). Academic groups are widely required to sharing research result by publication. However, due to the enormous investments in oil production and corresponding company secrecy policies, sharing of background information related to the sampled reservoir and the sample origin may be restricted. As a consequence of this, limited background information, for example, about the physicochemical characteristics of the reservoirs (even though existing), may complicate an interpretation of the results of analyses from these environments.

Also IPR issues might influence the research on oil reservoir microbial communities, since dissemination of results may be delayed due to patenting processes, or even be excluded from publication or patenting, and kept undisclosed. Findings in studies of oil reservoir microbes can be of high economical value for oil companies (or other companies), and hence, carefullness is indicated when sharing information from such findings. Since an in depth understanding of oil reservoirs as habitats for microbial life still is in its infancy, and in addition, such environments provide very special environmental conditions, new species with yet unknown but highly desired qualities or new enzymes with high industrial potential might be revealed in such studies.

4. Metabolic Capabilities of Oil Reservoir Microorganisms

As in many microbial habitats, the in situ microbial metabolic processes within oil reservoirs are likely to be very diverse. The processes are obviously dependent on the different groups of organisms present within the reservoirs at any given time, as well as on nutrients and other compounds available for consumption. These are parameters that differ from reservoir to reservoir and might also change within the same site due to natural processes and/or anthropogenic influences. In general, due to the absence of oxygen, microorganisms indigenous to oil reservoirs can be

expected to be capable of anaerobic metabolism. In turn, strictly aerobic species unambiguously detected in reservoir samples are likely to be contaminants either from oil production processes or sampling. Due to the relatively limited knowledge of oil reservoir microbial communities, the understanding of the true in situ processes is also limited and will in most cases be rather speculative. However, by increasing knowledge on the composition of these microbial consortia and comparison with related (and currently better understood) habitats, some main metabolic processes of oil reservoirs can be predicted, and the main groups of microbes expected to be involved are discussed below.

4.1. SULFATE REDUCERS

Sulfate-reducing bacteria (SRB) constitute a group frequently isolated from oil reservoir samples (e.g., Rosnes et al., 1991; Tardy-Jacquenod et al., 1996; Leu et al., 1998, 1999; Kaster et al., 2007; Kotlar et al., 2011). SRB taxa are in many cases also likely to be able to reduce other sulfur-containing compounds (like sulfite or thiosulfate), and hence the name sulfate-reducing bacteria might in some cases be misleading. SRB recovered from oil reservoir samples do seemingly belong to different phyla; Proteobacteria, mainly Deltaproteobacteria as, for example, Desulfovibrionales and Desulfomonadales species (Beeder et al., 1995; Rees et al., 1995; Rozanova et al., 2001b), Firmicutes (Nilsen et al., 1996a, b; Magot et al., 2000; Dahle et al., 2008; Youssef et al., 2009; Kotlar et al., 2011), and Thermodesulfobacteria species (Beeder et al., 1995; Yamane et al., 2011). There are also several archaeal species recovered from oil reservoirs that exhibit sulfate-reducing capabilities, exemplified by *Archaeoglobus fulgidus* (Beeder et al., 1994), *Archaeoglobus lithotrophicus* (Stetter et al., 1993), and *Thermococcus* sp. (Slobodkin et al., 1999; Orphan et al., 2000).

SRB can partly or completely oxidize a broad variety of substrates, including aromatic and aliphatic constituents of petroleum oil, coupled to the reduction of sulfate to hydrogen sulfide (H_2S) (Rosnes et al., 1991; Nilsen et al., 1996b; Rabus et al., 1996). H_2S accumulation promoted by SRB activities might in some cases result in reservoir souring (Sect. 4.6.1). SRB strains normally grow by using sulfate, sulfite, or thiosulfate as electron acceptors; otherwise, their growth is linked to fermentative processes in the absence of these electron acceptors.

4.2. METHANOGENS

Methanogenesis is the final step in the complex processes involved in the anaerobic degradation of organic matter (such as petroleum oil), and methanogens are microbes able to convert hydrogen (H_2), carbon dioxide (CO_2), and fermentative substrates (e.g., acetate) into methane (Magot et al., 2000; Gray et al., 2009). These organisms do all belong to the archaeal domain and typically live in

anaerobic habitats, often attributing extreme conditions. Methanogens are generally divided into three distinct groups depending on their substrates: hydrogenotrophs (H_2/CO_2), methylotrophs (methylated compounds), and acetoclasts (acetate) (Garcia et al., 2000).

There are numerous examples of methanogens recovered from, or identified in, oil reservoir samples, and several different genera have frequently been identified, such as *Methanocalculus* (Ollivier et al., 1998; Li et al., 2007a, 2012; Yamane et al., 2011; Mbadinga et al., 2012), *Methanoculleus* (Ollivier et al., 1998; Orphan et al., 2000; Li et al., 2007a, 2012; Brakstad et al., 2008; Cheng et al., 2008; Lan et al., 2011; Yamane et al., 2011; Mbadinga et al., 2012; Tang et al., 2012), *Methanobacterium* (Belyaev et al., 1983; Orphan et al., 2000; Bonch-Osmolovskaya et al., 2003; Li et al., 2007a, b, 2012; Ren et al., 2011; Yamane et al., 2011; Tang et al., 2012), *Methanothermobacter* (Bonch-Osmolovskaya et al., 2003; Nazina et al., 2006; Li et al., 2007a, b; Cheng et al., 2011; Lan et al., 2011; Ren et al., 2011; Yamane et al., 2011; Mbadinga et al., 2012; Tang et al., 2012), and *Methanococcus* (Nilsen and Torsvik, 1996; Orphan et al., 2000; Li et al., 2007a, b, 2012; Kaster et al., 2009; Kotlar et al., 2011). The different species seemingly use different substrates for growth but all produce methane, either by their own metabolism completely, but in many cases in syntrophic interactions with other microorganisms, for example, SRB and/or fermentative bacteria (Garcia et al., 2000; Scholten et al., 2007; Dar et al., 2008; Wintermute and Silver, 2010); see Sect. 4.5 below.

4.3. FERMENTATIVE BACTERIA

Fermentative microorganisms constitute an important part of oil reservoir microbial communities. Several types of mesophilic fermentative bacteria have been isolated from low-temperature oil reservoirs (Magot et al., 2000), but for the thermophilic fermenters, the largest fraction of recovered species are members of the Thermotogae phylum (Davey et al., 1993; Jeanthon et al., 1995; Ravot et al., 1995; Fardeau et al., 1997; Lien et al., 1998; Orphan et al., 2000; l'Haridon et al., 2001, 2002; Takahata et al., 2001; Miranda-Tello et al., 2004, 2007; DiPippo et al., 2009; Youssef et al., 2009; Jayasinghearachchi and Lal, 2011; Mbadinga et al., 2012) or the family of Thermoanaerobiaceae (Fardeau et al., 1993, 2000; Cayol et al., 1995; l'Haridon et al., 1995; Leu et al., 1998; Li et al., 2007b).

Most Thermotogales species isolated are able to grow on complex substrates (e.g., amino acids, sugars, and peptides), reducing sulfur and/or thiosulfate (Fardeau et al., 1997; Takahata et al., 2001). The Thermoanaerobacteriales ferment sugars (e.g., Cayol et al., 1995; Grassia et al., 1996), organic acids (e.g., Rees et al., 1997), and/or amino acids (e.g., Dahle and Birkeland, 2006) and typically reduce thiosulfate to sulfide or elemental sulfur, using electrons from carbohydrates or hydrogen (Magot et al., 2000). Common end products are H_2 , CO_2 , and acetate, compounds that are often used as substrates by other microbes within the habitat, indicating

interactions and syntrophy involving fermentative microbes within the oil reservoirs (Sect. 4.5). There are also data indicating presence of fermentative archaeal species, like Thermococci and Pyrococci, within the oil reservoir habitats (e.g., l'Haridon et al., 1995; Miroschnichenko et al., 2001; Kaster et al., 2009; Kotlar et al., 2011; Lan et al., 2011; Ren et al., 2011; Lewin et al., 2013). When isolated and cultivated in the laboratory, such strains usually grow on complex carbon sources and reduce elemental sulfur to sulfide (Stetter et al., 1993).

4.4. OTHER GROUPS

Oil reservoir microbial communities may contain species belonging to other groups than those described above. Several studies have detected iron-reducing bacteria like *Deferribacter thermophilus* able to reduce iron or manganese (Greene et al., 1997; Orphan et al., 2000), *Alteromonas/Shewanella* species capable of iron, elemental sulfur, sulfite, and thiosulfate reduction (Magot et al., 2000; Brakstad et al., 2008), as well as nitrate-reducing bacteria (NRB), like different *Geobacillus* species (Nazina et al., 2001; Bonch-Osmolovskaya et al., 2003; Li et al., 2007b; Shestakova et al., 2011). However, due to the lack of data about availability of, for example, nitrate, iron, or manganese levels in oil reservoirs (as exists in higher extent for sulfate and methane), the relevance of metabolic processes related to these compounds is harder to evaluate and therefore becomes very speculative.

4.5. SYNTROPHIC INTERACTIONS WITHIN OIL RESERVOIR MICROBIAL CONSORTIA

Microbial processes within environmental habitats are usually not independent of each other, but rather connected, and many microbes are likely to form syntrophic interactions (Wintermute and Silver, 2010). The complexity of such interactions and their dependence on microbial growth and available nutrients make most discussions speculative. However, based on existing data and processes likely to occur, some syntrophic interactions can be expected within oil reservoir habitats. Furthermore, it has been shown that syntrophic processes in general result in low-energy yields and consequently slow growth of the microbes involved (McInerney et al., 2009). This is consistent with reports indicating slow growth of subsurface microbial communities (e.g., Price and Sowers, 2004; Jørgensen and D'Hondt, 2006; Morono et al., 2011), which is likely to also be true for microbes prevailing in oil reservoirs.

The presence of SRB and methanogens within the habitat and the indication of active methanogenic hydrocarbon metabolism within the oil fields (e.g., Jones et al., 2008) suggest the presence of syntrophic interactions between SRB and methanogens and possibly with additional microbial groups (Jones et al., 2008; Pernthaler et al., 2008; Gray et al., 2009; Mayumi et al., 2011). It has been shown

that SRB and methanogens under some conditions compete for the same substrates (electrons and H_2) produced by fermentative microbes (Dar et al., 2008). At high-sulfate levels, SRB growth will be favored (due to their thermodynamically favorable process), whereas at lower-sulfate concentrations, substrates will be used by methanogens. However, SRB do not compete for methylated substrates; hence, methylotrophic methanogens are then favored even at high-sulfate concentrations (Cetecioglu et al., 2009; Lazar et al., 2011).

Methanogens are capable of using different substrates; hence, methanogenesis is likely to occur under several conditions. In environments containing complex organic compounds, such as oil reservoirs, and with low levels of sulfate and nitrate, methanogens are reported to be linked to chemoheterotrophic bacteria for organic substrate degradation (Garcia et al., 2000). Polymers are microbiologically degraded, resulting in simpler organic compounds utilized for acidogenesis by fermentative bacteria, which in turn produce substrates for methanogens or for syntrophic bacteria. The resulting simple methylated compounds, acetate, alcohols, and H_2/CO_2 are then consumed by methanogenic *Archaea* in methanogenesis. The syntrophic interactions are dependent on a H_2 -consuming part in the interaction, keeping H_2 -levels low and hence making the whole reaction thermodynamically favorable (McInerney and Bryant, 1981).

4.6. IMPACT OF OIL RESERVOIR MICROBIAL PROCESSES ON PETROLEUM OIL AND OIL PRODUCTION

Metabolic processes attributed by oil reservoir microbial communities will, to a smaller or larger extent, have an impact on the petroleum oil and oil production from the reservoir. Different groups of microbes are proposed to have different effects and can affect reservoir souring, oil recovery, or corrosion of infrastructures (Youssef et al., 2009). Some processes do seemingly have negative effects on oil production, whereas others are suggested to aid oil recovery, normally referred to as Microbial Enhanced Oil Recovery (MEOR) or Biologically activated Enhanced Oil Recovery (Bio-EOR). Due to the constant need for increased oil recovery, progression within this field is naturally desirable. However, this is a complex and challenging research area. There are supposed differences between lab-scale experiments and actual oil field effects, as well as various challenges (e.g., long-term effects) connected to field studies, both being examples of factors complicating these studies.

4.6.1. Negative Effects of Microbial Processes in Oil Production

SRB (Sect. 4.1) are one of the most well-known microbial groups of oil reservoirs due to their possible different effects on petroleum oil and oil recovery. In situ growth of SRB using sulfate as electron acceptor can result in accumulation of H_2S and consequently in reservoir souring (Bødtker et al., 2008). This might be a consequence of reservoir flooding, since seawater introduced into the reservoir usually is high in sulfate levels, promoting growth of SRB and consumption of H_2

for H_2S production over other processes (e.g., methanogenesis, see Sect. 4.5). However, not all flooded reservoirs are soured, and hence the level of reservoir souring also seems to depend on additional factors. Reservoir souring is often associated with plugging (due to accumulation of sulfide minerals), problems with corrosion of pipes, and platform structures and with risks associated with the toxicity of H_2S (Cord-Ruwisch et al., 1987; Myhr et al., 2002; Duncan et al., 2009). Naturally, petroleum oil is rich in hydrocarbons and may hence be seen as microbial growth substrates within the reservoir. Several species of the microbial communities are able to degrade oil constituents and use them as carbon source. However, due to the expected lack of other nutrients, growth (and thereby oil consumption in situ) is limited. Different microorganisms and potential genes expected to play a role in oil degradation have been identified and analyzed (e.g., Head et al., 2006). One specific example is methanogens degrading petroleum hydrocarbons and producing methane gas (Jones et al., 2008; Gieg et al., 2010). SRB and Fe(III)-reducing bacteria are also expected to degrade hydrocarbons in oil reservoirs considering the presence of sulfate and Fe(III) oxides and the reported capacity of these microorganisms to degrade hydrocarbons (Van Hamme et al., 2003).

4.6.2. MEOR and Bio-EOR

Many oil fields are approaching tail production, and hence, various tertiary methods for enhanced oil recovery (EOR) are wished for and desirable to apply within these reservoirs. There are different microbial processes and products derived from microbial metabolism that are suggested to have positive effects on oil recovery from a reservoir site (MEOR/Bio-EOR processes). These involve the reduction in oil viscosity by production of solvents or gases, increase in oil mobilization by hydrocarbon metabolism, or production of emulsifiers (Belyaev et al., 2004; Sen, 2008). Potentially relevant EOR methods also include reservoir flooding using alkaline solutions or additives such as biologically derived polymers and surfactants. Several studies indicate promising results; however, actual in situ processes are often difficult to monitor, and field studies might be complex to perform and interpret, which makes this a very challenging research area.

A common problem in oil production is immobilized oil trapped within reservoir sediments. Several end products from microbial processes, like gases (CO_2 , H_2), acids, and different solvents, have been proposed to reduce oil viscosity, dissolve deposits, and alter wettability, which might result in enhanced mobilization and transportation of oil and thus in increased oil recovery (Belyaev et al., 2004; Salehi et al., 2008; Youssef et al., 2009). Microbial groups potentially involved in such processes are mainly fermentative bacteria and methanogens. Oil mobilization is also believed to be aided by microbial production of biosurfactants (low molecular weight surface active agents with amphiphilic properties forming micelles) acting on the oil-water interphase and lowering the surface or interface tensions (e.g., Banat, 1995; Bordoloi and Konwar, 2009). Microbial species suggested to produce biosurfactants within oil reservoirs might be various, but

main groups are *Bacillus* sp., *Pseudomonas* sp., and *Rhodococcus* sp. (Aburuwaida et al., 1991; Li et al., 2002; Mukherjee and Das, 2005; Youssef et al., 2009). Recent findings indicate that an increased recovery might be a combined effect of both reduced viscosity by scission of heavy molecules and a strong local bio-surfactant production from activated microbial consortia. Additionally, oil biodegradation can promote the conversion of heavy (and “hard-to-recover”) oil fractions to lighter oil fractions, thus increasing oil mobilization, for example, by microorganisms able to degrade n-alkanes (Wentzel et al., 2007). Another oil production problem might be plugging of pipes by paraffin or by other deposits. These are often treated using chemical injections but might be removed by hydrocarbon degrading microorganisms injected together with or without nutrients (e.g., Lazar et al., 1999).

As mentioned (Sect. 4.6.1), SRB can in some cases cause problems in oil production, and one strategy that has been used to counteract their effects is to stimulate nitrate-reducing bacteria (NRB, Sect. 4.4) in situ. This can be done by introduction of nitrate and potentially of NRB (Bødtker et al., 2009; Lysnes et al., 2009). NRB might then compete with SRB for electron donors, oxidize undesirable high levels of sulfide, and increase the redox potential in the habitat, leading to inhibition of SRB growth (Jenneman et al., 1986; Telang et al., 1997; Nemati et al., 2001; Myhr et al., 2002; Voordouw et al., 2009). Hence, NRB stimulation might then outcompete SRB growth in situ, with reduced reservoir souring as a positive effect (Grigoryan and Voordouw, 2008).

5. Future Perspectives and Biotechnological Exploitation of High-Temperature Oil Reservoir Microbiology Research

The highest reported temperature supporting life of microorganisms is very close to 120 °C (Takai et al., 2008), and in the deep biosphere, within sediments buried 200–500 million years ago, extraordinary new types of organisms may be found. These microbes are truly poly-extremophiles, being highly thermophilic, halophilic, piezotolerant, and solventophilic. Studies of these (belonging to both the bacterial and the archaeal domain) can provide new knowledge and understanding of various oil reservoir-associated mechanisms and characteristics, as well as reveal very exciting properties since the genetic materials of these microbes may encode biocatalysts with potentially highly relevant industrial implications. Gene mining and bioprospecting for a variety of new properties in proteins and metabolites may lead to new industrial applications, including those for enhanced oil recovery: Bio-EOR (Sect. 4.6.2). The use of extremophiles in various biocatalytic processes has already provided a new wave in the biotech industry, with bioprocesses performable at temperature and pressure conditions never before considered possible, and enzymes isolated from organisms originating from oil reservoirs might furnish new incentives for the development of entirely new processes. In addition, the genetic information of these microbes also has the

potential to be developed into new tools for searching for new oil deposits in sensitive areas (like the Arctic, Antarctica, or jungle areas) using novel detection systems.

Obtaining new reserves of oil answers the increasing need of oil products and ensures a sustainable development of oil companies. Obtaining new reserves implies either to discover and develop new oil and gas fields by exploration or to increase the recovery rate of existing fields. One important research aim today is to develop biotechnological methods to enhance oil recovery (EOR). Two-thirds of the world's extractable fossil fuels lay within the category of heavy to extra heavy oil, and the world's average recovery rate from this type of oil reservoirs is only at about 7 %. Therefore, technologies that could boost these recoveries would have a tremendous economic impact. Today, different process technologies exist to extract these oils. However, these are all high-cost, high-energy, and high-emission technologies, and they are also associated with other environmental concerns. In addition to limitations in recovery of heavy oils, there are other concerns in oil recovery from present reservoir sites, including oil immobilization and plugging (Sect. 4.6.2), issues also being targets for combinations of conventional methods, and Bio-EOR processes. Further and deepened characterization of oil reservoir microbial communities is therefore very important. Not only are there numerous applicable features (suitable for both oil and biotechnology industry) to discover but also a need for an increased understanding of these communities. In order to access high quality samples for such studies, extensive collaborations with the oil industry are crucial and require well-designed and accurate sampling methods, limiting the risks of contamination and loss of sample representativeness to an absolute minimum.

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7. References

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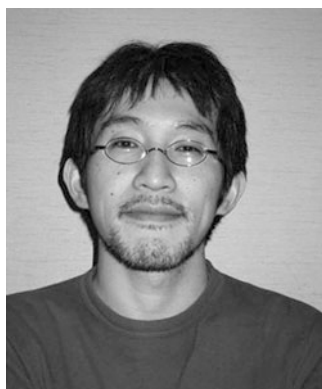
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Biodata of **Shigeru Deguchi** and **Koki Horikoshi**, authors of “*Expanding Limits for Life to a New Dimension: Microbial Growth at Hypergravity.*”

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EXPANDING LIMITS FOR LIFE TO A NEW DIMENSION: MICROBIAL GROWTH AT HYPERGRAVITY

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1. Life on a Neutron Star

Limits for life were extended dramatically in the late twentieth century with a progress in extremophiles research (Rothschild and Mancinelli, 2001; Takai, 2011). For example, Brock isolated a thermophilic bacterium, *Thermus aquaticus*, in a hot spring of Yellowstone National Park (Brock and Freeze, 1969). *T. aquaticus* broke the upper temperature limit for life at that time (73 °C) and extended it to 82–88 °C (Horikoshi and Bull, 2011). Subsequent discovery of microbial communities around deep-sea hydrothermal vents, which provide temperatures much greater than 100 °C, extended the limit even further. Currently, *Methanopyrus kandleri* strain 116, isolated from the Kairei hydrothermal field on the Central Indian Ridge, holds the record temperature of 122 °C (Takai et al., 2008).

The robustness of prokaryotic life to physical extremes has led to their ubiquitous presence on Earth (Whitman et al., 1998; Schleifer, 2004). Resilience to physical extremes is also extremely likely to be required for the existence of life beyond this planet (Rothschild and Mancinelli, 2001; Des Marais et al., 2008; Takai, 2011). Thus, exploring the physical limits of organismic viability has important implications in considering the emergence, transport, adaptation, and evolution of life in extraterrestrial habitats (Fajardo-Cavazos et al., 2006; Des Marais et al., 2008), and is also crucial in the search for life in extraterrestrial habitats, as the knowledge effectively helps in narrowing down possible targets to search (Des Marais et al., 2008).

When it comes to extraterrestrial environments, however, expanded sets of criteria should be taken into account. *Halomonas* strain GFAJ-1 is a bacterium isolated from Mono Lake in California and grows in the presence of high concentrations of arsenic. Wolfe-Simon et al., who discovered the organism, claimed that it incorporated arsenic instead of phosphorus to synthesize biopolymers, notably DNA, and challenged the hypothesis on six essential elements for life (carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus) (Wolfe-Simon et al., 2011). Although the claim has been recently refuted by two independent studies, both of which showed that GFAJ-1 is nothing more than an arsenic-tolerant bacterium and does not utilize arsenic to synthesize its DNA (Erb et al., 2012;

Reaves et al., 2012), extensive discussion over the claim illustrates the relevance that extremophiles research may potentially have on astrobiology.

Environmental parameters other than elemental composition of life also have to be addressed, and gravity is one of them. As far as terrestrial environments are concerned, gravity can be considered to be constant, but it becomes a variable when you start considering the habitability of extraterrestrial environments. An ultradense neutron star was first hypothesized in the 1930s and later realized with the discovery of a pulsar (Hewish et al., 1968). Extreme physical conditions of neutron stars, such as densities as high as 5–10 times the nuclear equilibrium density of neutrons and protons, gravities up to $10^{11} \times g$, and magnetic fields up to 10^{11} T (Potekhin et al., 2003; Lattimer and Prakash, 2004; Manchester, 2004), make these ideal test benches for existing theories of condensed matter physics (Lattimer and Prakash, 2004). Biology at such physical extremes, immense gravities in particular, also stirred imagination of scientific-fiction authors, and life in hypergravitational habitats such as neutron stars is a favorite theme for science fiction novels (Drake, 1973; Forward, 1980). However, life under ultrahigh gravities has only attracted sparse scientific attention, despite the robustness of extremophiles against other physical extremes (Rothschild and Mancinelli, 2001; Takai, 2011).

2. Microorganisms Under Hypogravity

Research on biological responses to gravity is divided into those under hypogravity (below Earth's gravity) and hypergravity (above Earth's gravity). Most previous efforts have been devoted to the former, because the effect of hypogravity on biological processes, microgravity in particular, is relevant to human health during space flight (Todd, 1989; Nickerson et al., 2004). Using microorganisms as model life forms (Nickerson et al., 2004), numerous experiments have been performed both in orbit and in Earth-based clinostats that simulate microgravity. The results demonstrate that microgravity affects microorganisms in a wide variety of ways related to their growth, physiology, pathogenesis, stress resistance, and gene expression.

The majority of the studies indicate that microgravity stimulates the growth of microorganisms (e.g., *Escherichia coli*, *Bacillus subtilis*, *Salmonella enterica* serovar Typhimurium) compared with $1 \times g$ controls (Taylor, 1974; Ciferri et al., 1986; Mennigmann and Lange, 1986; Bouloc and D'Ari, 1991; Gasset et al., 1994; Fang et al., 1997; Klaus et al., 1997; Kacena et al., 1999; Brown et al., 2002; Wilson et al., 2002a). In the case of *E. coli*, for example, the lag phase was shortened, the duration of exponential growth was increased, and the final cell population density was approximately doubled during space flights (Klaus et al., 1997).

S. enterica serovar Typhimurium showed enhanced virulence in a murine infection model (Nickerson et al., 2000; Wilson et al., 2007) conducted in space flight and under modeled microgravity compared with conditions of normal gravity (Nickerson et al., 2000; Wilson et al., 2007). These microorganisms also

showed increased resistance to environmental stresses, increased survival in macrophages, and significant changes in protein expression levels (Nickerson et al., 2000). 2D gel electrophoresis and DNA microarray analysis have been used to elucidate the molecular mechanisms of microbial responses to microgravity (Nickerson et al., 2000; Johanson et al., 2002; Wilson et al., 2002b, 2007; Purevdorj-Gage et al., 2006). Recent analysis of *S. enterica* serovar Typhimurium grown in space identified 167 transcripts and 73 proteins that changed expression compared with ground controls, and conserved RNA-binding protein Hfq was identified as a likely global regulator (Wilson et al., 2007). Gene expression of eukaryotic *Saccharomyces cerevisiae* is also affected by simulated microgravity (Johanson et al., 2002; Purevdorj-Gage et al., 2006).

3. Survival of Microorganisms Under Hypergravity

Studies on microorganisms under hypergravity have had a close tie to astrobiology from the beginning. Microorganisms subjected to hyperaccelerations on the order of $10^5 g$ have attracted scientific attention in terms of bacterial transport between planets (panspermia). The hypothesized process begins with an asteroidal impact on a donating planet followed by consequent ejection of bacteria-bearing rocks (Fajardo-Cavazos et al., 2006). Under impact conditions, ejected rocks typically experience maximum accelerations of $3 \times 10^5 \times g$ and rise times of 0.5 ms in the case of (ejection from) Mars (Mastrapa et al., 2001). Bacteria have to survive extremes in both acceleration and rate of change of acceleration (Nicholson et al., 2000).

Accordingly, previous studies that dealt with microorganisms under accelerations much greater than $1 \times g$ focused mostly on survival. Unlike in microgravity, experiments in hypergravity were performed exclusively in simulated environments and primarily by subjecting microorganisms to centrifugal acceleration in centrifuges. Spores of *B. subtilis* tolerate accelerations exceeding 10,000–15,000 $\times g$ for indefinite periods of time but were inactivated to a 10 % survival rate when they were subjected for 65 h to 436,000 $\times g$ (Nicholson et al., 2000; Mastrapa et al., 2001). Inactivation of various microorganisms, including prokaryotic *E. coli*, *Thiobacillus intermedius*, *Bacillus amyloliquefaciens*, *Staphylococcus aureus* and eukaryotic *S. cerevisiae*, was also studied after they were subjected to 450,000 $\times g$ (Yoshida et al., 1999).

4. Growth of Microorganisms Under Hypergravity

Microbial proliferation, and not simply survival, has to be studied at hyperaccelerations to address the fundamental biological question of what are the physical limits of organismic viability (Rothschild and Mancinelli, 2001) under a range of gravitational accelerations larger than those found on Earth. Compared with the relatively active research on microbial responses to microgravity, however, there are

fewer studies that report growth behavior of microorganisms under hypergravity. The studies on microbial survival under hypergravity were conducted in phosphate buffered or physiological saline at 4 °C, wherein microbial proliferation was not possible even at 1 × *g*.

Bouloc and D'Ari reported that hyperaccelerations of 3 and 5 × *g* did not affect the growth of *E. coli* (Bouloc and D'Ari, 1991), whereas Brown et al. (2002) observed growth suppression at 50 × *g*. Similar observations were reported for *Paramecium tetraurelia*, which showed no effect at 10 × *g* but a significantly lower proliferation rate and a lower population density at 20 × *g* (Kato et al., 2003).

The only study that had dealt with the proliferation of microorganisms under hyperaccelerative conditions much larger than 1 × *g* was that of Montgomery et al., in which *E. coli* suspended in nutrient broth at 35 °C was subjected to centrifugation at 1,000 or 110,000 × *g* for 24 h (Montgomery et al., 1963). They reported that the growth pattern of *E. coli* was not altered at 1,000 × *g*, but was disturbed at 110,000 × *g*. The growth at 110,000 × *g* was characterized by an increased duration of the lag phase, prolonged generation time, and decreased maximal cell concentration compared with 1 × *g* controls.

We have recently showed that various microorganisms are proliferative under hypergravity conditions by culturing five microbial strains (Gram-negative *E. coli*, *Paracoccus denitrificans*, *Shewanella amazonensis*; Gram-positive *Lactobacillus delbrueckii* ssp. *delbrueckii*; and eukaryotic *S. cerevisiae*) in nutrient media under hyperaccelerations in centrifuges (Deguchi et al., 2011). *P. denitrificans* and *E. coli* were most tolerant to hyperaccelerations among the organisms tested and showed robust growth even at 403,627 × *g*, which was the highest acceleration achievable by an ultracentrifuge used in the experiment.

Figure 1 shows the growth curves of *P. denitrificans* at different accelerations (Deguchi et al., 2011). The growth curves at 1 × *g* and 7,500 × *g* were identical within experimental error, indicating that hyperacceleration up to 7,500 × *g* did not affect the growth of *P. denitrificans* at all. The growth was slightly retarded when the incubation acceleration was increased to 74,558 × *g* and 134,425 × *g*, as judged by the slight decreases in respective final cell concentrations. At 403,627 × *g*, a distinct lag phase appeared and the growth was retarded significantly. The final cell concentration was significantly smaller at this acceleration compared with those at lower values.

Photographs in Fig. 2 show cultures of *P. denitrificans* in Luria-Bertani (LB) broth containing 25 mM KNO₃ after spinning in an ultracentrifuge at 403,627 × *g* and at 30 °C (Deguchi et al., 2011). At this acceleration, *P. denitrificans* cells sedimented and formed a pellet at the bottom of a centrifuge tube soon after centrifugation began. Initially, a pellet was not visible (Fig. 2a) because the total number of *P. denitrificans* cells in the culture was small (~10⁶ cells). However, a pellet of a visible size formed after spinning the culture for 6 h (Fig. 2b), and it increased in size with time (Fig. 2c, d).

Comparison of the generation time that was measured for all organisms showed that hyperacceleration had either no effect or a relatively small effect

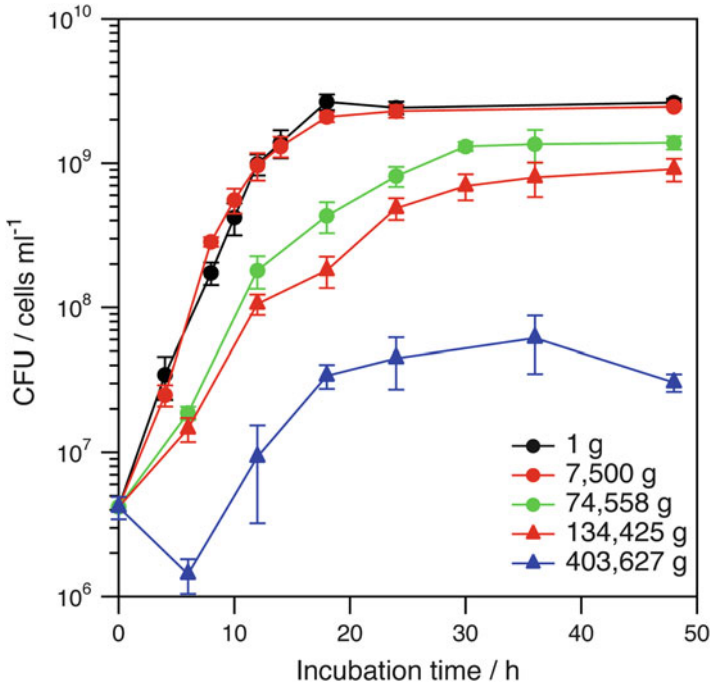


Figure 1. Growth curves of *P. denitrificans* at 30 °C and hyperaccelerations up to $403,627 \times g$ (Modified and reprinted from Deguchi et al., 2011).

on growth below $7,500 \times g$, whereas growth was significantly retarded for above $\sim 2 \times 10^4 \times g$. The effect of hyperacceleration on growth was lowest for *P. denitrificans*. For the eukaryotic *S. cerevisiae*, the observed growth behavior stands in contrast to that of prokaryotes in that the generation time became progressively longer with increased acceleration (Fig. 3).

5. Mechanical Deformation Under Hyperacceleration

Macroorganisms easily collapse under accelerations of only a few times that of Earth's surface gravity (Nicholson et al., 2000). In contrast, microorganisms are likely to show higher resistance to mechanical deformation under hyperaccelerations because the gravitational potential is proportional to the size of an object. Indeed, Yoshida et al. reported no change in the cell shape for *E. coli* and *B. amyloliquefaciens* after ultracentrifugation in physiological saline at $450,000 \times g$ and 4 °C for 24 h (Yoshida et al., 1999). We also found that the effect of mechanical deformation on cells that replicate via binary fission at hyperacceleration was negligible (Deguchi et al., 2011). Comparison of TEM images

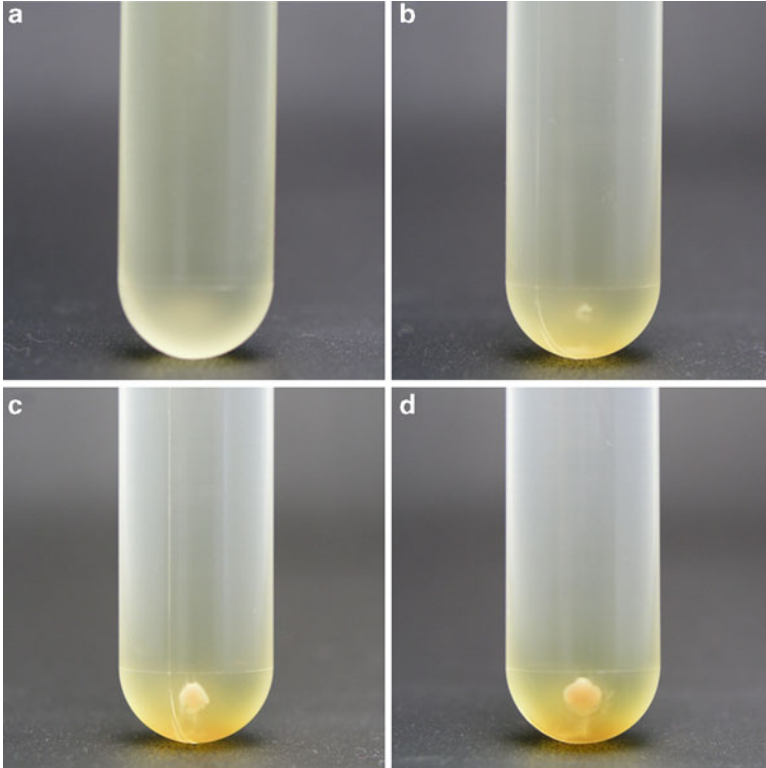


Figure 2. Growth of *Paracoccus denitrificans* at $403,627 \times g$. Photographs of pellet of *P. denitrificans* cells after incubation at $403,627 \times g$ and $30\text{ }^{\circ}\text{C}$ for 0 h (a), 6 h (b), 24 h (c), and 48 h (d). The outer diameter of the tube is 18 mm (Reprinted from Deguchi et al., 2011).

(Fig. 4a, b) of *P. denitrificans* cells incubated at $30\text{ }^{\circ}\text{C}$ for 48 h at both $1 \times g$ and $134,425 \times g$ and histograms of cell dimensions (Fig. 4c, d) shows no significant differences in the morphology of both sets of cells. These results suggest that prokaryotic cells are small enough ($\sim 1\text{ }\mu\text{m}$) that irreversible mechanical deformation due to gravity is not significant.

6. Sedimentation of Inside Cells Under Hyperaccelerations

A distinct concentration gradient of nutrient components in LB broth was observed when the culture of *P. denitrificans* was spun at $403,627 \times g$ for longer than 6 h (Fig. 2). Yellow-colored components formed a sediment at the bottom of the centrifuge tube, leaving a colorless supernatant. A similar concentration gradient of cytoplasmic components may form within microbial cells at hyperaccelerations (Fig. 5) and negatively affect growth under hyperaccelerative conditions.

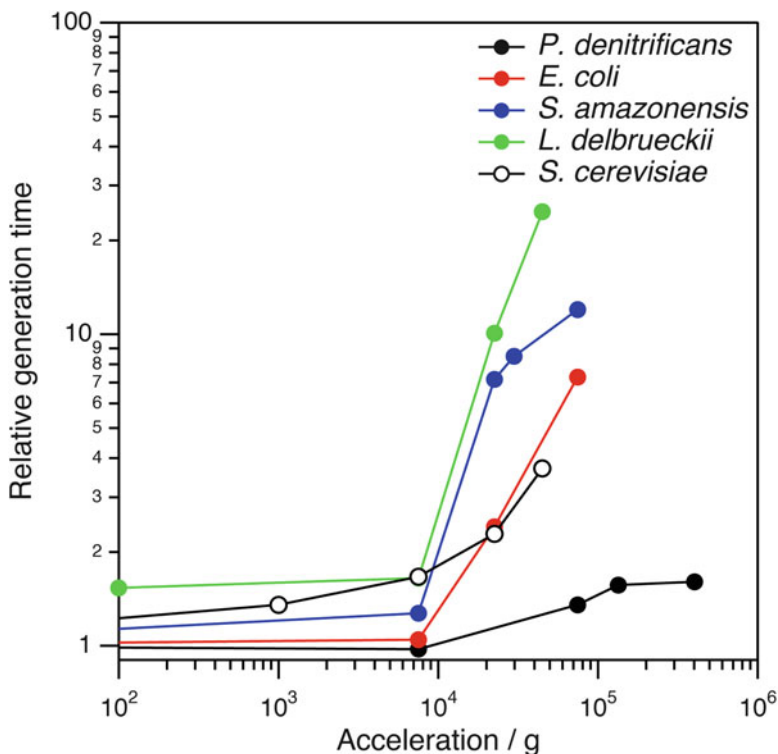


Figure 3. Change in relative generation time of various microorganisms as a function of incubation acceleration (Modified and reprinted from Deguchi et al., 2011).

Numerical calculations showed that differential sedimentation of subcellular moieties and the formation of other molecular concentration gradients do not occur within cells due to the small size of prokaryotic cells (Deguchi et al., 2011). Calculated sedimentation over 1 μm , which is a typical size for prokaryotic cells, showed that sedimentation is negligible for relatively small proteins. Even a 100-kDa protein had a concentration at the top of the cell that was 96 % of the value at the bottom. In contrast, a very profound effect of sedimentation was calculated for a 1-MDa protein, where the concentration at the top was 69 % of that at the bottom. It was also shown that the sedimentation of a 1-MDa protein within a prokaryotic cell became noticeable above $\sim 10^4 \times g$. It is interesting to point out that the very significant retardation of growth by hyperacceleration was also observed above $\sim 10^4 \times g$ for all of the prokaryotic microorganisms studied (Fig. 3). These analyses indicate that intracellular sedimentation of large molecular complexes, for example, bacterial 70S ribosome, may be one reason for growth retardation under hyperaccelerations above $\sim 10^4 \times g$.

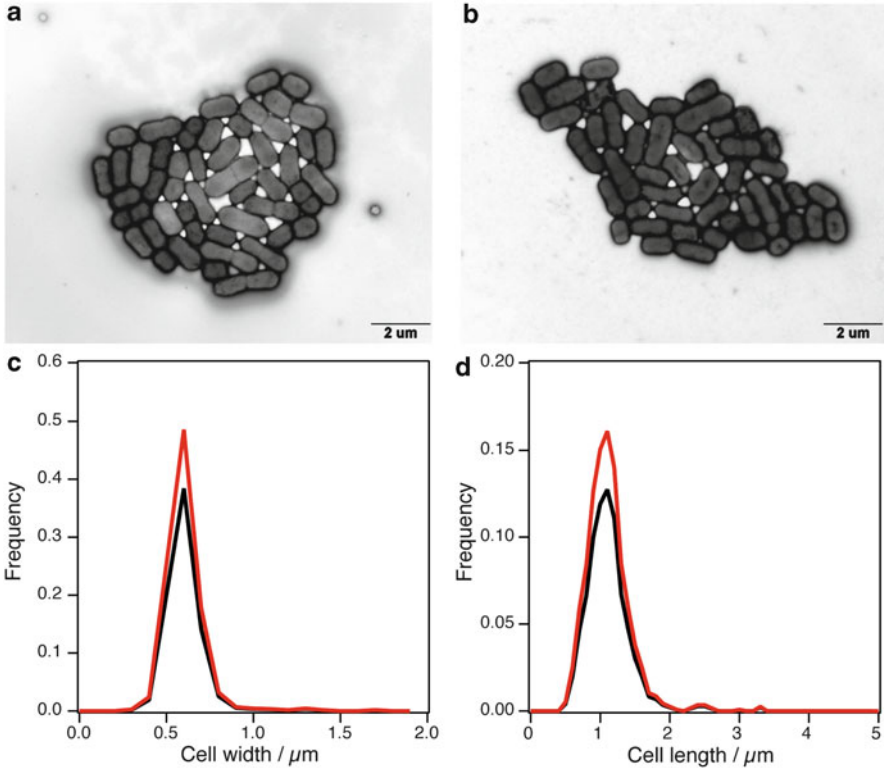


Figure 4. (a and b) Transmission electron microscopy images of *P. denitrificans* cells after incubation at 30 °C and at $1 \times g$ for 4 h (a) and $134,425 \times g$ for 48 h (b). (Scale bar: 2 μm .) (c and d) Size distribution of *P. denitrificans* cells after incubation at $1 \times g$ (black) and $134,425 \times g$ (red) (Modified and reprinted from Deguchi et al., 2011).

The situation becomes completely different when sedimentation over a distance of 10 μm , which is a characteristic size for eukaryotic cells, was considered. In this case, a profound sedimentation effect was calculated even for 100-kDa proteins. Thus, intracellular sedimentation effects may be more significant in the nearly spherical cells of *S. cerevisiae* (ca. 8- μm diameter) (Madigan et al., 1997) compared with smaller prokaryotes.

The generation time of eukaryotic *S. cerevisiae* showed different acceleration dependence compared with the prokaryotes (Fig. 3). The difference could be attributed in part to the larger cell size of *S. cerevisiae*. In the case of eukaryotic cells, however, sedimentation of their organelles is more likely, since these are significantly larger than either proteins or ribosomal complexes. Nuclei and mitochondria of *S. cerevisiae*, for example, can be pelleted easily by centrifugation at $600 \times g$ for 5 min and at $10,000 \times g$ for 2 min, respectively (Diekert et al., 2001).

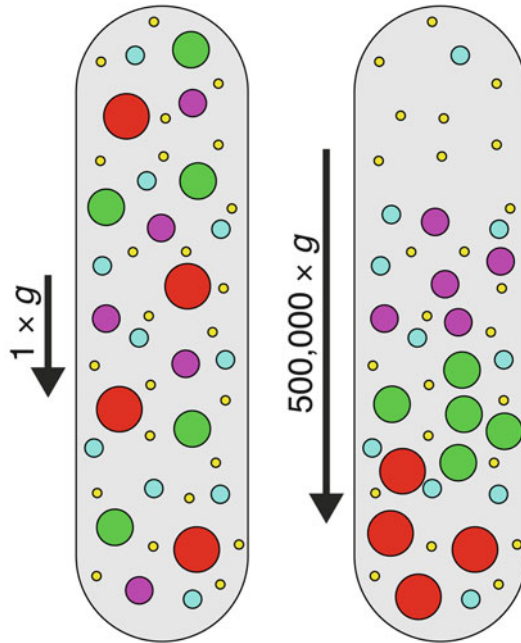


Figure 5. Schematic representation of distribution of cellular components within a microbial cell. The components are homogeneously distributed at $1 \times g$ (left), whereas gradient may form at hyperaccelerations (right).

Thus, during growth of *S. cerevisiae* at hyperaccelerations above $10,000 \times g$, these organelles could not have been distributed homogeneously in the cytoplasm as they are at $1 \times g$. Rather, they should have separated and clustered at the bottom of the cell. Although the effect was not lethal enough to suppress the growth completely, organellar sedimentation and clustering should have affected and retarded the cell growth.

7. Multiple Forms of Physical Stresses in a Centrifuge Tube

Microorganisms that are grown in centrifuges also experience physical stresses other than hyperacceleration (Deguchi et al., 2011). First, microbial cells in the pellet at the bottom of the centrifuge tube (Fig. 2) were subjected to a hydrostatic pressure due to the weight of the water column above the pellet. The pressure is negligibly small at $1 \times g$, but becomes significant at hyperaccelerations (e.g., 42.2 MPa at $134,425 \times g$ and 126.5 MPa at $403,627 \times g$) and may affect the microbial growth (Ishii et al., 2004; Abe, 2007).

Very interestingly, we found that the growth of *P. denitrificans* was completely inhibited above 40 MPa at $1 \times g$ (Deguchi et al., 2011), which in turn suggests that the growth should not have occurred above $134,425 \times g$. The reason for this discrepancy is not clear at present, but it may be ascribed to the difference in cell density during growth at elevated pressures compared with hyperaccelerations. *P. denitrificans* grew planktonically when it was cultured at elevated pressures, while the growth occurred in a densely packed pellet at hyperaccelerations. The pressure effect on cellular processes depends on cell density in some cases (Koyama et al., 2002). Human dermal fibroblasts undergo a significant morphological change and become rounded when they are subjected to 70 MPa at a low cell density (subconfluence). However, no such change is observed when they are pressurized at a high cell density (full confluence). Higher cell density at hyperacceleration may therefore offset the pressure effect to some extent. The compositional gradient in the medium induced during sedimentation may be another possibility.

High cell density in sedimented, congested pellets under conditions of hyperacceleration may have negatively impacted the growth. The density of cells within these pellets increased as gravity increased, leading to relatively smaller void volumes between cells in pellets formed at relatively high gravity. Therefore, diffusion rates of small molecules in these pellets became progressively slower due to an increasing obstruction effect, and consequently, nutrient uptake and waste disposal by cells in the growing pellets were inhibited, thereby affecting the growth rates.

8. Implications for Astrobiology and Biotechnology

Since the first extrasolar planet was identified around a pulsar (Wolszczan and Frail, 1992), nearly 800 extrasolar planets have been discovered to date (Schneider, 1995). Search for a habitable planet is one of the strong motivations for the search. Our results expand the limits for life into the hypergravity regime where this had not been seriously considered before (Rothschild and Mancinelli, 2001). Most significantly, our finding extends the possibility of life beyond planets to massive substellar objects such as brown dwarfs, the coldest of which has an effective surface temperature of ~ 400 K (Eisenhardt et al., 2010), extremely close in value to the known upper temperature limit for life (122°C or 395 K) (Takai et al., 2008). The relatively strong gravitational field associated with brown dwarfs is one of the several limiting factors in considering existence of life on brown dwarfs (Schulze-Makuch and Irwin, 2008). Our results unambiguously show that the $\sim 10\text{--}10^2 \times g$ gravitational fields existent on relatively cold (~ 600 K) brown dwarfs (Leggett et al., 2009) must not be a primary limiting factor in assessing their potential for harboring life as we know it. Our results also show that hyperacceleration of $\sim 10^5 \times g$ is within a habitable range for some microorganisms and significantly enhance the evidence that bacteria can remain robustly viable after asteroidal impact-style ejection.

The finding may also have practical implications. Industrial potentials of enzymes from extremophiles are important motivation to drive the field. Such enzymes, often called extremozymes (Hough and Danson, 1999), do not lose their enzymatic activities even under rather harsh process conditions at which industrial processes are often carried out. For instance, a thermostable DNA polymerase from *T. aquaticus* was a critical component to develop polymerase chain reaction (PCR) (Saiki et al., 1988). Alkaliphilic microorganisms are also important sources of enzymes for biotechnology (Horikoshi, 1996). Alkaline cellulases and protease are commonly found in laundry detergents, while alkaline cyclomalto-dextrin glucanotransferase is used in the industrial production of cyclodextrin.

It has been suggested that altering the accelerative environment could be used to manipulate bacterial fermentation processes (Klaus, 1998) via production and localization of microbial metabolites known to be affected by microgravity (Fang et al., 1997; Demain and Fang, 2001). It is likely that hyperaccelerative conditions may also be used to induce unique metabolite production in growing cultures. For example, production of β -lactam antibiotics by *Streptomyces clavuligerus*, production of rapamycin by *Streptomyces hygroscopicus*, and production of microcin B17 by *E. coli* were suppressed during culturing in simulated microgravity, whereas production of gramicidin S by *Bacillus brevis* was unaffected (Fang et al., 1997; Demain and Fang, 2001). The suppressed biosynthesis of antibiotics reported under microgravitational conditions compared with $1 \times g$ controls (Fang et al., 1997; Demain and Fang, 2001) may be enhanced at elevated gravities.

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**PART VI:
OXYGEN RELATIONSHIPS**

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Stock
Filker**

**Yakimov
Stoeck
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MICROBIAL EUKARYOTES IN MARINE OXYGEN MINIMUM ZONES

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1. Introduction

Nutrient and energy flow patterns within marine ecosystems are largely controlled by dissolved oxygen (O_2) concentrations (Diaz and Rosenberg, 2008; Diaz et al., 2009). Microbial groups capable of utilizing alternative electron acceptors such as nitrate (NO_3^-), nitrite (NO_2^-), manganese (Mn), iron (Fe), sulfate (SO_4^{2-}), or carbon dioxide (CO_2) are selected for under declining dissolved oxygen concentrations (Zehnder and Stumm, 1988). Oxygen minimum zones (OMZs) are midwater regions of the ocean that are depleted in dissolved oxygen (typically $<20 \mu M$) and are increasing worldwide due to natural and anthropogenic sources. Within oxygen-depleted water columns, microbial utilization of nitrite or nitrate as electron acceptors results in nitrous oxide (N_2O) and dinitrogen gas (N_2) production (Lam and Kuypers, 2010). N_2O released into the atmosphere contributes to depletion of ozone and is a greenhouse gas with 25 times the heat-trapping capability of CO_2 (Naqvi et al., 2010). An increase in concentration of hydrogen sulfide (H_2S), which is toxic to most eukaryotes, is also characteristic of marine oxygen-depleted water columns including OMZs. Hydrogen sulfide results from microbially mediated reduction of SO_4^{2-} (Teske, 2010). Reduction of CO_2 by methanogenic archaea under anoxic conditions results in the production of methane (CH_4) (Naqvi et al., 2010). Recent studies of microbial community metabolism and structure within marine OMZs indicate a strong capacity of microbial populations to buffer release of climate-active trace gases and accumulation of H_2S (Lavik et al., 2009; Walsh et al., 2009; Canfield et al., 2010; Zaikova et al., 2010). Bacteria and archaea are the primary drivers of these biogeochemical transformations (Arrigo, 2005), but it is likely that protists act as important biological controls on their abundance and distribution through predation on different microbes within marine OMZs. Furthermore, by hosting epi- or endo-bionts, protists may indirectly contribute to key biochemical processes through the activities of their bacterial and archaeal partners.

Minimal information exists regarding the influence of OMZ formation on protist communities. The responses of protists to changing levels of water column oxygen deficiency will likely have impacts on the abundance, distribution, and activity of bacterial and archaeal populations with resulting feedback on nutrient and climate-active trace gas cycling. Several recent studies of protists along oxygen

gradients provide an initial framework for understanding how protistan communities in oxygen-depleted water columns are structured (Behnke et al., 2006, 2010; Zuendorf et al., 2006; Edgcomb et al., 2011a, b; Orsi et al., 2011, 2012a). Work in the Cariaco Basin revealed an exceptionally high taxonomic richness of protists and specialization of many protistan taxa to different biogeochemical niches and sites in the basin (Orsi et al., 2011; Edgcomb et al., 2011a). Seasonal fluctuations in protistan community structure were found in the anoxic Framvaren Fjord, Norway (Behnke et al., 2010). Protistan subgroup diversity changed extensively across time and space, although major taxonomic lineages remained consistent throughout the time course of the studies. This is consistent with selection of diverse low-abundance populations upon the appearance of favorable environmental conditions.

We discuss here the responses of marine protists to OMZ formation in Saanich Inlet, British Columbia, Canada, in the context of what is currently known about two model end-member oxygen-depleted water column ecosystems: Cariaco Basin, Venezuela, and Framvaren Fjord, Norway. The spatiotemporal variability of protists in relation to dissolved gases and nutrients has been analyzed in these locations with multivariate statistical tools to identify potential relationships between environmental parameters and taxonomic groups at different stages of water column stratification and renewal. Common and unique patterns of protistan community composition between the three ecosystems are discussed here.

2. General Materials and Methods

Samples from Saanich Inlet, B.C., Canada, were collected and processed as described in Zaikova and colleagues (2010). 18S rRNA sequences from the Cariaco Basin (Edgcomb et al., 2011a; Orsi et al., 2011) and the Framvaren Fjord (Behnke et al., 2010) were obtained from the GenBank nt database. Quality control, sequence clustering, and taxonomic assignments of sequence data were performed in MOTHUR (Schloss, 2009). Canonical correspondence analysis (CCA) was used to elucidate relationships between protistan community structure and concentrations of dissolved nutrients and gases. Multiresponse permutation procedure (MRPP) was used to test for a statistically significant influence of season, depth, nitrate, sulfide, and oxygen on the observed OTU distribution. A Monte Carlo test was also used to assess the null hypothesis of no relationship between OTU distributions and environmental variables. Monte Carlo tests, MRPP, and CCA were implemented using the PC-ORD software package (MjM Software Design). The correlation between protistan community structure across the three ecosystems and the oxygen concentration in each sample was determined via principal component analysis (PCA) using the *FactoMineR* module (<http://factominer.free.fr>). The first two principal components calculated from the PCA analysis were used to hierarchically cluster samples using Manhattan distance with complete linkage.

3. The Protists of Saanich Inlet

3.1. PROTISTAN COMMUNITY STRUCTURE IN SAANICH INLET

Protistan populations associated with oxic ($>90 \mu\text{M}$), dysoxic ($20\text{--}90 \mu\text{M}$), suboxic ($1\text{--}20 \mu\text{M}$), or anoxic/sulfidic ($<1 \mu\text{M}/\pm\text{sulfide}$) water column conditions in the Saanich Inlet are clearly distinct (Fig. 1). This finding validates previous observations of oxygen and sulfide concentrations influencing microbial distributions in marine environments, such as the Cariaco Basin (Taylor et al., 2001; Li et al., 2008; Lin et al., 2008; Edgcomb et al., 2011a; Orsi et al., 2011) and the Framvaren Fjord (Behnke et al., 2006; Stoeck et al., 2009, 2010). The multivariate statistical analysis of protists in Saanich Inlet (Fig. 1) represents the first investigation into the influence of methane on protist distributions in low-oxygen marine environments. The differential length of the methane and sulfide vectors in the CCA (Fig. 1)

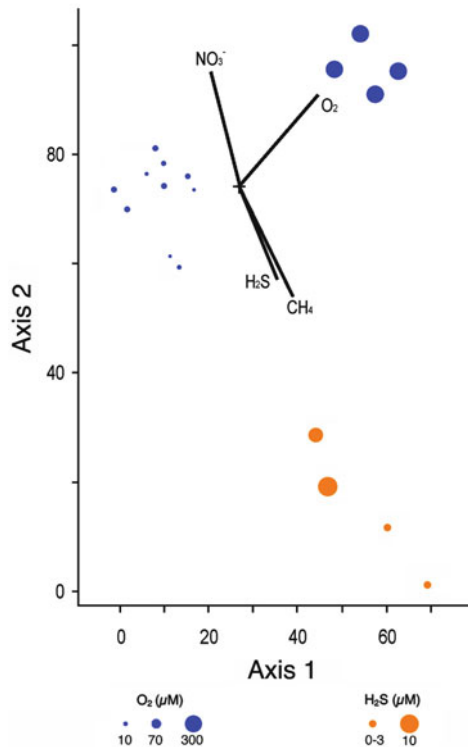


Figure 1. Canonical correspondence analysis of the Saanich Inlet 18S rRNA gene sequence dataset clustered at the 98 % identity threshold. Samples are represented in the biplot by dots, the size and color of which indicates the presence and concentration of dissolved oxygen (blue) or sulfide (yellow). Axes 1 and 2 explained 8 and 7.7 % of the variance in OTU distribution, respectively.

suggests that some protists distributions correlate stronger with methane than sulfide. This is an interesting finding because sulfide is known to be toxic to many eukaryotes. Curiously, methanogenic archaea were not detected in Saanich Inlet water samples from the same depths and times (Zaikova et al., 2010). One likely source of methane in the water column is diffusive flux from underlying sediments. However, new methane production in the water column originating from methanogenic symbionts of anaerobic ciliates, a well-known partnership in oxygen-depleted marine environments (e.g., Embley and Finlay, 1993; Fenchel and Finlay, 1995; van Hoek et al., 2000; Edgcomb et al., 2011c), may also contribute to methane accumulation in basin waters. We suggest that such symbioses may make a significant contribution to methane cycling in low-oxygen and anoxic marine environments.

In Saanich Inlet, selection of protistan populations in different niches along the redoxcline during the year is dependent on intensity of renewal events (Orsi et al., 2012a). Samples collected at 200 m during renewal (November) do not group with the other 200 m samples taken during April, February, and July when waters at this depth are ordinarily anoxic (Fig. 1). Rather, samples taken during renewal at this depth group with dysoxic samples. This is likely an effect of the physical movement of an oxygenated water mass into deep basin waters known to occur during renewal events (Zaikova et al., 2010). These findings confirm that marine OMZ formation can have a strong influence on the protistan communities, with different populations being selected for as a result of changes in dissolved oxygen concentrations.

At 200 m during winter months, an increase in dinoflagellates (90 % of which correspond to Syndiniales) and stramenopiles was observed relative to July and April in Saanich Inlet (Fig. 2 and Orsi et al., 2012a). The upwelling water that replaces deeper waters (200 m) at this time originates from coastal marine sources, a habitat in which representatives of the Syndiniales and stramenopiles have been detected previously (Lin et al., 2006; Massana et al., 2006). Syndiniales are common parasites of marine fauna and likely co-occur with another taxon host in these oxygen-depleted waters. After re-stratification of the water column that typically occurs during the summer, most protists in the anoxic portion of the water column belong to the Ciliophora, stramenopiles, and Euglenozoa (Fig. 2 and Orsi et al., 2012a). A similar observation has been made in the Cariaco Basin, in which waters below the oxic/anoxic interface contain over twice the number of ciliate and euglenozoan taxa relative to oxygenated waters (Orsi et al., 2011). However, it is unknown whether the dominant ciliates and euglenozoans found at 200 m during stratification (oxygen depletion) survive periodic exposure to oxygen during renewal events by migrating into low-oxygen sediments or by becoming less active (and less numerous) until favorable conditions are restored. Nevertheless, the results from studies of Saanich Inlet, Cariaco Basin, and Framvaren Fjord all indicate that Ciliophora and Euglenozoa are selected for by water column oxygen depletion (Behnke et al., 2010; Orsi et al., 2011, 2012a). Ciliophora and Euglenozoa contain many species of anaerobes and microaerophiles, and many

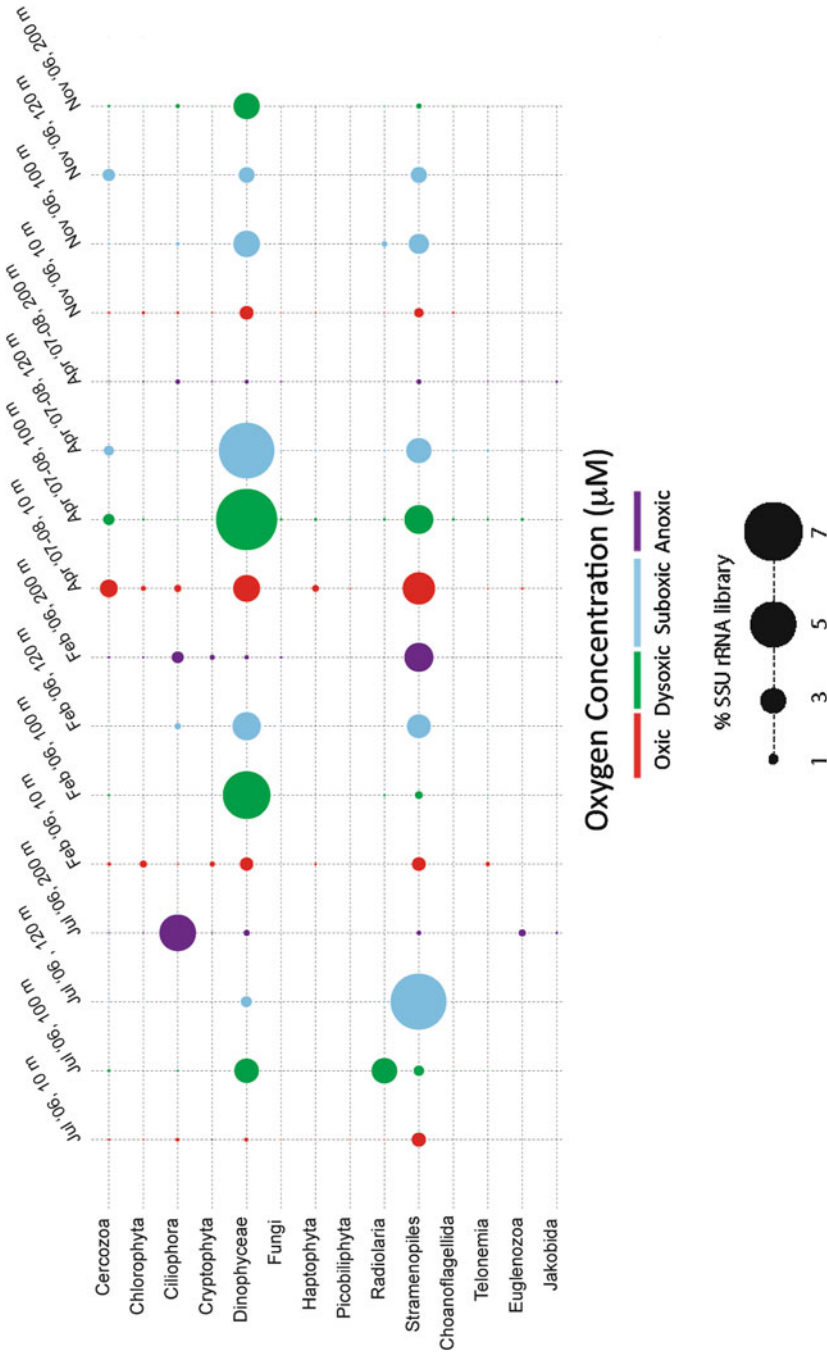


Figure 2. Phylum-level taxonomic affiliations of Saanich Inlet 18S rRNA gene sequences. Sampling dates and water depths (m) are indicated on the top of the figure (Jul July, Feb February, Apr April, Nov November). The relative abundance (% of total) of sequences affiliated with each taxonomic group within a sample is indicated by the area of the circle. The concentration of oxygen (in μM) within each sample is represented by colors: oxic (red), dysoxic (green), suboxic (blue), anoxic (purple).

of the closest described relatives of the sequences (i.e., *Calkinsia*, *Cyclidium*, *Strombidium*, and *Nyctotherus*) have been detected in other studies of marine oxygen-depleted water columns (Behnke et al., 2010; Orsi et al., 2011, 2012a).

Phylogenetic analyses of ciliate and euglenozoan-affiliated OTUs recovered from anoxic waters, as well as the relatively low (<92 %) identities to their closest described species in public databases, suggest that oxygen-depleted marine waters select for novel lineages within these phyla (Orsi et al., 2011, 2012a, b). The new Symbiontida-affiliated lineages with low (<90 %) identities to the euglenozoan *Calkinsia aureus* (Orsi et al., 2011, 2012a; Yubuki et al., 2009) likely correspond to euglenid protists exhibiting symbioses with bacteria. *C. aureus* has a cortex that is completely covered by bacteria belonging to the bacterial genus *Arcobacter*, a group capable of performing chemoautotrophy and chemoorganotrophy coupled to nitrate reduction and sulfide oxidization (Bernhard et al., 2000; Edgcomb et al., 2010; Yubuki et al., 2009). Phylogenetic analyses of ciliate-affiliated sequences recovered from both oxygen-depleted waters of the Cariaco Basin and Saanich Inlet (Orsi et al., 2012a) reveal a high diversity of the novel ciliate class Cariacotrichea (Orsi et al., 2012b) and suggest that this new taxon is restricted to oxygen-depleted marine habitats.

3.2. COMPARISON OF PROTIST COMMUNITIES IN SAANICH INLET, CARIACO BASIN, AND FRAMVAREN FJORD

To better understand how changing levels of oxygen and sulfide in marine waters affect protistan community structure and dynamics on a global scale, we compared available 18S rRNA sequence datasets from Saanich Inlet, Cariaco Basin, and Framvaren Fjord. Sequences affiliated with the Ciliophora, Euglenozoa, Choanoflagellata, stramenopiles, Fungi, and Dinophyceae were well represented from anoxic samples across locations, but many taxa within those groups had unequal representation (Orsi et al., 2012a). For example, stramenopile-affiliated sequences were less prevalent in Saanich Inlet compared to Cariaco Basin and Framvaren Fjord, and Polycystinea- and fungal-affiliated sequences were less abundant in Saanich Inlet and Framvaren compared to Cariaco (Orsi et al., 2012a).

Statistical estimates of taxonomic richness suggest that the Cariaco Basin contains roughly twice the number of species and genera than are estimated for the Saanich Inlet and roughly ten times the number of such taxa from the Framvaren Fjord (Orsi et al., 2012a). Combined principal component (PCA) and hierarchical cluster analyses of the Saanich Inlet, Cariaco Basin, and Framvaren Fjord datasets revealed biogeographic and niche-specific clustering patterns (Fig. 3, Orsi et al., 2012a). The majority of oxic, dysoxic, and suboxic samples from Saanich Inlet, Cariaco Basin, and Framvaren Fjord samples form independent clusters. Interestingly, one hyper-sulfidic Framvaren Fjord sample clusters with anoxic/sulfidic samples from Saanich Inlet and one anoxic deep sample from the Cariaco Basin (Fig. 3). Furthermore, almost all of the Cariaco samples cluster

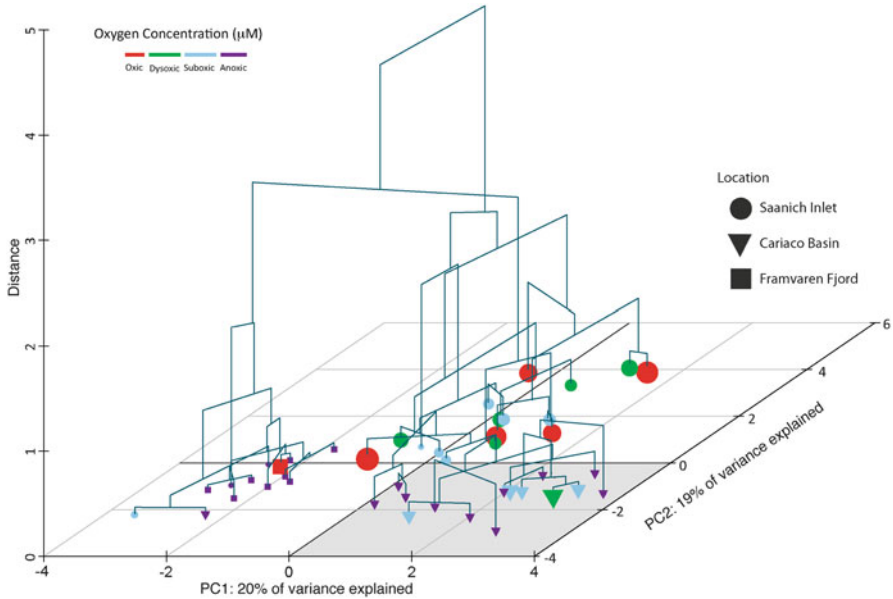


Figure 3. Principal component analysis and hierarchical clustering of samples from Saanich Inlet, Cariaco Basin, and Framvaren Fjord (Adapted from Orsi et al., 2012a). Each dot represents a sample and shapes correspond to sample location: Saanich Inlet (circles), Cariaco Basin (triangles), and Framvaren Fjord (squares). Colors indicate dissolved oxygen concentration, oxic (red), dysoxic (green), suboxic (light blue), and anoxic (purple). The size of each dot is scaled according to the value of the oxygen concentration (in μM). The clustering pattern is linked to a dendrogram generated from hierarchical clustering of the samples. Note that the majority of samples from Cariaco cluster together, separate from the other two locations, in the lower right (highlighted) quadrant of the grid. *PC1* principal component 1, *PC2* principal component 2.

together, separate from samples taken from the other two locations (Fig. 3). This suggests that protistan distributions are controlled largely by geographic location, water depth, and oxygen concentration.

The differences between protistan populations at the three sites are likely due to several differences between the oceanographic regimes. One likely reason is the large difference in physical size of the water masses. The eastern and western sub-basins of the Cariaco contain unique protistan communities, which are driven in part by differences in riverine inputs, trophic responses to differential prey items, and primary production (Orsi et al., 2011) in different parts of the Basin. Thus, the larger size of the Cariaco Basin provides additional niches that permit higher protist diversity relative to the smaller water masses in Saanich Inlet and Framvaren Fjord. Also, unlike Saanich Inlet and Cariaco, the oxic/anoxic interface of Framvaren Fjord lies in the photic zone and contains significantly higher sulfide levels. The Cariaco Basin has remained anoxic for millions of years (Schubert, 1982) and experiences only limited oxygen intrusion

events (Lin et al., 2008), while the Saanich Inlet experiences only seasonal anoxia. Because Cariaco has been anoxic for a longer period of time, this likely allowed for a higher amount of speciation of anaerobic protists relative to Saanich Inlet. Differences in seawater temperatures may contribute to the differences in community structure across the three locations, as temperature has been shown to control the distribution of marine microbial populations (Rutherford et al., 1999; Fuhrman et al., 2008).

3.3. FLUCTUATIONS IN RARE MICROBIAL POPULATIONS

An analysis of microbial populations in Saanich Inlet during water column anoxia and during oxygenated renewal suggests that many protists present at low abundance are selected for once the preferred conditions arise (Orsi et al., 2012a). The number of ciliate-, euglenozoan-, and stramenopile-affiliated sequences increased significantly during after OMZ formation (Fig. 2). This observation is likely a result of environmental selection for subgroups within the phyla adapted to oxygen-depleted and sulfidic conditions. Ciliates and stramenopiles typically graze bacteria (Massana et al., 2006; Sherr and Sherr, 2002) in the marine water column and likely respond to increasing numbers of selected prey species that occur after OMZ formation. Because of this grazing activity, ciliates and stramenopiles likely regulate abundances of denitrifying and anammox bacteria responsible for the production of nitrous oxide that are known to exist in marine OMZs (Zaikova et al., 2010). Nitrous oxide is a greenhouse gas that causes ozone depletion. Thus, a potentially important relationship may exist between ciliate and stramenopile grazers, denitrifying and anammox bacteria, and the fluxes of nitrous oxide from OMZs waters to the atmosphere.

The first investigation into the influence of methane on the distribution of protistan communities in marine OMZs revealed that methane is correlated with the distribution of many anaerobic ciliates (Orsi et al., 2012a). These results suggested that methane may explain these ciliates distributions more than sulfide (Fig. 1), a phenomena likely due in part to methane produced by methanogenic archaeal partners. Methanogenic archaea may not have been detected in the Saanich Inlet water column during periods of anoxia (Zaikova et al., 2010) because they were associated with anaerobic ciliates. Thus, methanogens associated with ciliates likely contribute the majority of methane produced in the water column of Saanich Inlet after OMZ formation. Fluctuations in populations of anaerobic ciliates (and their associated methanogenic symbionts), which are selected for as a result of OMZ formation, have the potential to make a significant contribution to methane cycling in OMZs.

These results reveal a linkage between OMZ formation and a response from low-abundance protistan populations. Also termed “the rare biosphere” (Pedrós-Alió, 2007), such populations may play an important role in ecosystem resilience and response. Fluctuations in abundances of protists affiliated with the stramenopiles,

Ciliophora, and Euglenozoa (Fig. 2) indicate that temporally rare populations of protists become abundant in Saanich Inlet upon OMZ formation.

4. Looking Forward

Several preliminary studies (e.g., Behnke et al., 2010; Orsi et al., 2011, 2012a; Edgcomb et al., 2011a) indicate that microbial eukaryotes likely play an important role within oxygen minimum zones. The most important role of protists within OMZs is likely their regulation, through grazing, of bacterial and archaeal populations that mediate biogeochemical transformations of dissolved gases and organic and inorganic nutrients. Symbioses may also represent an important ecological function of protists in OMZs as partnerships between anaerobic ciliates and methanogenic archaea may also make a significant contribution to methane cycling in OMZs (Orsi et al., 2012a). Despite these important functions performed by protists, essentially no quantitative information exists regarding the extent of protistan activities on biogeochemical cycling within OMZs. Limited information exists on grazing rates of different protists on specific microbial groups performing known biogeochemical transformations in OMZs and the resulting impact on fluxes of inorganic and organic substrates. Continued studies of specific grazing impacts, symbiosis, and bioremineralization—and the resulting impact on biogeochemical cycles are warranted. Such studies will benefit from an integrated research approach that synthesizes molecular approaches (i.e., metagenomics and metatranscriptomics) and culture-based physiological studies. Multidisciplinary collaborations between marine geochemists, protistologists, and molecular and microbial ecologists are required for such studies to be successful.

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DEEP HYPERSALINE ANOXIC BASINS AS MODEL SYSTEMS FOR ENVIRONMENTAL SELECTION OF MICROBIAL PLANKTON

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1. History and Origin of Deep Hypersaline Anoxic Basins

In the second half of the twentieth century, the Mediterranean Sea became of tremendous interest for seismological, micropaleontological, and geochemical investigations. The presence of Messinian evaporites below the clastic sediment layers as well as the periodic appearance of sapropels attracted the attention of a multitude of researchers (de Lange and Ten Haven, 1983). On various expeditions in the early 1980s, scientists discovered unusual structures during seafloor surveys using seismic reflection profiles and backscatter imaging (De Lange and Ten Haven, 1983; Jongsma et al., 1983; Cita et al., 1985). Bathymetric mapping uncovered abyssal depressions of more than 3,000 m depth (Fig. 1). Chemical analyses of bottom water samples revealed the existence of lakes in these basins, displaying extremely high ionic concentrations compared to normal seawater.

These lakes are distributed over several regions of the eastern part of the Mediterranean Sea. The l'Atalante, Discovery, and Urania Basins are located at the Medriff Corridor (Medriff Consortium, 1995), the Bannock, Thetis, and Medee Basins in the northwestern part of the Mediterranean Ridge and the Tyro Basin in the southwestern part of the Strabo Trench (Fig. 2b).

The appearance of hypersaline anoxic basins is not exclusively restricted to the Mediterranean Sea. Scientists discovered brine-filled depressions well before the Mediterranean deep-sea brine lakes were found. In 1965, two hydrothermal sites, the Atlantis II Basin and the Discovery Basin, were detected in the Red Sea (not to be mistaken for the Discovery Basin in the eastern Mediterranean Sea). A first systematic survey of these basins and their brines was conducted 1 year later (results presented in Degens and Ross, 1969). Twelve years later, another group of scientists discovered a 400 km² depression in the continental slope of the Gulf of Mexico, named Orca Basin (Fig. 2a), showing similar characteristics as the Mediterranean DHABs and the Red Sea brine (Shokes et al., 1977).

The origin of the brine lakes in the Mediterranean basins is based on a sequence of geological processes, starting with the formation of the basins

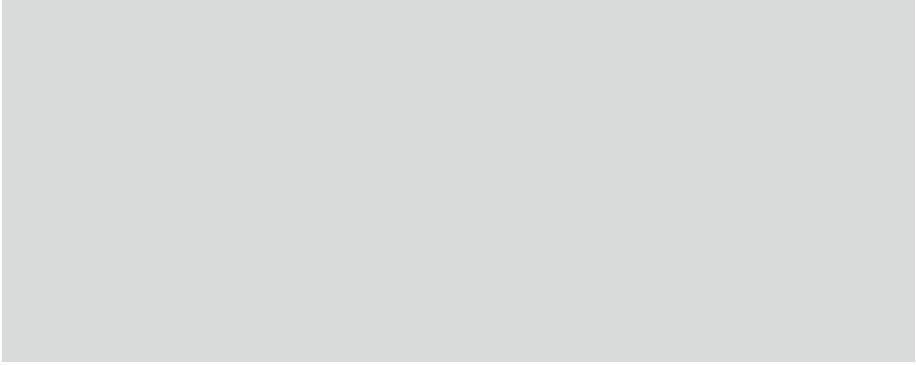


Figure 1. Bathymetric maps of two Mediterranean DHABs. Brines occur in a depth of around 3,550 and 3,500 m for (a) Discovery Basin and (b) Urania Basin, respectively.

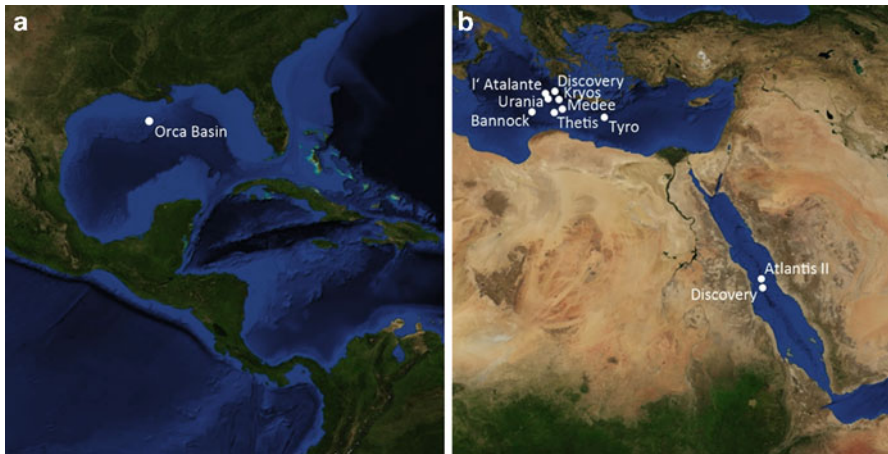


Figure 2. Locations of deep hypersaline anoxic basins (DHABs). (a) The Orca Basin is situated in the Gulf of Mexico (North America). (b) The brine pools of the Mediterranean Sea are distributed in the eastern part next to Crete. Both, the Atlantis II and Discovery Basin are located in the center of the Red Sea (Maps modified from <http://visibleearth.nasa.gov>).

themselves through tectonic activity between the African and European continental plates leading to accretionary structures, like the “cobblestone topography” of the Mediterranean Ridge (Kastens, 1981). Tensional stress in the hemipelagic sediments of the seafloor due to initial folding of the sediment layers caused fractures exposing the underlying evaporites to seawater (Camerlenghi, 1990).

In contrast, the basins of the Red Sea, which developed when Arabia was separated from Africa during the movement of the Red Sea Rift, and the Orca Basin show a similar manner in their evolution. Studies of both indicate tectonic

deformation together with the dissolution of near-surface Late Miocene salt deposits as the cause for their development (Ross et al., 1973; Shokes et al., 1977).

Although the basins in the Eastern Mediterranean, the Orca Basin and the Red Sea, differ in their geological settings, they all harbor evaporitic deposits closely beneath the seafloor. Whereas the evaporites in the Orca Basin are Jurassic in age, those of the Red Sea and the Mediterranean basins are quite young on a geological scale and formed in the late Miocene (Ross et al., 1973; Camerlenghi, 1990). The evaporites occurring in the Mediterranean area are a result of the Messinian salinity crisis. About six million years ago, the communication between the Atlantic Ocean and the Mediterranean Sea was temporarily interrupted, causing the desiccation of the Mediterranean Sea (the “desiccated deep-sea basin” model by Hsü et al., 1973). Along with the evaporation of the seawater minerals cropped out, forming the typical stratification of marine evaporites. Due to a global sea level rise, the connection to the Atlantic Ocean was reestablished, leading to a flooding event in the beginning of the Pliocene (Spezzaferri et al., 1998). Investigations revealed that during this ca. 600,000 years enduring event a salt layer of 1,500 m to more than 3,000 m thickness had been laid down over the Mediterranean Sea floor, dramatically changing the chemistry of the ocean (Hsü et al., 1973). Along with the returning seawater, dissolution processes started releasing minerals of the evaporitic beddings, until almost saturated salt concentrations were reached. The minerals considered to be of geological significance in the dissolution of evaporitic deposits are halite (NaCl), carnallite ($\text{KMgCl}_3 \cdot 6\text{H}_2\text{O}$), bischofite ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), and anhydrite (CaSO_4). They belong to the upper mineral layers and are first to redissolve, together with potassium and magnesium salts. Carbonates, such as dolomite ($\text{CaMg}(\text{CO}_3)_2$) and calcite (CaCO_3), exist in lower strata and are too insoluble in seawater to have a large influence on the hydrochemistry. Other minerals, like sylvite (KCl), that have a higher solubility are too rare in evaporitic deposits to have an effect on the salt concentration (Camerlenghi, 1990).

Although the brines of the Mediterranean DHABs are geographically not distant, they all show unique hydrochemical compositions (Table 1). Salinity ranges from 95 PSU (Discovery brine) up to 365 PSU (l'Atalante brine) and is therefore ten times higher than in normal seawater, permitting the classification of these environments as hypersaline. Sodium concentrations vary greatly from 68 mmol/l in the Discovery brine (van der Wielen et al., 2005) to 5,300 mmol/l in the Tyro brine (La Cono et al., 2011), whereas the magnesium and sulfate concentrations (71 and 53 mmol/l (La Cono et al., 2011), respectively) in this brine are the lowest encountered among the Mediterranean brines. The highest magnesium concentration (4,995 mmol/l) was detected in Discovery brine contrasting the lowest magnesium concentration (71 mmol/l) in the Tyro brine. The highest sodium concentration (397 mmol/l) can be measured in the l'Atalante brine (van der Wielen et al., 2005), while the lowest one occurs in the Discovery brine. Concentration of the toxic hydrogen sulfide ranges between 0.7 mmol/l (Discovery brine, van der Wielen et al., 2005) and 15 mmol/l (Urania brine).

The dissimilarities between the chemical compositions of the brine lakes are a consequence of the different compositions of the evaporitic salt deposits, which derive from the different depositional stages of the subterranean salt deposits (de Lange et al., 1990).

In contrast to the Mediterranean brines and the Orca brine, those from the Red Sea are termed hot brines, as they origin from seawater that obtains geothermal heat and dissolved minerals while circulating through subterranean evaporite deposits before reaching the surface and filling the basin depressions (Craig, 1969). Unlike the Mediterranean brines, those of the Red Sea do not show a variance in their particular hydrochemistry, with exception of the salinity and sodium concentration (Table 1).

Hypersaline lakes can be classified in either thalassohaline or athalassohaline environments based on their ionic composition. Thalassohaline environments originate from the evaporation of seawater and therefore show a seawater-like proportion of the major ions with high sodium, chloride, and sulfate concentrations and lower magnesium, potassium, and calcium concentrations. Changes in the ionic composition can occur as evaporation proceeds, and some minerals (e.g., calcite, aragonite, gypsum) precipitate long before NaCl saturation is reached. This applies to the Mediterranean Basins Urania, Bannock, l'Atalante, Thetis, Medee, and Tyro, as well as for both the Red Sea lakes and the Orca Basin. The brines of the Discovery and presumably the Kryos Basin show a distinct ionic composition, differing greatly from that of seawater (athalassohaline). These basins have high magnesium and calcium and low sodium, potassium, and sulfate concentrations.

The high concentrations of dissolved ions and the resulting high densities within the different brines (up to 1.23 kg/m^3 for Mediterranean DHABs, La Cono et al., 2011) prevent mixing with overlying seawater. The salt-induced stratification leads to the formation of a sharp, well-defined interphase that separates the brines from the normal saline seawater (Fig. 3). An exchange of oxygen and minerals over this native boundary is restricted. Additionally, due to the "salting-out" effect (the solubility of a substance in a solvent is reduced, when another substance is dissolved in the solvent), the solubility of oxygen in such brine lakes is downsized around one quarter compared to seawater (Sonnenfeld, 1984). The complete depletion of oxygen in the brines is also indicated by the redox potential decreasing rapidly from +200 mV in the seawater to -100 mV in the upper interphase, resulting in a highly reduced brine with negative values often exceeding -400 mV. Furthermore, influence of light, pH, and temperature changes are hampered, causing a physically stable and closed, high saline and anoxic environment.

The around 1–3 m thin interphase is considered a hotspot for biological activities (Daffonchio et al., 2006; Yakimov et al., 2007) and is highly interesting for science, as it displays steep gradients in hydrochemical and physical parameters such as oxygen, pH, salinity, density, temperature, and electron donors and acceptors on a very small spatial scale (Fig. 4).

Table 1. Hydrogeochemistry of different hypersaline, anoxic basins in the Mediterranean Sea, the Red Sea, and the Gulf of Mexico.

Basin	Layer	Depth (m)	Salinity ^a (PSU)	O ₂ ^a (ml/l)	Na ⁺ (mmol/l)	Mg ²⁺ (mmol/l)	SO ₄ ²⁻ (mmol/l)	HS ⁻ (mmol/l)
Mediterranean Sea	Bannock	IF	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		B	n.a.	n.a.	4,235 ^c	650 ^c	137 ^c	3 ^c
Discovery		IF	38	0.5	27.2	1,998	n.a.	n.a.
		B	3,581	0	68 ^c	4,995 ^c	96 ^c	0.7 ^c
Kryos*		IF	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		B	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
l'Atalante		IF	39	1.9	n.a.	n.a.	31	n.a.
		B	3,501	365	n.a.	4,674 ^e	397 ^e	2.9 ^e
Medee		IF	70	0.5	847	161	41	n.a.
		B	2,950	320	0	4,818	792	2.9
Thetis		IF	80	0.68	1,368	174	76	0.11
		B	3,380	348	0	4,760 ^b	265 ^b	2.1 ^b
Tyro		IF	67	0.5	1,111	15	11	0.07
		B	3,448	321	0	5,300 ^b	53 ^b	2.1 ^b
Urania		IF	63	1.22	876	79	42	0.66
		B	3,493	240	0	3,505 ^b	107 ^b	15
Red Sea	Atlantis II	B	252 ^f	0.3 ^f	5,100 ^d	36.9 ^d	10.8 ^d	n.a.
	Discovery	B	100 ^f	0.4 ^f	5,700 ^d	42.6 ^d	9.9 ^d	n.a.
Gulf of Mexico	Orca	B	260 ^g	0	4,155 ^b	40.9 ^h	38.7 ^h	n.a.

^aEdgcomb et al. (2011), ^bLa Cono et al. (2011), ^cvan der Wielen et al. (2005), ^dAntunes et al. (2011), ^evan der Wielen et al. (2005) Supporting Online Material, ^fSwift et al. (2012), ^gvan Capellen et al. (1998) and ^hSchiff (2007).

n.a. = not available.

*No data published on Kryos hydrochemistry, but for completeness, the basin is mentioned in this table.

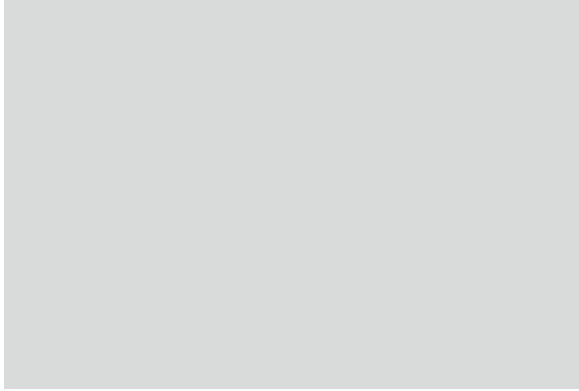


Figure 3. Image of the seafloor (*left*) showing the transition (interphase) from seawater into the brine layer (*right, dark area*) (The picture was taken during a mapping dive of the ROV Jason (WHOI)).

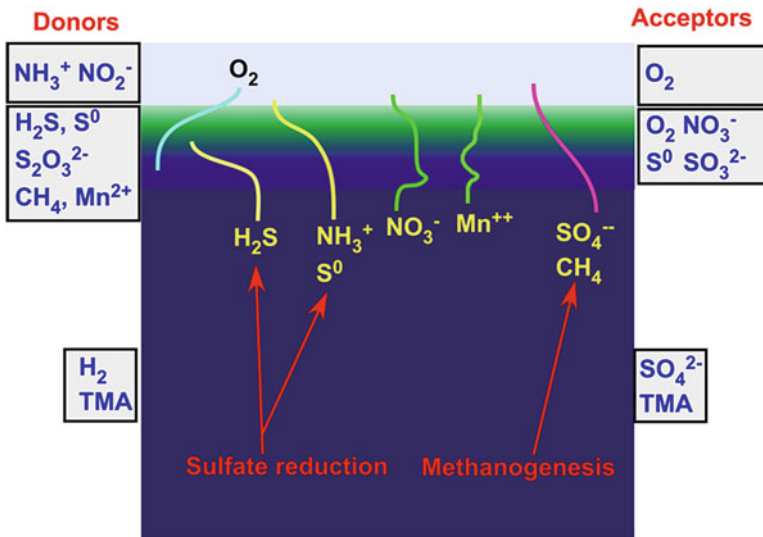


Figure 4. Pronounced changes in major electron acceptors and donors on a very small spatial scale occur within the 1–3 m thin interphase that separates the normal saline and normoxic deep-sea water from the high-density brine lake water, fostering changes in microbial communities along these gradients.

2. DHABs as a Model System for Environmental Selection and Allopatric Evolution

Considering the polyextreme character of the DHAB brines, the high diversity of microbial organisms thriving in these habitats is astonishing and raises a fundamental question: what are the adaptation strategies of the polyextremophiles living in

the harsh conditions of the DHABs? In this chapter, we will not review the different adaptation strategies of extremophiles to environmental parameters characterizing the DHAB brines (see above), as this has been done extensively in a number of excellent papers and books (e.g., Cavicchioli et al., 2011; Gunde-Cimerman et al., 2000, 2003; Oren, 2002, 2011). Our intention with this chapter is (1) to demonstrate the model character of DHABs for studies of environmental selection and allopatric evolution of microbes, (2) to review our current knowledge on this subject, and (3) to outline an avenue for further research towards understanding the role of environmental selection in contemporary distribution patterns of microbes.

The extreme environmental parameters in DHAB brines such as monovalent and divalent cations, high salinity up to saturation level, anoxia, methane, and hydrogen sulfide, ammonium, and other ions vary considerably in the individual DHAB brines (Table 1). Different combinations of these environmental parameters in the various DHAB brines require specific adaptations and thus exert a high selection pressure on plankton communities in these unique habitats. This selection pressure is enhanced through the island character of each basin, that is, their geographic separation from each other and unsurmountable environmental barriers (normsaline and normoxic deep-sea water) that prevents exchange of most, if not all strict anaerobe and halophilic microbes.

The “basin reservoir effect” as described by Algeo and Lyons (2006) for modern anoxic marine environments nicely describes isolation as an “engine to drive” evolutionary selection. A pronounced stratification of the water column above deep-sea basins (such as DHABs) causes the isolation of the deep water and leads to high variations of trace metal concentrations in the water column. The absence of water mixing supports that the restricted water mass is depleted in dissolved trace metals and this prevents – against all expectations – further authigenic enrichment of sediments (Tribovillard et al., 2008). This reservoir effect does not only influence the depletion and dissolution of metals or organic matter. As a consequence of the different physicochemical parameters caused by the isolation, there is an influence on the organisms that live and thrive in seclusion. To take this basin reservoir effect to extremes and transfer its meaning to DHABs, without or with very limited influence (emigration and immigration) from an “outside world,” the isolated water masses in the deep basins with their unique hydrochemistries form an ideal ground for the evolution of hitherto unknown and specially adapted life forms. These forms evolve under nearly complete isolation from the rest of the oceanic realm. Hence, allopatric evolution is the most likely common consequence for any living being in the deep hypersaline anoxic basins.

Indeed, comparisons of microbial communities within a number of brine basins have revealed decisive differences in their compositions not only among the individual brines but also compared to the overlying interphases. Before protistan specialists began to investigate microeukaryote communities in the basins, research in the brine lakes was already conducted on other microbial communities. Two pioneering studies (van der Wielen et al., 2005; Yakimov et al., 2007) characterized the bacterial and archaeal populations of diverse Mediterranean DHABs.

In 2005, van der Wielen and colleagues investigated the basins l'Atalante, Bannock, Discovery, and Urania. They targeted the geochemistry, uncultivated microbiota, and in situ microbial metabolic activities. The authors aimed to “determine the extent to which geochemical conditions influence the evolution of brine communities and to study whether life is possible under the hostile conditions of Discovery brine” (van der Wielen et al., 2005). As part of their work, phylogenetic analyses of bacterial taxonomic marker genes (small subunit ribosomal RNA, SSU rRNA) revealed that most detected gene sequences are not related to phylogenetic groups that are distributed in normal seawater and therefore indicate an adapted and specialized microbial community in the Discovery brine (see also Table 2). A cluster analysis of combined archaeal and bacterial 16S rRNA gene data from four different hypersaline basins highlighted the differences between the microbial communities, showing a clear separation of Discovery from the other investigated DHABs (Fig. 5, left panel). Also a cluster analysis based on geochemical data results in a segregation of the Discovery basin, indicating an immediate effect of geochemical conditions on microbial communities (Fig. 5, right panel).

The unusually high amount of MgCl_2 in the Discovery Basin seems to produce different, more specialized communities than all the other basins. Reason for that was earlier given by Oren (1994): most known halophilic microorganisms thrive under high NaCl but not under high MgCl_2 concentrations.

Environmental selection in DHABs is not solely restricted to brines of different basins but can also be detected along the steep chemical gradients in the individual interphases. Yakimov et al. (2007) focused on such a prokaryote community analysis exclusively in the l'Atalante basin. Even just within l'Atalante's interphase, in a narrow layer of only 2 m, the archaeal community is dominated by Crenarchaeota group I, whereas the bacterial community mainly consists of Epsilonproteobacteria and sulfur-oxidizing Gammaproteobacteria in the upper part of the interphase and Deep-Sea Hydrothermal vent Euryarchaeota group 6, *Methanohalophilus* and Deltaproteobacteria, Kebrit Deep candidate division, and Shaban Deep candidate division in the lower part (Table 2). A recent analysis of bacterial communities and functional genes throughout a DHAB interphase showed the decisive changes in the phylogenetic structure of bacterial communities with increasing depth and salt concentration. Furthermore, the metabolic profiles of the bacterial communities change with increasing depth and salinity: a shift of functional genes appears throughout the DHAB interphase along a vertical gradient from ammonia monooxygenase subunit alpha (*amoA*) in the seawater fraction to large subunit of RubisCO form I (*cbbL*) and large subunit of ATP citrate lyase (*aclA*) in the upper interphase, followed by adenylylsulfate reductase subunit alpha (*aprA*) in the lower interphase, and finally sulfate reductase subunit alpha (*dsrA*) and methyl coenzyme m reductase subunit alpha (*mcrA*) in the brine layer (Fig. 6).

Only a few years later, scientists started to reveal the protistan community structures in the interphases and brines of the different DHABs (Alexander

Table 2. Key players of all three domains of life in the different basins.

	Basin	Layer	Archaea	Bacteria	Eukaryotes
Mediterranean Sea	Bannock	IF	MSBL1 ^a	MSBL2 ^a	Dinoflagellates ^b
		B	MSBL1 ^c	Gammaproteobacteria ^a	Dinoflagellates ^b
	Discovery	IF	MSBL1 ^c	Gammaproteobacteria ^d	Dinoflagellates ^b
		B	MSBL1 ^c	Gammaproteobacteria ^d	Dinoflagellates ^b
	Kryos*	IF	n.a.	n.a.	n.a.
		B	n.a.	n.a.	n.a.
	l'Atalante	IF	Gammaproteobacteria ^d	Epsilonproteobacteria ^e	Ciliates ^f
		B	MSBL1 ^c	n.a.	Ciliates ^f
	Medee	IF	n.a.	n.a.	n.a.
		B	n.a.	n.a.	n.a.
Thetis		IF	Thaumarchaeota	Epsilonproteobacteria ^g	Dinoflagellates ^b
		B	MSBL1 ^g	KBI ^h	Fungi ^h
	Tyro	IF	n.a.	n.a.	n.a.
		B	n.a.	n.a.	n.a.
Urania		IF	n.a.	Epsilonproteobacteria ⁱ	Ciliates ^j
		B	MSBL1 ^c	Deltaproteobacteria ⁱ	n.a.
	Atlantis II	B	n.a.	Deltaproteobacteria ^d	n.a.
Red Sea	Discovery	B	n.a.	n.a.	n.a.
	Orca	B	n.a.	n.a.	n.a.
Gulf of Mexico		B	n.a.	n.a.	n.a.

^aDaffonchio et al. (2006), ^bEdgcomb et al. (2009), ^cvan der Wielen et al. (2005) Supporting Online Material, ^dAntunes et al. (2011), ^eYakimov et al. (2007), ^fAlexander et al. (2009), ^gLa Cono et al. (2011), ^hStock et al. (2012), ⁱBorin et al. (2009) and ^jFilker (2011).

Note: For details on taxonomic identities of DHAB residence, we refer to the chapter of Edgcomb and Orsi. n.a. = not available.

*No data published on Kryos hydrochemistry, but for completeness, the basin is mentioned in this table.

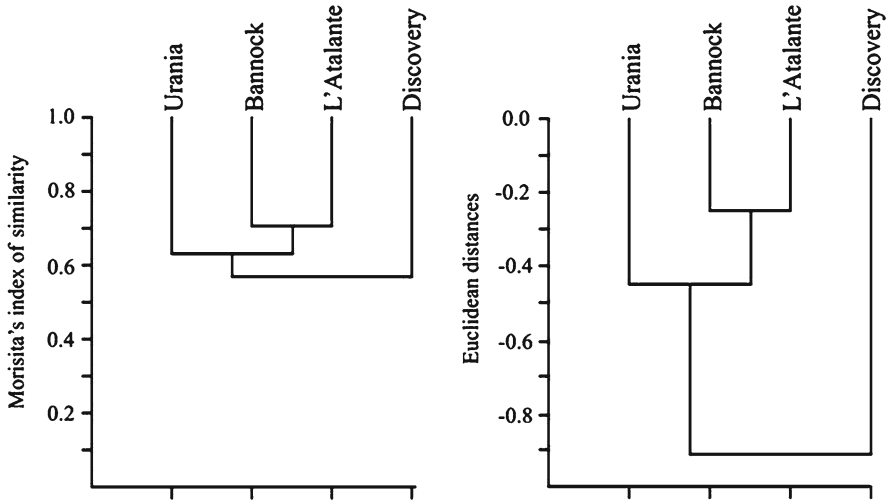


Figure 5. UPGMA cluster analysis based on the Morisita index, showing the similarity between the operational taxonomic unit distribution of four DHABs (>97 % 16S rRNA gene sequence similarity) (*left*). Euclidean distances between the hydrochemical data of the same basins (*right*) (From van der Wielen et al. (2005). Reprinted with permission from AAAS).

et al., 2009; Edgcomb et al., 2009; Stock et al., 2012). Soon, they also could assume that environmental selection may be the driving force for the formation of distinct protistan communities within the DHABs. Alexander et al. (2009), for example, compared protistan communities within the layers of the l'Atalante Basin and showed that phylotype richness, community membership, and community structure differed significantly between the brine and the interphase. Furthermore, the protistan community in the l'Atalante Basin was distinctively different from any previously described hypersaline community. The authors therefore hypothesized “that extreme environments may exert a high selection pressure possibly resulting in the evolution of an exceptional and distinctive assemblage of protists” (Alexander et al., 2009).

Another study comparing protistan communities between the brines and interphases of the Bannock and Discovery basins also showed little similarity in species composition between the communities (Edgcomb et al., 2009). This and the fact that a high proportion of the sequence clades in this study were unique to DHABs and also highly divergent to previously described 18S rRNA gene sequences were discussed by the authors (see also Table 2). They suggested the different biogeochemical conditions in the different basins to be responsible for specific selection pressure on the communities that supports evolution of biogeographically patterned, specialized protistan communities. Stock et al. (2012) focused on the Thetis Basin and detected closely related ciliates that were exclusive to Mediterranean brines including only two shared phylotypes between

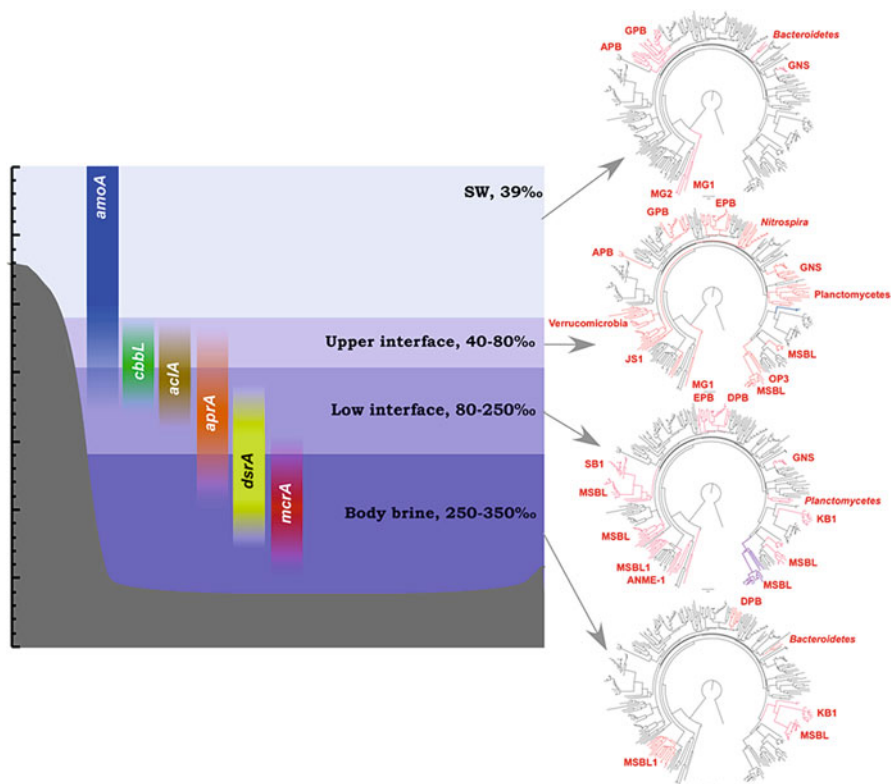


Figure 6. Stratification (salinity, bacteria, bacterial metabolic processes) of a DHAB interphase and brine. With increasing depth and salt concentration, the phylogenetic structure of bacterial communities (*right*) changes decisively as well as the metabolic profiles of the bacteria. The latter is indicated by the change of functional genes along the vertical depth gradient: *amoA* ammonia monooxygenase subunit alpha, *cbbL* large subunit of RubisCO form I, *acIA* large subunit of ATP citrate lyase, *aprA* adenylylsulfate reductase subunit alpha, *dsrA* sulfate reductase subunit alpha, *mcrA* methyl coenzyme m reductase subunit alpha.

interphase and brine. The authors discussed these ciliates to comprise special adaptations to DHAB habitats. In addition, several distinct morphotypes in the brine of the Thetis Basin were suggested to link the detected rRNA sequences (via fluorescence in situ hybridization) from the brine to indigenous polyextremophile protists (Stock et al., 2012). Because some important known halophiles could not be detected in any study of DHAB brines, the polyextreme conditions in the DHABs were assumed to most likely select for specialized communities that include different specifically adapted phylogenetic lineages in the DHABs (Stock et al., 2012). Similar results were achieved in a study targeting only one specific group of protists, the kinetoplastids. It was shown that different groups of kinetoplastids were unevenly represented in the different Mediterranean basins, which also was discussed as a result of environmental selection (Edgcomb et al., 2011).

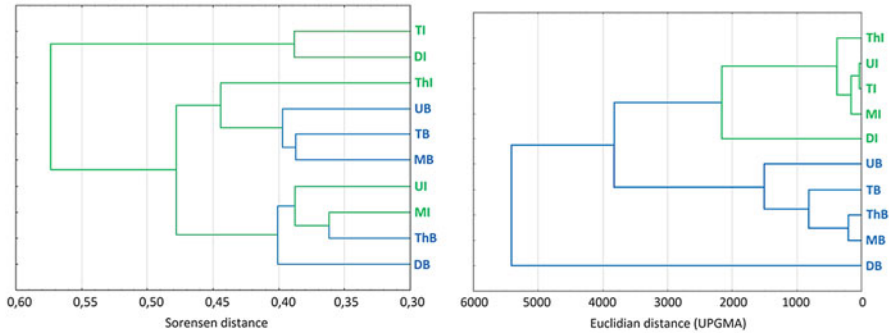


Figure 7. Hierarchical clustering (Chao-Sørensen distance) of brines (B) and interphases (I) of different Mediterranean DHABs based on T-RF size and relative abundance (*left*). Hierarchical clustering (Euclidian distance, unweighted pair group method with arithmetic mean, UPGMA) of the same samples based on physicochemical characteristics (see Table 1) (*right*). *D* Discovery, *M* Medee, *Th* Thetis, *T* Tyro, *U* Urania.

All these studies report significant dissimilarities in protistan community structures between the different DHAB layers and habitats. A fast and competitive method to investigate differences in (protistan) community compositions across many samples is terminal restriction fragment length polymorphism (T-RFLP), a molecular fingerprinting technique (Liu et al., 1997; Lüdemann et al., 2000). This strategy has been applied to plankton communities of five DHAB brines and interphases (Filker et al., 2013). Based on the resulting data, the Sørensen Index (Magurran, 2004) was calculated in order to study similarities and dissimilarities among the communities. This analysis revealed that the dissimilarity between brine communities in different basins is as pronounced as community dissimilarities within the interphases (Fig. 7, left panel). An UPGMA cluster analysis of corresponding hydrogeochemical data shows that the brine samples cluster together, clearly separating from the interphase samples. Interestingly, in both the community similarity clustering (Fig. 7, left panel) and the clustering based on hydrochemical parameters (Fig. 4, right panel), the Discovery brine with its extremely high magnesium and hydrogen sulfide concentrations stands out. This picture very well reflects the findings of van der Wielen et al. (2005) for prokaryotic communities (see above).

Concluding from these protistan diversity studies within the DHABs, we strongly emphasize the possibility of environmental selection as a major driving force in the formation of distinct protistan community structures. The findings support the idea of selection of microbial communities through environmental factors. Given the diverse and distinct combination of environmental factors in the DHABs, these habitats are ideal model systems. The unique island character in the Mediterranean Sea in the deep depressions in the seafloor formed thousands of years ago allows for the detection of environmental

selection driven by geochemical differences and physical separation in the anoxic hypersaline basins.

Further studies will be in order to provide deeper insights into these model systems, interactions between microbial communities and physiological adaptations to the polyextreme DHAB conditions in all three domains of life. Interesting questions regarding the evolution and spatial diversity patterns of microbes that could be applied to DHABs as model systems are: (1) Does the distance between basins have an influence on community structures? (2) What influence does the respective hydrochemistries of the DHABs have? (3) Do the detected hydrochemical gradients within the DHABs act as biogeographic barriers? (4) Are there special hydrochemical parameters that exert a higher selection pressure on the community composition than others?

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MICROBIAL EUKARYOTES IN HYPERSALINE ANOXIC DEEP-SEA BASINS

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1. Introduction

Deep hypersaline anoxic basins (DHABs) found in the Eastern Mediterranean Sea are thought to have formed several thousand years ago through the dissolution of buried Messinian evaporitic deposits, followed by accumulation of brines and collection in sea floor depressions (Cita, 2006 and references therein). Mediterranean and Red Sea DHABs have yielded exciting insights into novel microbial diversity across all three domains of life and have extended our knowledge of the environmental factors that define the limits of life (e.g., Alexander et al., 2009; Danovaro et al., 2010; Eder et al., 1999, 2001, 2002; Edgcomb et al., 2009, 2011b; Sass et al., 2001; van der Wielen et al., 2005; van der Wielen and Heijs, 2007; Yakimov et al., 2007b). Sequences of many taxonomic groups revealed by these studies have no known homologues in public databases, suggesting these habitats harbor organisms with possibly novel metabolic/physiological characteristics. Elucidation of the special adaptations of microbial eukaryotes (and organisms in general) to the extreme conditions found in DHABs will increase our understanding of the limits of tolerance and extent of life on Earth.

DHABs such as Urania, Discovery, Thetis, Bannock and L'Atalante (Fig. 1) are found more than 3,000 m below sea level, and the extremely high densities of these basins (typically ranging from 1.13 to 1.35×10^3 kg/m³) relative to Mediterranean seawater (1.03×10^3 kg/m³) result in minimal mixing with overlying seawater and the establishment of a steep halocline (van der Wielen et al., 2005). The chemistry of different DHABs is distinct (Table 1).

For example, Mg²⁺ concentrations in Discovery Basin can reach up to 5,000 mM compared with 300–650 mM in other basins and ca. 60 mM in regular seawater. Sodium concentrations can range from 70 mM (Discovery Basin) to 4,700 mM, compared with 500 mM in seawater above these basins (van der Wielen et al., 2005). Methane concentrations are known to vary from undetectable to over 5 mM, and sulfide can be as high as 16 mM (Urania Basin) compared with 2.6×10^{-6} mM in normal seawater (van der Wielen et al., 2005). Together with pressures associated with the depths of these basins, they are some of the most polyextreme habitats on Earth. Deep hypersaline anoxic marine habitats exist in other locations, such as the Red Sea and Orca Basin in the Gulf of



Figure 1. Map of several Eastern Mediterranean DHABs (Adapted from GoogleMaps©).

Mexico. The Orca Basin brines developed in a similar manner to E. Mediterranean brines, through the dissolution of underlying Jurassic age salt deposits, followed by accumulation in seafloor depressions. The Red Sea is an ocean in statu nascendi within the African Rift Valley system, and within this sea, numerous brine pools can be found along the bottom where tectonic activity exposes seawater to buried Miocene evaporite deposits (Eder et al., 2001).

Studies of bacteria and archaea in Eastern Mediterranean DHABs utilizing molecular approaches; measurements of microbial activities such as sulfate reduction, methanogenesis, and heterotrophic activity; and microscopy have revealed diverse and abundant communities in these habitats (e.g., Daffonchio et al., 2006; Hallsworth et al., 2007; van der Wielen et al., 2005; van der Wielen and Heijs, 2007; Yakimov et al., 2007a). Bacteria/archaeal microbial counts with 4',6-diamidino-2-phenylindole (DAPI) ranged from $1.9 \times 10^4 \text{ ml}^{-1}$ in the brine of Discovery to $1.5 \times 10^5 \text{ ml}^{-1}$ in Urania brine (van der Wielen et al., 2005). Counts of bacteria and archaea are higher in the haloclines of these basins. For example, in the halocline of Urania Basin, counts are ca. $4.3 \times 10^6 \text{ ml}^{-1}$, and this is much higher than in the seawater directly above the halocline, where counts are ca. $3.9 \times 10^4 \text{ ml}^{-1}$ (Borin et al., 2009). Based on activity measurements, results of cultivation studies, DNA fingerprinting, and 16S rRNA gene libraries, *Deltaproteobacteria* and *Epsilonproteobacteria*, predominantly sulfate-reducing bacteria and sulfur oxidizers, respectively, dominate the bacterial community, and methanogens dominate the

Table 1. Physicochemical data for several Eastern Mediterranean deep hypersaline anoxic basins and locations in the Kebrüt Deep of the Red Sea and Orca Basin.

Sample	Coordinates		Depth (m)	Total salinity		Conductivity (S/m)
				PSU	Oxygen (ml/l)	
Discovery Reference ^b	35°19'N	21°41'E	3,578	38	1.69	4.7
Discovery Interface ^b	35°19'N	21°41'E	3,580	70	0.50	7.1
Discovery Brine ^b	35°19'N	21°41'E	3,582	95 ^a	0	11.3 ^a
Urania Interface ^c	35°13'N	21°28'E	3,467	63	1.22	7.8
Urania Brine ^c	35°13'N	21°28'E	3,472	240	0	15.6
Thetis Interface ^d	34°40'N	22°08'E	3,259	80	0.68	8.2
Thetis Brine ^d	34°40'N	22°08'E	3,415	340	0	16.7
L'Atalante Upper Interface ^e	35°18'N	21°23'E	3,499	39	0.63	na
L'Atalante Lower Interface ^e	35°18'N	21°23'E	3501	365	0	na
Bannock Brine ^b	34°17'N	20°00'E	3,790	280	0	na
Bannock Interface ^b	34°17'N	20°00'E	3,300	246	0.50	na
Red Sea Kebrüt ^f	24.5°N	37.1°E	1,549	260	0	na
Orca Basin ^g	26°55'N	91°21'E	2,400	250	0	na

Discovery reference is seawater above Discovery Basin, E. Mediterranean.

na = not available.

^aUsing the conventional sensor mounted on CTD rosette, the measurement of conductivity is not reliable in athalassohaline brines enriched by divalent cations.

^bEdgcomb et al. (2009).

^cOrsi et al. (2012).

^dStock et al. (2011).

^eAlexander et al. (2009), salinity reported in ppt.

^fEder et al. (2001).

^gTribovillard et al. (2008).

archaeal community in at least some of these DHABs (Borin et al., 2009). This is consistent with the notion that elevated biomass observed within DHAB haloclines is sustained largely by sulfur cycling and methanogenesis.

Studies of chemosynthetic (anoxic) deep-sea systems have revealed a high abundance of bacteria, particularly along oxyclines, and this abundance usually supports a secondary food web of unicellular eukaryotes (e.g., Edgcomb et al., 2011a; Stoeck et al., 2006; Taylor et al., 2001, 2006). Moderately hypersaline systems are known to sustain rich and diverse communities of mostly halotolerant eukaryotes (Hauer and Rogerson, 2005). Habitats with salinities in excess of 30 ‰ are not thought to harbor many protists (Elloumi et al., 2006; Oren, 2000; Pedrós-Alió et al., 2000; Por, 1980; Ramos-Cormenzana, 1991) (for opposing viewpoint, see Finlay, 1990). However, initial investigations into protist diversity in several Eastern Mediterranean DHABs using DNA-based (Edgcomb et al., 2009) and RNA-based (Alexander et al., 2009) molecular approaches suggested that these habitats not only harbor diverse protistan communities but that these communities are largely unique to these basins and share little overlap with overlying waters with typical marine salinity and oxygen tension.

2. General Materials and Methods

Samples for data discussed here were collected during four expeditions, including a cruise in July 2009 on the *R/V Okeanus* to Discovery and Urania Basins, in September 2009 and 2010 on the *R/V Urania* (MAMBA program) to Thetis Basins, and in November 2011 on the *R/V Atlantis* to Urania, L'Atalante, and Discovery Basins (Edgcomb et al. 2009; Stock et al. 2011). The position of the halocline was determined during the *R/V Okeanus* cruise using a SBE9 CTD (Sea-Bird Electronics, USA) equipped with an SBE43 oxygen sensor (Sea-Bird Electronics, USA) and on the *R/V Urania* cruises using a Sea-Bird SBE-911 plus conductivity-temperature-depth (CTD) sensors (Sea-Bird Electronics, Inc., Bellevue, WA, USA). On the November 2011 cruise, the halocline position was determined using a newly developed water column sampler, the Submersion Incubation Device In Situ Microbial Sampler (SID-ISMS developed by C. Taylor and V. Edgcomb at WHOI and McLane Research Laboratories, Inc.). This instrument was equipped with a high-range CTD (Neil Brown Ocean Sensors, Inc.), a 4,330 oxygen optode (Aanderaa Data Instruments), and two turbidity sensors (WETLabs, Inc.). Samples were collected from the halocline and brine of each basin using a rosette equipped with Niskin bottles and from a norm-saline reference deep seawater sample. Table 1 presents coordinates, depth, total salinity, oxygen concentration, and conductivity for each sampling location/depth. The salinity gradient from the top to the bottom of individual Niskin bottles was confirmed on board the ship using a WTW portable sensor for conductivity, pH, and temperature (WTW, Weinheim, Germany). Samples were processed and preserved in RNAlater (Ambion) for molecular analyses or in formaldehyde (2 % final conc.) for fluorescence in situ hybridization (FISH) according to methods described in Edgcomb et al. (2011a, b, c). For combined FISH and scanning electron microscopy (SEM), sample processing followed the methods described in Stoeck et al. (2003).

RNA was extracted from min. 3 replicate filters per water sample as described previously in Alexander et al. (2009) using bead beating of filters in an extraction buffer and E-Matrix tubes (QBiogene, MP Biomedicals, USA), followed by the RNA/DNA Allprep kit (Qiagen, Hildesheim, Germany) according to the manufacturer's instructions. Reverse transcription, PCR amplification of the small subunit ribosomal RNA gene (18S rRNA), and analyses of sequenced clone libraries followed the protocols described in Stock et al. (2011) and Edgcomb et al. (2011a). DNA preparations and 18S gene sequencing and analysis followed protocols described in Edgcomb et al. (2009).

3. Comparison of Microbial Eukaryotes in Different DHAB Environments

3.1. DNA AND RNA-BASED EVIDENCE FOR EUKARYOTIC LIFE IN DHABS

The first evidence that microbial eukaryotes were active and diverse members of the microbial community in DHABs of the Eastern Mediterranean came from a cDNA-based analysis of protistan communities in the halocline of L'Atalante

Basin (Alexander et al. 2009) and a DNA-based study of the haloclines and brines of Bannock and Discovery Basins (Edgcomb et al., 2009). Diverse protist signatures were found in both halocline and brine water samples of Discovery and Bannock, with 75 % of phylotypes (at 98 % sequence similarity) affiliating with the alveolates, 12 % of which represented ciliates, and 62 % dinoflagellates (Edgcomb et al., 2009). Both groups of alveolates are successful phagotrophs and likely feed on the abundant bacteria present, particularly along the halocline. Fungi were also fairly abundant in all samples (17 % of phylotypes), particularly in brine samples, consistent with the notion that fungi are active remineralizers of organic material (dead cells and detritus) that accumulates at the halocline, and some of which sinks into the brine.

Since it is difficult to interpret activity from DNA-based surveys, the study by Alexander et al. (2009) was particularly important in showing that eukaryotic RNA could be recovered from L'Atalante Basin halocline water samples. This provided evidence of active eukaryotes. This study compared 18S rRNA gene signatures recovered from the upper (3,499 m) and lower (3,501 m) halocline of L'Atalante Basin. Alexander et al. (2009) recovered 43 phylotypes at 99 % sequence similarity from the upper halocline and 42 phylotypes from the lower halocline. Interestingly, even though ciliates represented the largest proportion of phylotypes in both libraries (18 in upper halocline and 21 in lower), the number of shared phylotypes was very low (12) between the two halocline samples which were collected at depths separated by only ~1.5 m. Differences in the protist community composition likely reflect the sharp gradient of electron donors and acceptors, salinity, and perhaps the gradient in ammonia concentrations, which increases from 5.5 μM in the upper halocline to 3,000 μM in the lower. The shared taxa included ciliates (7), choanoflagellates (2), fungi, Radiolaria, and jakobids (1 operational taxonomic unit defined at 97 % sequence similarity for each group). Fungi and Radiolaria made up a large proportion of the upper halocline community, and only one representative of each group was detected in the lower halocline. Dinoflagellates were diverse in the lower halocline, and none were detected in the upper halocline. Furthermore, cryptophytes were found exclusively in the lower, more hypersaline waters, and stramenopiles, haptophytes, rhizarians, and chlorophytes were detected exclusively in the upper halocline library. Jaccard indices supported the conclusion that these two closely located communities were very different from one another (Alexander et al., 2009). In both the study of Alexander et al. (2009) and Edgcomb et al. (2009), signatures of novel taxonomic groupings were detected, particularly within alveolates, and many sequences affiliated with sequences from other environmental diversity surveys from a variety of habitats. When community membership was compared for Bannock and Discovery, Jaccard indices suggested that the communities were unique from one another and shared little (0.8–2.8 %) in species composition with overlying waters with typical marine salinity and oxygen. These observations produced the hypothesis that differences in basin chemistry are selecting for unique protist communities.

Further evidence for environmental selection for unique protist communities in these DHABs comes from a study of Thetis Basin, which has one of the highest

salt concentrations reported for DHABs (348‰), yet supports protist counts of ca. $0.6 \times 10^4 \text{ l}^{-1}$ of anoxic brine (Stock et al., 2011). This cDNA-based study identified fungi as the most diverse taxonomic group of eukaryotes in the brine (38 % of OTUs based on 98 % sequence similarity), followed by ciliates and stramenopiles, each accounting for 20 % of phylotypes. Many of the ciliate sequences detected in this study were closely related to sequences detected in surveys of other DHABs, suggesting specific adaptations to these habitats. In addition, marine stramenopile (MAST) clades were detected in the Thetis brine samples, and this expands the known salinity range of these organisms. Also detected were signatures of dinoflagellates, haptophytes, choanoflagellates, and jakobids, and beta-diversity analyses supported the uniqueness of brine vs. halocline communities (Stock et al., 2011).

The first two studies of protistan diversity in DHABs indicated that the communities were enriched with dinoflagellates, ciliates, and other alveolates, as well as fungi, but were conspicuously poor in stramenopiles. Neither study indicated the presence of kinetoplastid flagellates, which have been reported previously from anoxic and high-salt environments (Hauer and Rogerson, 2005). Kinetoplastid-specific primers were applied to nucleic acid samples from six different DHABs with distinct geochemical settings, and diverse kinetoplastid sequences were detected in three of the six DHABs, plus the normal seawater sample above one of the basins (Edgcomb et al., 2011b). While abundance in clone libraries must be interpreted with caution, it was noticed that an “unidentified clade” of kinetoplastids dominated the clone library from the Discovery Basin halocline (Fig. 2). Sequences affiliating with this unidentified clade were only recovered from halocline samples from Urania, Discovery, and another newly discovered basin, and not from brine samples or the reference seawater sample, suggestive of a halocline specialist.

3.2. LINKING SIGNATURES TO CELLS USING MICROSCOPY

Since it is difficult based on DNA- or RNA-based markers to prove that signatures we recovered represent active/living cells, we therefore applied fluorescent *in situ* hybridization (FISH) to link molecules with indigenous organisms. FISH probes developed using the 18S rRNA gene sequence of this “unidentified clade” of kinetoplastids allowed the counting and visualization of this group, which represented as much as 10 % of the total protist community in the Discovery Basin halocline (6.4×10^3 “unidentified” kinetoplastids per liter) and is most likely a new genus (Fig. 3k). The fact that kinetoplastids were not detected in all basins and that this novel group of kinetoplastids was only found in halocline samples from 3/6 basins supports the notion that unique basin chemistry is driving environmental selection of DHAB protist communities. So far this has been demonstrated only for kinetoplastids, but with available molecular signatures from a wide range of taxonomic groups detected in DHABs, it is possible to utilize these sequence data to test additional hypotheses.

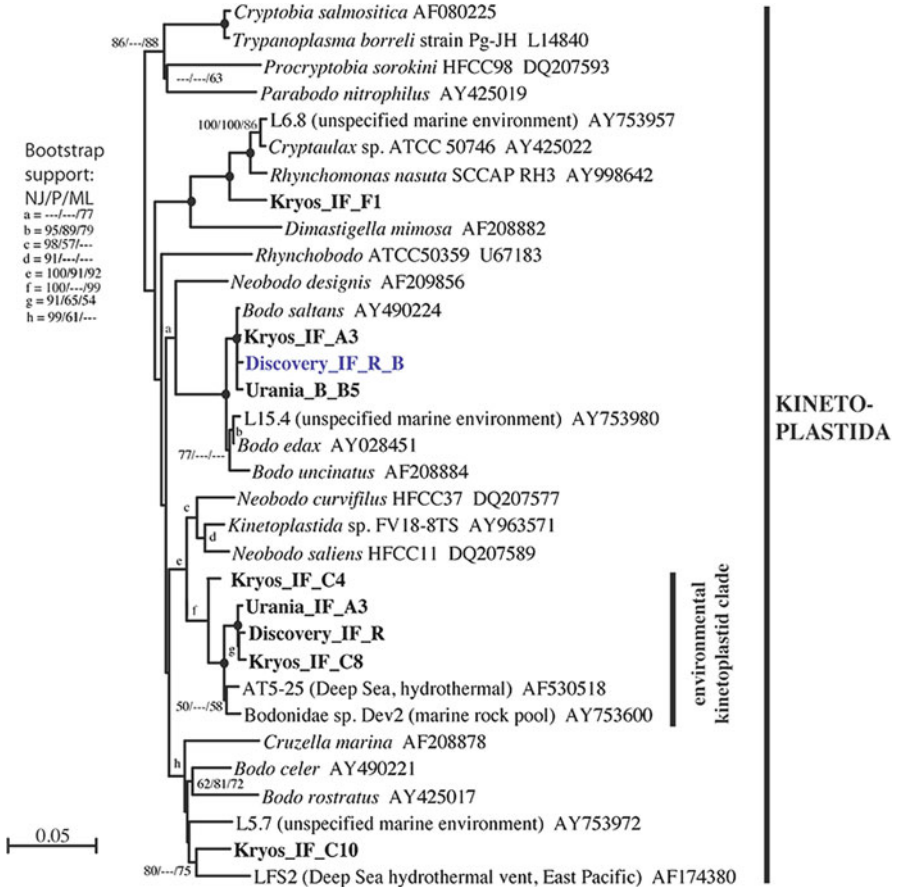


Figure 2. Minimum evolution (NJ distance) phylogenetic tree of small subunit ribosomal RNA phylotypes obtained for kinetoplastids (Adapted from Edgcomb et al., 2011b). DHAB sequences in bold. “IF” in the phylotypes name refers to the halocline, “B” refers to the brine, and “R” refers to the normoxic and norm-saline deep-sea reference seawater. Numbers at nodes correspond to NJ distance, parsimony, and maximum likelihood bootstrap values, respectively, (1,000 replicates, only values >50 % shown). Dots at nodes designate nodes with significant support under all three methods.

3.3. ABUNDANT PUTATIVE SYMBIOSES BETWEEN PROTISTS AND BACTERIA AND/OR ARCHAEA IN DHABS

Endosymbiotic associations are common within protists (for recent review, see Nowack and Melkonin, 2010), and in the case of micro-oxic (up to 0.1 ml/l; Bernhard and Sen Gupta, 1999) or anoxic and potentially sulfidic marine environments, symbiosis between protists and bacteria and/or archaea may represent a strategy for exploiting these otherwise inhospitable environments. Symbiotic relationships between organisms affiliated with the Bacteria, Archaea, and Metazoa

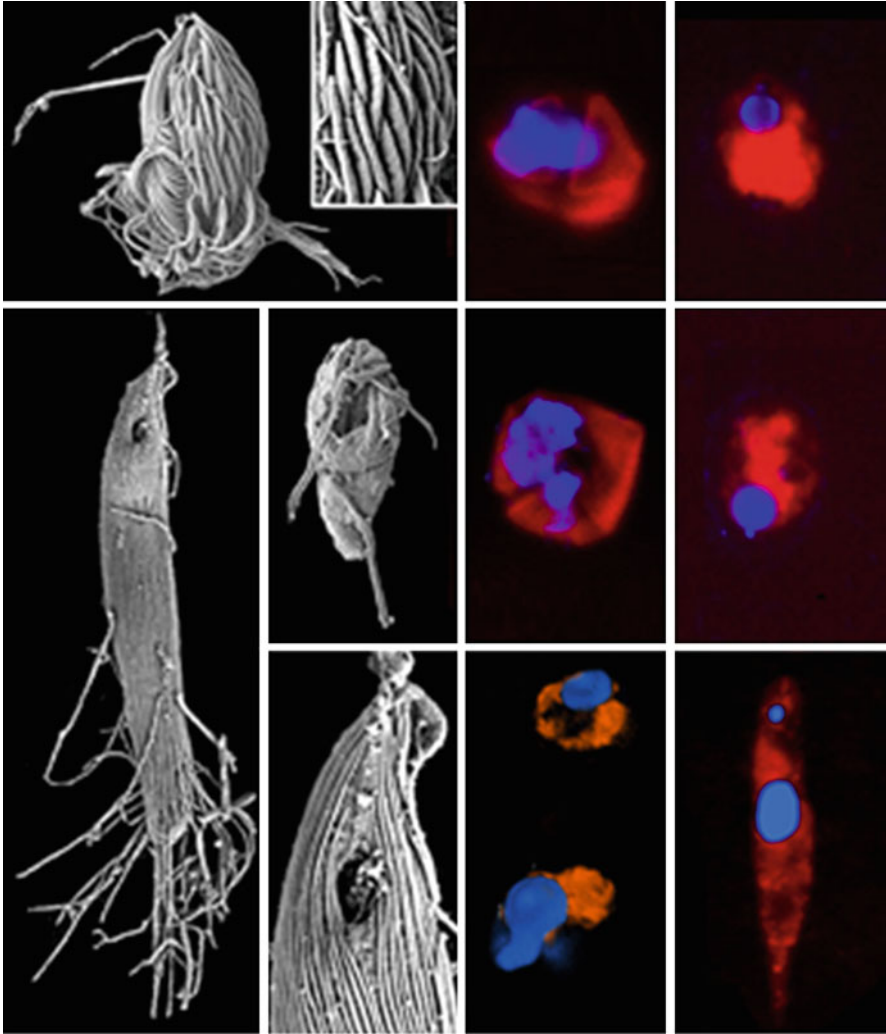


Figure 3. Scanning electron and epifluorescent micrographs of microbial eukaryotes from the haloclines of Urania and Discovery Basins in the Eastern Mediterranean Sea. **(a)** A scuticociliate morphotype consistently associated with epibiotic bacteria **(b)** that has been found to be the most abundant eukaryotic morphotype in the Urania halocline (Orsi et al., 2012). **(g, i)** The same scuticociliate morphotype stained with a fluorescent oligonucleotide probe specific to eukaryotic rRNA (*red*) and counterstained with DAPI revealing the macro- and micronuclei (*blue*). **(c)** A larger ciliate associated with long (10–20 μm), thin, filamentous bacteria **(d)**; this morphotype is the most abundant eukaryotic morphotype in the Discovery halocline (Orsi et al., 2012). **(e)** A small flagellate, possibly corresponding to the MAST stramenopile clade, recovered from the Urania halocline. **(f, h)** Dinoflagellates from Urania halocline, stained with a fluorescent oligonucleotide probe (*red*) specific to large subunit of dinoflagellate rRNA that likely correspond to either *Gymnodinium* or *Gyrodinium* genera (nuclei are stained blue with DAPI). **(j)** Smaller dinoflagellates stained with the same dinoflagellate-specific probe and DAPI. **(k)** A cell corresponding to a novel kinetoplastid clade (see Edgcomb et al., 2011b for discussion) recovered from the Discovery halocline. *Scale bars:* **(a, j)** = 10 μm ; **(b)** = 2 μm ; **(c)** = 35 μm ; **(d)** = 5 μm ; **(f, g, h, i)** = 7 μm ; **(k)** = 25 μm .

in oxygen-depleted marine habitats also are known in a variety of hydrothermal habitats, silled basins, seeps (e.g., Cavanaugh et al., 1981; Cavanaugh, 1994; Barry et al., 1996; Distel and Felbeck, 1988), and shallow water hypersaline environments (discussed in Hickman, 2005). Ciliates and flagellates with bacterial and/or archaeal partners are commonly observed in norm-saline, oxygen-depleted, and anoxic/sulfidic sedimentary habitats such as the Santa Barbara Basin (e.g., Bernhard et al., 2000; Edgcomb et al., 2010) and water columns such as the Cariaco Basin (Edgcomb et al., 2011c). Although steep chemoclines with gradients in available electron donors and acceptors likely select for different types of symbioses, the symbionts of many free-living ciliates in anoxic marine habitats are known to be methanogens (e.g., Fenchel and Finlay, 1991; van Hoek et al., 2000; Embley and Finlay, 1993, 1994). In many cases, associations between hydrogenosomes and endosymbiotic methanogens are observed (Embley and Finlay, 1994), suggesting a cooperative metabolism in anoxic environments. Other types of bacterial and archaeal partners are observed in ciliates and flagellates in anoxic/sulfidic habitats. For example, sulfur- or sulfide-oxidizing epsilonproteobacterial epibionts were observed to cover the external cell surface of a group of euglenozoans from Santa Barbara Basin (Edgcomb et al., 2010). In addition to possible nutritional roles for the host, these epibionts may play a role in the adaptation of these Euglenozoa to otherwise toxic, sulfidic habitats. The haloclines and anoxic and frequently sulfidic brines of DHABs are logical environments to look for similar putative symbioses between protists and bacteria/archaea.

Formaldehyde-fixed samples from Discovery and Urania Basins (collected November 2011) were examined with scanning electron microscopy (SEM). Based on SEM observations, ciliates dominated the eukaryotic microbial community in the halocline of Discovery and Urania Basins, and >80% of those ciliates had visible epibionts (Fig. 3a–d). The detection of intact nuclei with DAPI (e.g., ciliates; Fig. 3g, i) and positive FISH hybridization is further proof that these eukaryotes are alive in this habitat. It is also unlikely that epibionts would remain associated with dead cells or that flagella would remain intact on dead flagellates. Other types of intact eukaryotic cells were also observed on SEM filters from Discovery and Urania Basin halocline waters, including dinoflagellates (Fig. 3f, h, j), flagellates (Fig. 2e), and kinetoplastids (Fig. 3k).

4. Looking Forward

Microbial eukaryotes are diverse and abundant members of the microbial community across Eastern Mediterranean DHABs based on 18S rRNA molecular signatures and cell counts of eukaryotes in limited samples using FISH/DAPI. High-resolution sampling is needed in order to precisely target the narrow haloclines typical of DHABs. Traditional Niskin sampling captures a column of water that can span almost half the thickness of the halocline, and it is difficult to maintain the integrity of such steep gradients within the Niskin bottles during

transport to the surface. The SID-ISMS sampler allowed for the first time in November 2011 high-resolution sampling of these habitats and in situ sample preservation. The next step toward understanding the adaptations and activities of microbial eukaryotes in these polyextreme habitats will be to examine gene expression. Because transport from such deep and extreme habitats in Niskin bottles is likely to introduce artifacts, new in situ sampling and preservation capabilities are likely to enhance our ability to understand adaptations of eukaryotes to DHAB environments.

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Biodata of **Aharon Oren**, author of “*Life at High Salt and Low Oxygen: How Do the Halobacteriaceae Cope with Low Oxygen Concentrations in Their Environment?*”

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LIFE AT HIGH SALT AND LOW OXYGEN: HOW DO THE *HALOBACTERIACEAE* COPE WITH LOW OXYGEN CONCENTRATIONS IN THEIR ENVIRONMENT?

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1. Introduction

The halophilic Archaea of the order *Halobacteriales*, family *Halobacteriaceae*, are the halophiles par excellence. As of December 2011, this family encompassed 36 genera with 129 species with standing in the prokaryote nomenclature (Oren, 2012). They all require high NaCl concentrations for growth, most species do not grow at salt concentrations below 150–200 g/l, and many also thrive in salt-saturated brines (Oren, 2002, 2006). Only a few species can tolerate salt concentrations below 100 g/l, conditions that cause lysis of most members of the family.

With one notable exception (*Halorhabdus tiamatea*, see below), all members of the *Halobacteriaceae* are aerobes that oxidize amino acids, sugars (in most species), and other simple carbon sources, using oxygen as terminal electron acceptor in respiration (Oren, 2006). As a result, these organisms can be expected to be exposed to two types of stress in salt lakes and brines: they have to deal not only with the osmotic problems caused by the extremely high salt concentration in their medium, but they also must cope with the stress caused by limited availability of molecular oxygen. The higher the salt concentration, the lower the solubility of oxygen and other gases is in water. In NaCl-saturated brines, the saturation concentration of oxygen is less than one-fifth of that in freshwater (Sherwood et al., 1991, 1992) (Fig. 1). Using a method based on the Winkler titration, Rodriguez-Valera et al. (1985) measured oxygen concentrations as low as 0.50 mg/l in saltern crystallizer ponds in Spain. The often high temperatures of natural hypersaline brines, as well as the presence of dense communities of halophilic Archaea that can take up oxygen at a high rate, may quickly lead to oxygen depletion (Tindall and Trüper, 1986). Measurements of respiration rates in saltern crystallizer brines typically populated by 10^7 – 10^8 prokaryotic cells/ml confirm that this is indeed the case (Warkentin et al., 2009; see also Fig. 2).

In “athalassohaline” brines in which NaCl is not the dominant salt, dissolved oxygen are likewise low. Oxygen-saturated Dead Sea water (salinity ~275–280 ppt or ~340–345 g/l total dissolved salts, dominated by Mg^{2+} and Cl^- as major ions) contains only about 0.7–0.9 ml O_2 /l (\approx 1–1.3 mg/l) (Levy, 1980). Dead Sea water

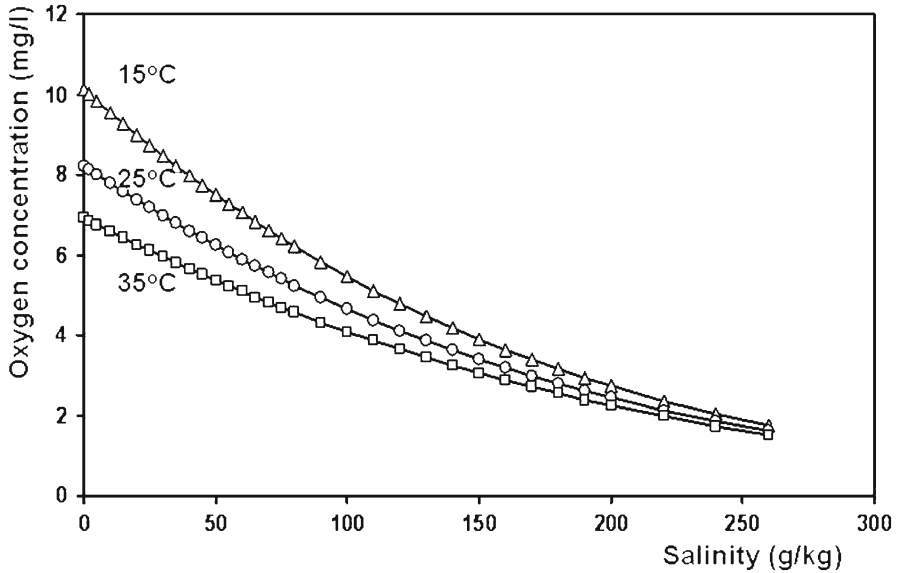


Figure 1. The relationship between oxygen solubility (mg/l) and salinity (ppt of NaCl in g/kg; a salinity of 260 g/kg corresponds with a salt-saturation at about 320 g/l) (Based on data presented by Sherwood et al., 1992).

of 280 ppt (345 g/l total dissolved salts) in equilibrium with air could contain 1.54 mg/l (0.99 ml/l) oxygen at 25 °C or 1.14 mg/l (~0.79 ml/l) at 46 °C (Shatkay, 1991; Shatkay et al., 1993).

To cope with the potential lack of molecular oxygen in many places where halophilic Archaea thrive, many members of the *Halobacteriaceae* have developed different methods for growth and survival in the absence of oxygen and/or ways to move toward more oxygen-rich niches. This chapter explores the diverse strategies used by the extremely halophilic Archaea to deal with low-oxygen situations while living in salt-saturated or nearly salt-saturated environments.

2. Denitrification

Many prokaryotes can use nitrate as terminal electron acceptor in respiration, reducing the nitrate to gaseous products: dinitrogen and sometimes minor amounts of nitrous oxide as well. This process is known as denitrification. Anaerobic growth in the presence of nitrate is not widespread among the members of the *Halobacteriaceae*, but some species are true denitrifiers that evolve N_2 and N_2O from nitrate when oxygen is in short supply. These include *Haloarcula marismortui*,

Har. vallismortis,¹ *Haloferax denitrificans*, *Hfx. mediterranei*, and *Halogeometricum borinquense* (Hochstein and Tomlinson, 1985; Mancinelli and Hochstein, 1986; Montalvo-Rodríguez et al., 1998; Oren et al., 1990; Tomlinson et al., 1986). In *Hfx. denitrificans*, the dissimilatory nitrate reductase is induced already under low oxygen concentrations, not only when oxygen is completely depleted (Hochstein, 1991). *Har. marismortui* and *Har. vallismortis* produce small amounts of nitrous oxide from nitrate in addition to dinitrogen; *Hfx. mediterranei* does not make N₂O (Mancinelli and Hochstein, 1986).

Thus, only a small minority of the *Halobacteriaceae* have the ability to grow by denitrification. The question then arises where in their natural environment they may encounter conditions where this ability may be advantageous. It can be argued that the presence of genes enabling anaerobic growth in the presence of nitrate signifies that some halophilic Archaea may occasionally encounter suitable conditions to express these genes and have a selective advantage. However, nitrate is rarely found at high concentrations in hypersaline environments. Hochstein and Tomlinson (1985) mention the virtual absence of nitrate from some hypersaline lakes (the Dead Sea, Great Salt Lake, Utah) as a possible indication for denitrification as allegedly nitrate-rich agricultural waters drain into them. However, there is a much more obvious reason why nitrate is seldom encountered in hypersaline brines: the lack of autotrophic nitrification. In soils and freshwater and marine environments, ammonium ions are oxidized under aerobic conditions to nitrite and then further to nitrate by different types of chemolithoautotrophic nitrifying bacteria. Autotrophic nitrification was never shown to occur at the highest salt concentrations. The upper salinity limit for autotrophic oxidation of ammonium ions to nitrite is around 120–150 g/l, and for oxidation of nitrite to nitrate the limit is probably even lower. The absence of nitrification at high salt concentrations was explained as being due to bioenergetic constraints: these processes yield very little energy only – probably not enough to support the energetically expensive modes of osmotic adaptation to provide osmotic balance of the cytoplasm with the external medium (Oren, 1999, 2001, 2011).

3. Use of Other Terminal Electron Acceptors: Dimethyl Sulfoxide, Trimethylamine *N*-Oxide, and Fumarate

Some members of the *Halobacteriaceae* can couple the reduction of other alternative electron acceptors with growth: dimethyl sulfoxide (DMSO), trimethylamine *N*-oxide (TMAO) (Oren and Trüper, 1990), and fumarate (Oren, 1991). The reduction products are dimethyl sulfide, trimethylamine, and succinate, respectively. Anaerobic growth in the presence of DMSO or TMAO was shown in *Hbt. salinarum*, *Har.*

¹In this chapter, three-letter abbreviations for genus names are used as recommended by the ICSP Subcommittee on the taxonomy of *Halobacteriaceae* (<http://www.the-icsp.org/taxa/halobacterlist.htm>): *Haloarcula* (*Har.*), *Halobacterium* (*Hbt.*), *Haloferax* (*Hfx.*), *Halogeometricum* (*Hgm.*), *Haloquadratum* (*Hqr.*), *Haloplanus* (*Hpn.*), *Halorhabdus* (*Hrd.*), and *Halorubrum* (*Hrr.*).

marismortui, *Har. vallismortis*, and *Hfx. mediterranei*. In *Hfx. volcanii*, DMSO supported anaerobic growth, while TMAO did not. No significant growth was observed in the presence of these electron acceptors in *Halorubrum sodomense*, *Hfx. gibbonsii*, and *Natronomonas pharaonis* (Oren and Trüper, 1990). An operon encoding the genes responsible for DMSO and TMAO reduction was identified during genomic analysis of *Halobacterium* NRC-1 (Müller and DasSarma, 2005).

The possible ecological importance of DMSO reduction under anaerobic conditions by halophilic Archaea in their natural environment is not clear. There is no reason to assume DMSO to be present in their habitat. In fact, the same is true for most non-halophilic prokaryotes that can reduce DMSO, including *Escherichia coli*. In the case of TMAO reduction, the situation may be different. TMAO is accumulated at high concentrations within the tissues of marine fish. Teleost fish may contain 3–4 % TMAO by dry weight; elasmobranchs may even have up to 7 % TMAO (Bickel-Sandkötter et al., 1996; Oren and Trüper, 1990). The formation of bad-smelling trimethylamine from trimethylamine oxide during the deterioration of fish is known for a long time already. Reduction of TMAO as a novel mechanism of anaerobic respiration, enabling anaerobic growth on non-fermentable substrates, was recognized in the 1970s (Strøm and Larsen, 1979; Strøm et al., 1979). One of the environments where halophilic Archaea often develop is salted fish, and therefore, TMAO may be available as an alternative electron acceptor enabling continuation of growth when oxygen supply is limited (see also Oren et al., 2011).

Another substrate known as a terminal electron acceptor is fumarate. Some facultative anaerobic prokaryotes such as *E. coli* have a dissimilatory fumarate reductase enabling anaerobic growth in the presence of fumarate. Also some halophilic Archaea grow anaerobically when fumarate is added to the growth medium: fumarate-driven anaerobic growth was reported in some strains of *Hbt. salinarum*, in *Hfx. volcanii*, and in *Hfx. denitrificans*. No such growth was obtained for *Hfx. mediterranei*, *Hfx. gibbonsii*, *Har. marismortui*, and *Har. vallismortis*. The ability to grow anaerobically in the presence of fumarate was not correlated with the above-described ability to use DMSO or TMAO as terminal electron acceptors (Oren, 1991).

Indications that fumarate can indeed be used as a terminal electron acceptor by natural communities of halophilic Archaea were obtained from respiration studies. If indeed fumarate can relieve the need for molecular oxygen, then fumarate addition may be expected to cause a decrease in respiration rates. This was indeed found in a study of the community respiration of the crystallizer brines of the salterns of Eilat, Israel. These studies were carried out in the dark to abolish photosynthetic oxygen production by the unicellular alga *Dunaliella salina*, which is the sole primary producer in the system. Dark incubation also prevents energy generation from light by the halophilic Archaea themselves, based on absorption of photons by the light-driven proton pump bacteriorhodopsin (see Sect. 5),

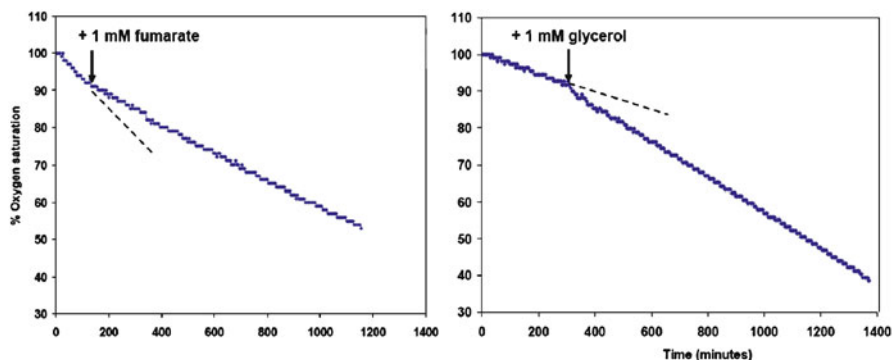


Figure 2. Decrease of the respiration rate by the microbial community from a saltern crystallizer pond in Eilat, Israel, following addition of 1 mM fumarate (*left panel*). A portion of 620 ml NaCl-saturated brine from a crystallizer pond sampled in May 2011, and containing 2.9×10^7 prokaryote cells/ml and 1,200 *Dunaliella salina* cells/ml, was incubated in the dark in a completely filled Plexiglas chamber provided with a Yellow Springs Instrument optical oxygen electrode (Pro20 Lab/Field Dissolved Oxygen Meter) and a magnetic stirring bar, the temperature being controlled at 30–31 °C. The oxygen concentration was recorded every 5 min. At the time indicated, fumarate was added to a final concentration of 1 mM by injection of 0.62 ml of a 1 M solution of NaOH-neutralized fumaric acid. For comparison, the *right panel* shows the stimulatory action of glycerol on the respiration rate in a parallel experiment. With thanks to the students Rael Horwitz, Natasha Belkin, Itay Doliansky, Shany Gefen-Treves, Michael Peer, and Haim Treves who participated in these experiments.

thereby decreasing the need for ATP generation by respiratory electron transport. In the NaCl-saturated brines, the oxygen concentration was estimated at around 25 μM , and the endogenous respiration rate of the microbial community (about 3.3×10^7 prokaryotic cells/ml and ~ 110 *Dunaliella* cells/ml) was about 0.5 $\mu\text{mol O}_2/\text{l}\cdot\text{h}$, so that oxygen could be depleted after 50 h. Experiments in which the decrease in oxygen concentration was monitored using the Winkler titration method showed that addition of 1 mM fumarate caused a drop of 16–34 % in respiration rate (Warkentin et al., 2009). The sparing effect of fumarate on the community respiration was confirmed in recent experiments in which an optical oxygen electrode (“optode”) was used to continuously monitor the oxygen in an incubation chamber in the dark (Fig. 2, left panel). The right panel of the figure shows a control experiment in which glycerol, a suitable substrate for growth of most halophilic Archaea, was added: here a significant stimulation of the respiratory activity was observed. Microscopic examination of the crystallizer brine used in these experiments showed that flat, square cells resembling *Haloquadratum walsbyi* dominated the community. Whether *Hqr. walsbyi* can use fumarate as electron acceptor is unknown. The property was not examined at the time the species description was prepared (Burns et al., 2007), and no gene for fumarate dehydrogenase was annotated in its genome (Bolhuis et al., 2006).

The ecological relevance of the use of fumarate as electron acceptor in respiration remains to be ascertained, as there is no clear reason why fumarate would be available in significant concentrations in any ecosystem in situations where molecular oxygen is in short supply.

Elemental sulfur also was suggested to serve as a potential electron acceptor in some members of the *Halobacteriaceae* (Tindall and Trüper, 1986). Under microaerophilic conditions, a number of strains reduce elemental sulfur to sulfide, but little information is available about the nature of the process.

4. Fermentation

Another way to cope with life in the absence of molecular oxygen is by fermentation. Fermentative growth appears to be rare in the *Halobacteriaceae*. There is, however, one notable exception, and that is *Halorhabdus tiamatea*. It was isolated from Shaban Deep, a hypersaline anoxic deep-sea brine 1,447 m below the surface of the Red Sea. In contrast to the earlier-described *Hrb. utahensis*, which is a red aerobic species isolated from Great Salt Lake, Utah, *Hrb. tiamatea* is not pigmented and does not readily grow under aerobic conditions (Antunes et al., 2008). The exact mode of fermentation is still to be elucidated, but a gene coding for L-lactate dehydrogenase was identified in its genome. It is therefore possible that lactate may be one of the fermentation products (Antunes et al., 2011a). *Hrb. tiamatea* can also grow anaerobically in the presence of elemental sulfur or nitrate as electron acceptors (Antunes et al., 2011b). Representatives of the genus *Halorhabdus* with an anaerobic mode of life may be more widespread in deep-sea brines: 16S rRNA gene sequences affiliated with the genus *Halorhabdus* were recovered with a remarkably high frequency from Discovery Basin, an anoxic hypersaline basin found at a depth of 3,580 m in the Eastern Mediterranean Sea (van der Wielen et al., 2005).

A well-documented mode of fermentative growth of a halophilic archaeon is that of *Hbt. salinarum* using arginine as energy source. The first indications that arginine may be metabolized by *Halobacterium* in a fermentative pathway came from a study by Dundas and Halvorson (1966) who showed that an isolate designated *Hbt. salinarum* strain 1 required arginine for growth. It converted arginine to citrulline which was further metabolized to ornithine and carbamoylphosphate, which is further split into CO₂ and ammonia with the gain of ATP (Ducharme et al., 1972; Dundas and Halvorson, 1966). This reaction was found to support anaerobic growth of *Hbt. salinarum* in the dark (Bickel-Sandkötter et al., 1996; Hartmann et al., 1980; Oesterhelt, 1982). The enzymes involved in the pathway were characterized in depth: arginine deiminase (Monstadt and Holldorf, 1991) and ornithine carbamoyltransferase (Ruepp et al., 1995). The genes involved in the fermentation pathway, encoding arginine deiminase (*arcA*), carbamate kinase (*arcC*), and catabolic ornithine carbamoyltransferase (*arcB*), are located together with the gene for a putative regulatory protein in an operon (*arcRACB*) (Ruepp and Soppa, 1996).

Anaerobic growth on arginine is not common among the halophilic Archaea: of all genera tested only *Halobacterium* strains showed fermentative growth on arginine (Oren, 2006). This enabled the design of a specific enrichment procedure for members of the genus *Halobacterium*: inoculation of a hypersaline growth medium with yeast extract as carbon source followed by incubation in the dark in the presence of 5 g/l L-arginine per liter specifically stimulates growth of strains of *Halobacterium*, a genus not abundantly found in most hypersaline environments. This method was successfully applied to obtain *Halobacterium* strains from the salterns of San Francisco Bay and of Eilat, Israel (Oren and Litchfield, 1999). To what extent the ability to grow anaerobically on arginine is ecologically relevant is not clear: no large amounts of this amino acid can be expected to accumulate to become available as a substrate for fermentation in any anaerobic ecosystem, not only in hypersaline ones.

There are a few additional reports suggesting that some members of the *Halobacteriaceae* may have a limited ability to grow in the absence of molecular oxygen using other, not further specified modes of fermentation. *Har. vallismortis* was described as a facultative anaerobe (Gonzalez et al., 1978), but the mode of its anaerobic metabolism was not investigated. Javor (1984) isolated a number of red halophilic Archaea from the saltern crystallizer ponds in California and Mexico and reported a slight increase in optical density of anaerobically incubated cultures when supplemented with glucose, a few other sugars, and a limited number of other substrates. Surprisingly, these other compounds included acetate, which is not a fermentable substrate. However, the optical densities of the cultures were always low, and therefore it may be questioned whether indeed anaerobic fermentative growth occurred.

5. Anaerobic Photoheterotrophic Growth

Another well-documented mode of anaerobic growth, at least in *Hbt. salinarum* but possibly functional in a few other members of the *Halobacteriaceae* family as well, is photoheterotrophic growth based on the presence of the light-driven proton pump bacteriorhodopsin. Excitation of bacteriorhodopsin generates a proton gradient across the cell membrane, and this proton gradient then drives the formation of ATP by the membrane-bound ATP synthase complex (Bickel-Sandkötter et al., 1996; Hartmann et al., 1980; Oesterhelt, 1982; Oesterhelt and Krippahl, 1983). Low oxygen concentrations together with the availability of light induce the *bop* gene cluster of *Hbt. salinarum* that encodes the genes for the biosynthesis of the protein moiety (bacterio-opsin) of the bacteriorhodopsin proton pump (Shand and Betlach, 1991).

However, the formation of retinal, the 20-carbon chromophore of bacteriorhodopsin, from its 40-carbon precursor β -carotene is oxygen dependent. Therefore, at least trace amounts of molecular oxygen are needed to enable sustained growth of *Halobacterium* in the light, or alternatively retinal or its derivatives should be supplied to the medium (Oesterhelt and Krippahl, 1983).

Bacteriorhodopsin is not formed by all members of the *Halobacteriaceae*. Among the species that do have the ability to synthesize the light-driven proton pump are *Hqr. walsbyi*, *Hrr. sodomense*, and *Har. marismortui*. Anaerobic photoheterotrophic growth has not yet been documented in these species.

6. Active and Passive Movement Toward Higher Oxygen Concentrations

An entirely different strategy to cope with stressful situations caused by low oxygen concentrations is to move toward areas where more oxygen may be available. This can be achieved in two different ways: actively by the use of flagella (aerotaxis) and passively by flotation, using gas vesicles.

Aerotaxis in a halophilic archaeon was reported in *Hbt. salinarum*: in a preparation of cells for microscopy, cells were seen to accumulate near air bubbles trapped between the microscope slide and the coverslip (Stoeckenius et al., 1988). Aerotaxis was less pronounced when the culture was illuminated with green light, absorbed by bacteriorhodopsin. When light energy is available for ATP production, there is less need for respiratory electron transport for energy generation, and the cells do not need to move actively toward the oxygen (Bibikov and Skulachev, 1989; Lindbeck et al., 1995). To what extent aerotaxis is common among the *Halobacteriaceae* is not known. Many species do not possess flagella, so they lack the ability of active movement altogether.

An altogether different strategy to move toward the oxygen is passive flotation by means of gas vesicles, gas-filled hollow structures that are produced by some prokaryotes, including a few representatives of the *Halobacteriaceae*, to regulate their buoyancy and to help them position themselves at the desired depth in aquatic systems. The presence of gas vesicles was first described by Klebahn (1919, see also DasSarma et al., 2010). Petter (1932) argued that gas vesicles might be important to aerobic halophilic prokaryotes, buoying them to the water surface where the oxygen concentration is highest. The early electron micrographs of *Hbt. salinarum* by Houwink (1956) provided pictures of surprisingly high quality, even allowing three-dimensional visualization of the gas vesicles within the rod-shaped cells.

Production of gas vesicles is not a general phenomenon among the halophilic Archaea. Out of the 129 species of *Halobacteriaceae* described (as of December 2011), only 7 were recorded to possess these structures:

- *Halobacterium salinarum*, the organism observed in the above-discussed early studies by Klebahn, Petter, and Houwink
- *Haloferax mediterranei* from a Spanish saltern pond (Rodríguez-Valera et al., 1983)
- *Halorubrum vacuolatum* (Mwatha and Grant, 1993), an alkaliphilic species of small cells originally described as *Natronobacterium vacuolatum* (*vacuolata*) from Lake Magadi, Kenya
- *Halogeometricum borinquense* from a saltern pond in Puerto Rico (Montalvo-Rodríguez et al., 1998)

- *Haloquadratum walsbyi*, flat square gas-vacuolated cells first observed in the brine pool on the Sinai peninsula, Egypt (Walsby, 1980), and isolated later from saltern ponds in Spain and Australia (Burns et al., 2007)
- *Haloplanus natans*, isolated from an experimental mesocosm containing a mixture of Dead Sea and Red Sea water (Elevi Bardavid et al., 2007)
- *Haloplanus vesicus*, a recent isolate from a marine solar saltern in China (Cui et al., 2010)

In-depth studies of the genes involved in gas vesicle production and their regulation in halophilic Archaea were thus far performed only with *Hbt. salinarum* and *Hfx. mediterranei*. Early studies of *Hbt. salinarum* showed that gas vesicles are mostly produced in the beginning of the stationary growth phase (Larsen et al., 1967). The fact that buoyancy is achieved only in the late exponential and stationary phases suggests that gas vesicles may be mainly important in the survival and dispersal of this organism. In *Hfx. mediterranei*, the induction of gas vesicle formation depends on light, salt, and oxygen concentration. Gas vesicles are only produced when the salt concentration in the medium exceeds 170 g/l and oxygen levels are low (Englert et al., 1990, 1992; Offner et al., 1998; Pfeifer et al., 1997, 2002; Röder and Pfeifer, 1996). How oxygen concentration is transduced to influence gas vesicle synthesis is not yet known (Pfeifer et al., 2002).

To elucidate the selective advantage that gas vesicles may bestow upon *Hbt. salinarum*, Beard et al. (1997) performed competition experiments between a gas-vacuolated strain and a mutant deficient in gas vesicle synthesis. In shaken cultures, both strains grew equally well, but in deep static cultures, where steep vertical oxygen concentration gradients were established, cells of the wild type floated and became dominant. In shallow static cultures, the gas-vesicle-deficient mutant won the competition. A possible explanation is that under the conditions employed, the wild type wasted much energy to produce unnecessary gas vesicles.

A key question is whether in natural aquatic environments halophilic Archaea indeed float toward the water surface and thus obtain a selective advantage by their increased access to oxygen. Thus far there is very little evidence that this may indeed be the case. Gas-vacuolated *Halobacterium* were generally isolated not from salt lakes but from salted fish and salted hides (Oren, 2002, 2006), and the genus *Halobacterium* contributes very little to the prokaryote community in saltern ponds and salt lakes (Oren and Litchfield, 1999). Cultivation studies as well as culture-independent environmental genomics studies have never shown *Hfx. mediterranei* to be a quantitative important organism in the salterns from which it was isolated or in any other hypersaline environment, this in spite of the great metabolic versatility displayed by this species (Rodriguez-Valera et al., 1983). It must be noted, however, that the presence of gas vesicles cannot be assessed by microscopic examination of samples in which the cells have been concentrated by high-speed centrifugation, as such treatment causes collapse of the vesicles.

The only gas-vacuolated extreme halophile that appears to contribute significantly to the prokaryote community in hypersaline lakes, both natural and artificial, is the flat square *Hqr. walsbyi*. There have occasionally been reports that its cells were seen floating on the surface of natural brines. In the brine pool on the Sinai Peninsula where the species was first observed, the cells buoyed up by their gas vesicles (Parkes and Walsby, 1981; Walsby, 1980). Thanks to their buoyancy, the cells could be further concentrated by leaving brine samples for a few days, collecting cells from the surface, followed by further concentration by low-speed centrifugation (Parkes and Walsby, 1981). Romanenko (1981) collected similar square-vacuolated cells (incorrectly interpreted by the author as being square microcolonies, the gas vesicles erroneously considered to be cells) from the surface water film of Saxkoye Lake, Russia, by floating electron microscope grids on the brine. But whether indeed these square structures were present at a higher density at the brine surface than in the deeper waters was not reported.

It must be doubted whether indeed the square Archaea of the genus *Haloquadratum* are sufficiently buoyant to efficiently float to the surface of salt lakes and saltern ponds. In a study of the brines of the saltern crystallizer ponds of Eilat, Israel, the cells showed neutral and not positive buoyancy. In laboratory simulations, the cells did not float to the surface, not even after low-speed centrifugation while taking care not to exceed the critical pressure at which the gas vesicles collapse. And if the square Archaea do not float in a test tube in the laboratory, they surely cannot be expected to buoy up in the natural environment where wind and water currents will tend to disperse them equally at all depths (Oren et al., 2006). Still, as shown by the above-discussed study by Beard et al. (1997), there must be some selective advantage to the presence of gas vacuoles, as their synthesis has an energy cost. A possible explanation was suggested by Bolhuis et al. (2006). They noted that in the square Archaea, the gas vesicles are mainly located close to the cell periphery (Burns et al., 2004; Parkes and Walsby, 1981; Walsby, 1980). A similar arrangement of peripheral gas vesicles in the larger cells was found in *Hpn. natans* (Fig. 3). Bolhuis and coworkers postulated that this arrangement of the gas vesicles may aid the cells to position themselves parallel to the surface. Horizontal positioning may then aid the cells in collecting as many photons as possible to be absorbed by the bacteriorhodopsin proton pump, present in *Haloquadratum*, to generate ATP. However, no experimental evidence was ever brought forward that indeed such cells tend to arrange themselves perpendicular to the water surface. Based on the theoretical considerations presented by Oren et al. (2006) related to the smallness of the cells and the implications of life at low Reynolds number, it is highly improbable that indeed the square cells can efficiently orient themselves toward the light. *Haloquadratum* lacks flagella, so that active movements of the cells are not possible. The explanation that the gas vesicles will aid the cells in positioning themselves for optimal light scavenging also does not hold for organisms such as *Hfx. mediterranei* and *Hgm. borinquense*, as these do not produce bacteriorhodopsin.

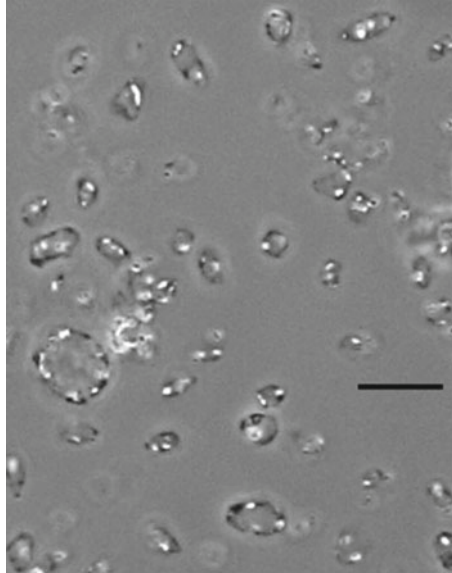


Figure 3. Phase-contrast micrograph of gas-vacuolated cells of *Haloplanus natans* strain RE-101T. Note the concentrations of gas vesicles at the periphery of the cells (From Elevi Bardavid et al., 2007, reproduced with permission from the Society for General Microbiology, Reading, UK).

7. Do Halophilic Archaea Also Experience Stress by High Oxygen Concentrations?

Although, as documented above, hypersaline environments are generally characterized by low oxygen tensions, reactive oxygen intermediates such as hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) may still be formed as byproducts of aerobic metabolism. Photosynthetic activity of halophilic or halotolerant algae such as *Dunaliella* spp. can locally lead to increased oxygen levels. Therefore, the halophilic Archaea, like all other aerobes, possess the means to detoxify potentially lethal active oxygen intermediates: catalase, peroxidases, and superoxide dismutase (SOD) (Salin and Brown-Peterson, 1993). Following incubation of *Hbt. salinarum* at low, suboptimal salt concentrations (1–1.5 M NaCl) at which oxygen concentrations at air saturation are much higher than in salt-saturated brines (Fig. 1), a nearly 100-fold increase in catalase activity was observed after 12 h, as compared with control cells kept at high NaCl concentrations. Peroxidase activity increased four- to five-fold following induction of a catalase distinct from the constitutive catalase-peroxidase present (Brown-Peterson and Salin, 1994; Brown-Peterson et al., 1994, 1995). The catalase-peroxidase gene of *Hbt. salinarum* was subcloned in shuttle vectors and expressed under different archaeal promoters. No induction was observed by a variety of environmental stress factors such as

exposure to H_2O_2 , high light intensity, high temperature, or heavy metals (Long and Salin, 2000). *Hbt. salinarum* also contains a separate peroxidase with a lower molecular mass than the combined catalase-peroxidase (Fukumori et al., 1985).

SOD activity is also found in halophilic Archaea. Bubbling of oxygen through a culture of *Hbt. salinarum* led to the induction of a manganese-containing SOD (Salin and Oesterhelt, 1988). *Hbt. salinarum* and *Hfx. volcanii* have genes encoding Mn-SODs that show a high degree of sequence similarity with bacterial genes, suggesting that they may have been acquired by lateral gene transmission (May and Dennis, 1987; May et al., 1989). In the presence of the herbicide paraquat which generates superoxide radicals, growth of *Hfx. volcanii* was slowed down, and the level of SOD was highly increased (May et al., 1989). In *Hbt. salinarum*, paraquat also caused an initial increase in SOD levels, but prolonged exposure led to a subsequent decrease in activity (May et al., 1989; Salin and Brown-Peterson, 1993). In *Hbt. salinarum*, the SOD gene is located adjacent to that of photolyase, an enzyme that repairs pyrimidine dimers (Takao et al., 1989).

8. Final Comments

The above survey shows that at least some members of the *Halobacteriaceae* have different modes of coping with stress caused by the limited availability of oxygen, this in addition to their ability to withstand the high salt concentrations in their habitat. Possible strategies on how to deal with low-oxygen situations are very diverse: anaerobic respiration with electron acceptors such as nitrate, DMSO, TMAO, or fumarate, photoheterotrophic growth using the light-driven proton pump bacteriorhodopsin, fermentative growth on arginine or possibly other fermentable substrates, active movement toward gaseous oxygen in a gradient, or passive flotation to the brine surface.

It is tempting to speculate that all these strategies may help halophilic Archaea to grow and survive in their hostile environment where the solubility of gaseous oxygen is extremely low and oxygen can thus easily become a limiting nutrient for these prokaryotes that normally lead an aerobic chemoheterotrophic life style. However, upon closer examination, it becomes evident that things are not that simple. Nitrate is seldom available in significant concentrations in hypersaline lakes (also due to the absence of autotrophic nitrification at high salt concentrations), and there is no reason to assume that DMSO, fumarate, or arginine may accumulate anywhere to concentrations that may support anaerobic growth of members of the *Halobacteriaceae* in nature. TMAO may be available during the decay of salted fish but hardly elsewhere. Only a few species produce gas vesicles, and there is little evidence, if any, that those that do can efficiently buoy up to the brine surface in natural salt lakes and saltern ponds to reach the oxygen. What the true ecological advantage of these gas vesicles may be still remains to be assessed.

We know little about the affinity of halophilic Archaea for oxygen, so to what extent their communities are truly oxygen limited in their natural environments is not yet clear. The fact that these cells also possess genes encoding enzymes important in protection against peroxides and superoxide radicals suggests that at least from time to time, they may be exposed not only to low oxygen stress but to high oxygen stress as well. In summary, our understanding of the relationships of the *Halobacteriaceae* to oxygen is still quite limited.

9. References

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**PART VII:
SELECTED ORGANISMS**

**Grube
Muggia
Gostinčar
Reisser**

**Lefèvre
Bazylnski
Schulze-Makuch
Seckbach**

Biodata of **Prof. Martin Grube**, **Dr. Lucia Muggia** and **Dr. Cene Gostinčar**, coauthors of “*Niches and Adaptations of Polyextremotolerant Black Fungi.*”

Martin Grube is an Associate Professor at the Institute of Plant Sciences, Graz, Austria. He received his PhD in biology from Karl-Franzens-University Graz in 1995. During his early career, he specialised on classification and taxonomy of lichens and lichenicolous fungi. After finishing a thesis on a group of tropical lichens in 1995, he established the molecular systematics laboratory at the Institute of Plant Sciences in Graz, Austria, where he introduced lichenological fellows to molecular techniques. In recent years, he focused his research on the aspects of symbiont composition and balance in lichens and in other host-associated microbial communities. Since several years, he is also interested in the evolution and ecology of extremotolerant fungi and the link between stress tolerance and symbiosis.

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Lucia Muggia graduated in biology at the University of Trieste (Italy) and received her PhD in molecular biology at the Karl-Franzens-University Graz. She is now a postdoctoral fellow at the Graz University. Her research focuses on the interactions and phylogenetic relationship among different organisms building the lichen symbiosis. In particular, she studies the symbiotic evolution of mycobiont and photobiont in the *Tephromela atra* lichen species complex and the evolution and specificity of lichenicolous fungi in alpine habitats.

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NICHES AND ADAPTATIONS OF POLYEXTREMOTOLERANT BLACK FUNGI

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1. Introduction

Most research of fungal biology is centred on relatively fast-growing fungi that can easily be cultured in mesophilic and axenic conditions. These conditions are in contrast to those more often present in the natural environment, where organisms compete for resources and often thrive persistently under poor nutrient conditions. Because specialists for those habitats are often (but not always) slow growing and not suitable for genetic manipulation, their biology is still poorly understood. We tend to regard the environments of oligotrophs as stressful when they comprise conditions that are not suitable for a majority of species. This includes conditions of extreme temperatures, osmotic stress and extreme pH values or the more or less rapid change of such parameters. Moderately stressful habitats can be tolerated by a diversity of microbial species, especially prokaryotes, but also by a range of fungi. In this chapter we focus on fungi as examples of polyextremotolerant eukaryotes. More specifically we review black meristematic fungi, which have a variety of interesting adaptations that allow the colonisation of a wide range of environments. This capacity is particularly significant when facing effects of environmental change and global warming.

2. Adaptations of Black Fungi: Growth Flexibility, Melanin and Protective Compounds

Many oligotrophic black fungi respond to stress conditions by expressing a high degree of phenotypic plasticity (Gostinčar et al., 2010). Depending on the environmental conditions, these fungi can shift between yeast-like, filamentous and meristematic growth (Butler and Day, 1998). Slepecky and Starmer (2009) have

recently demonstrated an enormous diversity of phenotypes in the ubiquitous black fungus *Aureobasidium pullulans*.

The most obvious and visually prominent adaptation of black fungi is the production of melanin. Melanins of black fungi comprise various types of polymeric pigments that result from the coupling of phenolic precursors, including 3,4-dihydroxyphenylalanine, γ -glutaminy-3,4-dihydroxybenzene, catechol and 1,8-dihydroxynaphthalene. Several enzymes are possibly involved in the polymerisation of the precursors (extracellular phenoloxidases, laccases, tyrosinases, catalases, peroxidases; Butler and Day, 1998). Melanin is a protective dark pigment in the cell walls that facilitates the persistence in hostile environments. It is involved in resistance to excessive heat or cold, extreme pH or osmotic conditions, polychromatic UV radiation and conditions of outer space, and it also seems to mediate tolerance towards metals (Gadd and Derome, 1988; Gunde-Cimerman et al., 2000; Onofri et al., 2008; Sterflinger et al., 2012). There is also evidence for the capacity of melanin to scatter and convert ionising gamma radiation into chemical energy, leading to growth benefits of black fungi (Dadachova et al., 2007). Metabolic responses of melanin-producing fungi to ionising radiation include increasing rates of electron transfer, measured as reduction of ferricyanide by NADH (Dadachova et al., 2007). Gamma radiation-induced oxidation of melanin resulted in electric current production, especially in the presence of a reducing agent (Turick et al., 2011). The biological significance of this phenomenon is still unclear. Gamma radiation, UV and visible light seem to cause a reduction of ATP levels in melanised cells of the fungus *Cryptococcus neoformans* (Bryan et al., 2011).

Other protective compounds are also involved in stress tolerance of black fungi (Oren and Gunde-Cimerman, 2007). These fungi have a significantly higher content of mycosporine-glutaminol-glucoside when grown in 10 % salt than in a salt-free medium. Mycosporines are efficient absorbers of UV radiation, with the maximum absorbance between 310 and 365 nm. Mycosporines and mycosporine-like amino acids (MAAs) comprise low-molecular-weight (generally less than 400 Da), water-soluble molecules composed of either an aminocyclohexenone or an aminocycloheximine ring, carrying nitrogen or imino alcohol substituents. When substituted with amino acid residues, they are designated MAAs. They act as shields against UV radiation, but some MAAs may also protect the cell by scavenging reactive oxygen species such as singlet oxygen, superoxide anions, hydroperoxyl radicals and hydroxyl radicals. Additionally, a function of mycosporines as compatible solutes has been suggested in fungi (Gorbushina et al., 2003; Kogej et al., 2006).

The role of polyphosphates in stress response of polyextremophiles has been reviewed recently by Orell et al. (2012). However, it is not yet known if these play a role also in black fungi. Present knowledge suggests that inorganic polyphosphates could be of relevance in metal resistance, salt tolerance, oxidative stress adaptation, temperature tolerance and other conditions of stress. Other potential protective compounds known from other species include polyalcohols, betaine, proline, carotenoid pigments or manganese compounds (Blomberg and Adler, 1992;

Daly et al., 2010; Lapinskas et al., 1995; Madhour et al., 2005; Shima and Takagi, 2009; Takagi, 2008). Research on lichen-forming relatives of black fungi indicates that the diversity of such chemicals might be even wider (Boustie et al., 2011).

3. Antifragility of Black Fungi

As resilientists persist in their niche, in contrast to sensitive/mesic organisms, they display a behaviour in the course of evolution which can be termed ‘antifragile’. Antifragility is a concept recently proposed by economist Nassim Taleb. While fragility describes a system breakdown under uncertain, changing and stressful events, antifragility means the opposite, that is, an ability to actually benefit from such conditions (Danchin et al., 2011; Taleb, 2011). The concept can be applied to biological systems as well. Living organisms need a certain amount of disorder to function properly; however, this amount is not the same for every species. Black fungi function in extreme uncertainty – and they do it extremely well. According to Taleb, biological systems are fragile when they are optimised for efficiency and antifragile when they accumulate functional redundancy. This can happen by maintaining cryptic genetic variation, by mechanisms such as canalisation, genetic redundancy or alternative splicing (Schlichting, 2008). It has been noted before that environmental heterogeneity and stress cultivate genetic polymorphisms, especially in dynamically cycling environments (Nevo, 2001). Environmentally induced phenotypic variation may be an important source of evolutionary novelty, since selection acts on a phenotype, not on a genotype (West-Eberhard, 2005). It appears to facilitate fungal colonisation of extreme environments as well as pathogenesis (Gostinčar et al., 2010, 2011). Some species/groups are specialised and almost invincible in their preferred habitat (but very fragile), while others are polyextremotolerant generalists, which can survive in different habitats. They do not just survive unchanged due to their robustness; instead, they efficiently adapt to catastrophic events and benefit from using the resources available due to destruction of the fragile species. Black fungi are a good example of such antifragile polyextremotolerant species (reviewed in Gostinčar et al., 2011). The signs of constant tinkering, an important aspect of antifragility, are clearly visible even in the standardised laboratory conditions. The morphology and melanisation of many species change constantly, resulting in strikingly different appearance even on the same Petri dish (our unpublished results; Slepecky and Starmer, 2009). Facultative associations/symbioses with other organisms (see below) are further examples of tinkering within black fungi in oligotrophic conditions.

4. Environmental Ubiquity of Polyextremotolerant Black Fungi

Ancestors of black fungi from the large lineages of Dothideomycetes and Chaetothyriomycetidae (which evolved much later) were presumably oligotrophic organisms living on rock surface or subsurface (Gueidan et al., 2011). Rocks, as

the most abundant natural substrate of oligotrophic black fungi, are colonised in all climatic zones, including the most hostile environments on Earth such as Antarctic dry valleys (Selbmann et al., 2005), the Atacama Desert or high alpine habitats in the Himalayas (Onofri et al., 2007; Selbmann, 2011, personal communication). Their radiotolerance could have helped them to survive and proliferate during historic periods of increased cosmic radiation, for example, due to weakened or absent magnetic field of the Earth (Dadachova and Casadevall, 2008). Similar to bacterium *Deinococcus radiodurans*, radiotolerance in black fungi is tightly linked to pronounced desiccation tolerance (which may have actually been the primary function of the adaptations; Daly, 2012). Consequently, these fungi appear to prevail in habitats with only sporadic availability of water (alternating humidity, rain, condensation).

Tolerance to low availability of water is also important in many other habitats, for example, in coastal hypersaline environments, Arctic glacial ice and on bathroom surfaces. There is an overlap of microbial diversity between these habitats: the black fungi *Cladosporium halotolerans* and *Aureobasidium pullulans*, for example, were isolated in all of them (Gunde-Cimerman et al., 2000; Zalar et al., 2007, 2008). *A. pullulans* seems to be particularly adaptive, and this species is also exceptional due to its relatively fast growth in comparison with other black fungi. Its distribution spans all climatic zones, and besides the already mentioned hypersaline and glacial environments, it is abundant in the phyllosphere (Andrews et al., 2002), particularly on plant leaves in organic agriculture (e.g. Grube et al., 2011, and references therein). It further occurs in polluted water (Vadkertiova and Slavikova, 1995), in salt-preserved food (Nisiotou et al., 2010), olive fermentation (Bevilacqua et al., 2012) and in harvested barley (Olstorpe et al., 2010), in aviation fuel tanks (Rauch et al., 2006), in various indoor habitats (Kaarakainen et al., 2009; Summerbell et al., 1992), on synthetic polymers (Cappitelli and Sorlini, 2008) and on degrading polyurethane and PVC plastics (Shah et al., 2008). *A. pullulans* has also been reported in a clinical context and is sometimes even regarded as an emerging pathogen (Chan et al., 2011). It causes a variety of localised infections, as well as rare systemic infections, including peritonitis in patients on peritoneal dialysis, splenic abscess, meningitis, skin and soft tissue infections and septicaemia in patients with malignancies, receiving major surgery or catheters (reviewed in Chan et al., 2011; Hawkes et al., 2005; Huang et al., 2008). Its increased indoor concentrations have also been correlated to various health symptoms (Su et al., 1992). Other black fungi are already a health concern and known as pathogens. We will focus on these further below.

5. Presence of Black Fungi in Unusual and Human-Made Niches

Black fungi have been detected in a range of unusual and human-made niches, which are also sources of new species. In the Lascaux caves – famous for their prehistoric paintings – two new *Ochroconis* species were described (Martin-Sanchez

et al., 2012). *Exophiala sideris* was isolated from arsenic mine polluted with alkylbenzenes (Seyedmousavi et al., 2011). The creosote or diesel or kerosene fungus *Amorphotheca resinae* is well-known for causing problems by colonising fuel tanks and valves. The species can degrade alkanes (branched and unbranched), alkenes, cyclic alkanes and aromatic hydrocarbons. It grows best on alkanes with C10–C18; however, no growth is observed on C29–C34 (Fürst, 2000). *Acidomyces acidophilus* is another species exhibiting exceptional stress tolerance (Selbmann et al., 2008). The habitats from which this species was isolated include extremely acidic, sulfate-containing industrial water, sulphur-containing soil (pH 1.1), uranium mine drain water and acidophilic moss species. Cultures grow well when adjusting the pH to 0.5 with HCl (Ivarson and Morita, 1982). This species has been found in a galvanisation jar at pH=0.5 and 20 % copper sulfate (W. Buzina, Graz, 2010, personal communication).

Oligotrophic fungi can readily grow in anthropogenic habitats: they are very common on monuments, concrete walls and similar rock-like surfaces where they can cause undesirable coloration. Kitchens and bathrooms harbour the largest numbers of microbes in the household (Beumer and Kusumaningrum, 2003; Ojima et al., 2002), and pathogenic species are not uncommon among them (Feazel et al., 2009; Nishiuchi et al., 2009). The conditions in many indoor habitats select for polyextremotolerants and result in a limited species diversity. Fungi growing on bathroom walls, for example, experience alternating periods of high humidity and desiccation, lack of nutrients and the presence of aggressive chemicals. The presence of high temperatures and physical and chemical characteristics of substrate materials are a further factor in selection of diversity. Fungi are present on glass, metals or silicon and on a wide variety of more or less durable organic surfaces such as plastic materials and other polymers (Gostinčar et al., 2011), which they might help to degrade. Some of these materials may promote the occurrence of some clinically relevant fungi or their closely related strains.

At least some oligotrophic fungi can use complex phenolic hydrocarbons from the environment as the sole source of carbon and energy. Such fungi are commonly found in unusual habitats, for example, biofilters or distilleries (Prenafeta-Boldu et al., 2006; Scott et al., 2007). Because several of these species are closely related to human pathogens (e.g. de Hoog et al., 2000, 2006), it has even been argued that there is a physiological connection between aromatic hydrocarbon assimilation and capability of mammalian infection (Prenafeta-Boldu et al., 2006, 2012). Some of these fungi include agents of deep skin lesions (*Cladophialophora carrionii* and *Exophiala spinifera*) as well as infections of the brain (*Cladophialophora bantiana* and *Exophiala dermatitidis*). The occurrence of highly similar sibling species in extreme environments that seem to evolve towards virulence and saprotrophy, respectively, suggests ongoing speciation (Badali et al., 2011).

A larger share of the oligotrophic black fungi encountered in humid indoor environments has a significant potential to cause human infection (Lian and de Hoog, 2010), due to their environmental preadaptations. With novel ways of living, we are unconsciously enriching for potentially problematic species in our own

homes. This is exemplified by a recent discovery that more than half of dishwashers contain a known fungal pathogen *Exophiala dermatitidis*. Not only that, the most virulent genotype A dominates in frequency over other genotypes (Zalar et al., 2011). It appears that fluctuations of pH, temperature, salinity and humidity in dishwashers select for a few species of polyextremotolerant generalists and against mesophilic and less harmful fungi. Stress tolerance traits that are selected in certain indoor environments may serve as preadaptations for pathogenicity by combating unfavourable conditions that are created by the animal immune response (Gostinčar et al., 2011; Casadevall, 2007). Tolerance to elevated temperatures, excellent oxidative stress response, production of melanin, extracellular polysaccharides, biofilm formation and yeast/mycelium shifts are all known virulence factors that have evolved as parts of polyextremotolerance of black fungi (Gostinčar et al., 2011). It is therefore not surprising that the adaptations to certain extreme environments and emerging fungal pathogens can frequently be found in the same phylogenetic groups (de Hoog et al., 2005).

6. Facultative Symbiotic Associations of Black Fungi: A Link to Lichen Symbioses

How do oligotrophic black fungi gain energy when they are growing on surfaces with low amounts to almost no usable organic carbon? Using fungal isolates from Antarctic cryptoendolithic communities, Palmer and Friedmann (1988) suggested aerial CO₂ uptake by a black fungus after ¹⁴C-labelling experiments. These experiments still need to be confirmed – although several alternative pathways of carbon dioxide uptake are known from prokaryotes (Bar-Even et al., 2012; Fuchs, 2011), such mechanisms have not yet been demonstrated for eukaryotes. Some other observations indicate that attachment to microscopic algae is an alternative strategy of black fungi to improve their carbon supply. In cocultures with lichen algae, rock-inhabiting and lichen-inhabiting microcolonial fungi need only a few months to develop lichenoid structures (Brunauer et al., 2007; Gorbushina et al., 2005). Cocultures of *Nostoc* sp. and a rock-inhabiting fungus (*Sarcinomyces* sp.) also develop a specific spatial arrangement of both organisms, together with the changes in cyanobacterial growth, suggesting a specific interaction (Gorbushina and Broughton, 2009). Such coexistence happens outside of laboratory as well. Black fungi and lichens often grow on the same pieces of rock, and in arid habitats, black fungi frequently colonise lichens (Harutyunyan et al., 2008). Rock surface can thus be thought of as a ‘symbiotic playground’, where detrimental interactions between species (antibiosis) are harmful and selected against (Gorbushina and Broughton, 2009).

Certain associations of fungi and algae have been termed ‘borderline lichens’ (Kohlmeyer et al., 2004). In these associations, no complex symbiotic structures, as typical for real lichens, are developed by the fungal partner (Fig. 1a). However, sexual fruiting bodies are formed in this symbiotic stage, and borderline lichens

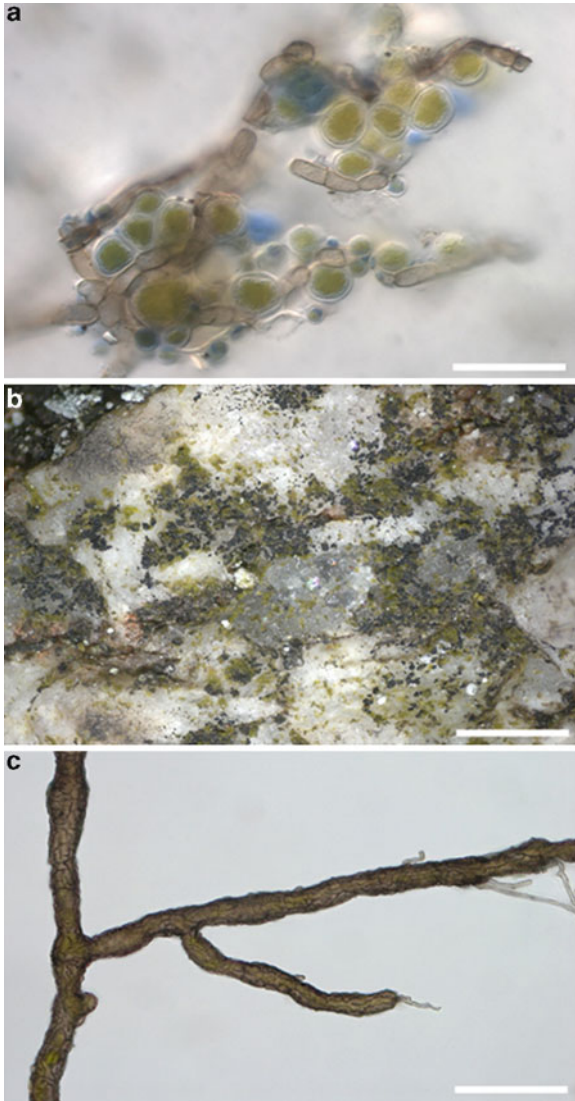


Figure 1. (a) Algal association of *Cladosporium*-like fungus (according to ITS sequences) on plastic surface (Botanical Garden Graz, 2011); Bar=30 μm . (b) *Lichenothelia* sp. on calcareous rocks; Bar=1 cm. (c) *Cystocoleus ebeneus*; Bar=50 μm .

can be recognised phenotypically. Associations of black fungi with algae are less structured, and in many cases sexual structures are not known. Exceptions are, among few others, represented by species assigned to the peculiar genus *Lichenothelia* (Henssen, 1987; Fig. 1b), a cosmopolitan genus of rock-inhabiting

fungi that encompasses currently 24 species (Robert et al., 2005). Some species, which have been found in association with algae or with lichen thalli, do produce fertile structures with asci and ascospores, but these are not sufficient for proper phylogenetic classification. The monophyly and relationships of species in this genus with other rock-inhabiting lineages and lichenised fungal lineages have not been studied and are therefore still poorly understood.

The capacity of oligotrophic black fungi for association with algae may have marked the beginning of evolution which led to the lichens as we know today. The last common ancestors of lichenised fungi and different lineages of black fungi lived in a not so distant evolutionary past, as shown by reconstructing their molecular phylogeny. Rock inhabitants are basal to the large lichenised lineages of Arthoniomycetes and Verrucariales (Gueidan et al., 2008; Ruibal et al., 2009), whereas both lifestyles are found in various clades of Dothideomycetes (Muggia et al., 2008; Nelsen et al., 2009). In Dothideomycetes, the lichen thallus morphology remains generally simple, compared to predominantly lichenised ascomycete lineages. In *Cystocoleus ebeneus*, for example, the thallus is formed by colonies of filamentous *Trentepohlia* algae, which are densely entwined by dark pigmented fungal hyphae (Fig. 1c).

Besides the nutritional benefits that the fungal partner gains in oligotrophic environments, transition from the rock inhabiting to the lichenised lifestyle of fungi in the early ascomycetous evolution also helped both fungi and algae to survive periods of extreme stress. While desiccation and irradiation increase the formation of reactive oxygen species in both symbiotic partners, the efficiency of their protective mechanisms is much higher when they are living in lichen symbiosis compared to encountering stress alone (Kranner et al., 2005). These mechanisms involve the glutathione redox system, which is also known from black fungi (Jürgensen et al., 2001). Various small protective molecules that accumulate in black fungi to alleviate the effects of stress have taken additional roles during the shift to the lichen lifestyle. Polyols such as ribitol, sorbitol and erythritol, as well as glucose, provided by algae and cyanobacteria, respectively, are taken up by lichen fungi as food molecules and then transformed to mannitol (Friedl and Büdel, 2008). Thus, efficient management of osmolytes by stress-tolerant fungi could have served as a preadaptation for the vigorous exchange of metabolites in the lichen symbiosis. On the other hand, diverse polyketides, often of variably combined phenolic units, largely replaced melanin in cell walls of lichen-forming fungi.

7. Potential Uses of Adaptive Black Fungi

Polyextremotolerant black fungi frequently have a diverse metabolism, capable of degrading exotic substrates and producing a variety of metabolites that can be used in industry, medicine and food production. Various black fungal species are good candidates for biological degradation of plastics (Shah et al., 2008;

Webb et al., 2000) and volatile aromatic hydrocarbons (Badali et al., 2011; Prenafeta-Boldu et al., 2006). Strains that are useful for degradation of aromatic hydrocarbons can be enriched by special isolation methods (Zhao et al., 2010).

Due to its good growth on exposed surfaces and ability to compete with other organisms, *Aureobasidium pullulans* can be used as a biocontrol agent on plants. *A. pullulans* can reduce postharvest decay of pears (Robiglio et al., 2011) and lower the concentration of ochratoxin A in wine grapes (de Felice et al., 2008). By using temporary high temperatures, UV radiation and low water availability in combination with a water-repellent layer, biofilms of *A. pullulans* can be used to cover timber and protect its surface over several years. The fungus survives the exposure to outdoor conditions even though the surface temperatures of the wood can exceed 70 °C during daytime (Sailer et al., 2010). Furthermore, due to its ability to degrade and produce a variety of substances, the species is used to produce the polysaccharide pullulan and antifungal aureobasidin A and is a source of amylases, proteinases, lipases, cellulases, xylanases, mannanases and siderophores (Chi et al., 2009). First evidence suggests that lichen-associated relatives of black fungi could also represent a still poorly investigated reservoir of valuable compounds (Boustie et al., 2011).

8. Conclusions

Certain fungal species excel at antifragility. They have chosen a generalistic survival strategy, which enables them to survive in a wide range of habitats, especially those characterised by unfavourable conditions for growth of most other microorganisms. This strategy relies on the abilities to tolerate different kinds of stress (polyextremotolerance) and to tap into diverse and frequently unconventional sources of energy. Many black fungi are good examples of this lifestyle. They are phenotypically plastic, able to quickly switch between different morphologies, adjust the production of protective compounds such as melanin and mycosporines, they produce large amounts of extracellular polysaccharides and can aggregate into biofilms. They seem to be able to form rudimentary lichen-like symbioses and can possibly convert ionising and UV/visible radiation into chemical energy. This combination of traits may have enabled their ancestors to colonise the extremely stressful (but also widely available) rock surface and subsurface. Later, they used it to invade all kinds of extreme environments, also anthropogenic ones, such as the surfaces of buildings and monuments, indoor habitats such as bathroom surfaces or dishwashers, novel polymer materials and areas contaminated with aromatic hydrocarbons. This contamination is often problematic, but the possibility of polyextremotolerant fungi to reuse their stress tolerance traits as preadaptations for combating or evading attacks of our immune responses is of an even larger concern. At the same time these species may represent a rich source of biotechnologically valuable compounds that can be used in medicine, industry and in other fields.

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POLYEXTREMOPHILIC PHOTOAUTOTROPHIC EUKARYOTIC ALGAE

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1. Introduction

In man, there is an inborn curiosity in all unusual, deviating from daily experience. This holds also for biological phenomena: organisms that live under conditions and in places that are unusual or inhospitable for man attract special interest and are categorized as “extreme.” This characterization clearly bases on an anthropocentric view of nature. Current biological research has shown that organisms that are endowed with a high ecological amplitude often are able to survive quite diverse and “extreme” ecological conditions for a considerable part of their life cycle, although their reproduction is bound to a much narrower range of environmental parameters that usually lies within the boundaries favorable for man. Those organisms are designated as “extremotolerant.” Organisms that are able to reproduce under “extreme” conditions or even need them for reproduction are designated as “extremophilic” (McElroy, 1974). It is obvious that there exist many more extremotolerant than extremophilic organisms, and careful examination is needed to separate them from each other.

The focus of this review shall be on polyextremophilic phototrophic eukaryotic algae. The term “polyextremophilic” (“poly” being the Greek term for many) is misleading, since usually organisms are able to deal with one, two, or sometimes also three types of extreme environmental conditions, such as extreme temperature (hot or cold) and, e.g., dryness (extreme lack of water). Thus, it would be more appropriate to talk about “diextremophilic” or “triextremophilic” organisms. It is also important to note that polyextremophily is proven by experiments and beyond any doubt only in few cases. A good example for this caveat is given by algae living in dry places such as hot or cold deserts. Usually, they are adapted to ambient temperature in being thermo- or psychrophilic. However, it is open for discussion whether they also should be designated as aridophilic or rather as aridotolerant. The same caveat holds for the impact of radiation. A chlorophycean alga detected in the storage pool of a nuclear reactor (Rivasseau et al., 2010) showing an intense protein repair activity is not necessarily radiophilic but rather radiotolerant. The same consideration also holds for algae producing special shielding pigments against elevated visible radiation. An aeroterrestrial alga living on a tree bark must be able to endure dry conditions and frequently is exposed to periods of high irradiance. However, it is not polyextremophilic but extremotolerant. It is conceivable that

there are more monoextremophilic algal species around than currently supposed. However, the suffix “-philic” should be given instead of “-tolerant” less generously and only after thorough experimental examination.

The following terms will be used to characterize various forms of extremophily (Canganella and Wiegel, 2011): Thermophiles have a T_{opt} for growth above 55 °C. Some authors (Canganella and Wiegel, 2011) add a category of hyperthermophiles with a T_{opt} above 80 °C. Psychrophiles show a T_{opt} for growth below 15 °C. Growth of acidophiles is best at pH below 3.0; alkaliphiles thrive best at pH above 8.5. Halophiles require salt concentrations of about 3 % at minimum for best growth.

It should be mentioned that there exist additional forms of extremophily such as piezophily (barophily), but this has been shown until now only for prokaryotes and some rhodophytes that live mainly in deep sea under aphotic or rather low light conditions (Stambler and Dubinsky, 2007). The same caveats hold for various reports on resistance to radiation or desiccation. Either they deal with prokaryotes or with algae that are specially adapted to harsh environmental conditions either by forming shielding pigments, by cooperation with fungi forming lichens, or by the retreat to an endolithic (Friedmann, 1980) or “endobone” (Reeb et al., 2011) environment. In neither case it could be shown that there is more than a tolerance of algae, i.e., that they really need elevated radiation or lack of water (xero- or aridophily) for reproduction.

In general, extremophily among autotrophic eukaryotic algae is yet a well-established phenomenon although it is difficult to give exact numbers of genera and species involved. Available data suggest that – although numbers are considerably lower than among prokaryotes – some extremophilic algal species are of great ecological importance. As to the phenomenon of polyextremophily, examples chosen for this chapter deal with psychrophilic and thermophilic specimens that have to master additional stress by dryness, radiation, low pH, or high salt concentrations.

It should also be mentioned that the term phototrophic excludes algae living heterotrophically. Also excluded are prokaryotic algae (cyanobacteria), algae that create an own favorable environment in an otherwise unfavorable and extreme environment (such as lichenized or endolithic algae) or algae that endure extreme conditions by forming resting stages (such as algae in permafrost or hot desert soils).

2. Life at Low Temperatures

2.1. ALGAL TAXA

It is interesting to note that about 85 % of the Earth’s biosphere is characterized by temperatures permanently below 5 °C and that sea ice covers about 13 % of the Earth’s surface, snow about 35 % of the land surface (Margesin, 2012). Thus, adaption to low temperatures is a fairly common challenge for life on our planet.

Different habitats are on and in ice (marine and freshwater) and glaciers, on and in snow and dry substrates such as soil and rocks.

As to marine ice, it differs from freshwater ice in containing small channels and fissures filled with brine of high salinity (3.5–20 %; Mock and Junge, 2007). Algae trying to grow there have to be psychrohalophilic by definition and also have to adapt to high pH (up to 11) and usually to irradiance (Plettner et al., 2001) that varies between very high (at and near surface of ice), very low (at the subsurface), or medium (in between). Thus, they may be characterized as “psychrohaloalkaliphilic.”

Most psychrophilic eukaryotic algae belong to chlorophyte (Chlorophytina and Streptophytina) and diatom taxa. Sea ice diatoms (Bacillariophyceae) are important primary producers in being responsible for about 50 % of the polar primary production and thus form the basis of polar food webs. As to numbers, among ice algae diatoms play a prominent role. Their biomass can amount to 350 mg C per 1 m³ of ice, and they cover about 20 % of global photosynthetic carbon fixation (Mock and Junge, 2007) being adapted to low light conditions and to different degrees of ambient salinity (Zhang et al., 1999). Other ice algal specimens belong to dinoflagellate and xanthophyceyan taxa.

Most polar diatoms are adapted to low light conditions growing at irradiance intensities as low as 0.01 % of incoming solar radiation, i.e., below 0.5 μmol photons m⁻² s⁻¹. However, diatoms growing at or near the upper surface of ice are also able to cope with 350 μmol photons m⁻² s⁻¹ (Mock and Junge, 2007).

In freshwater ice, viable diatom cells such as of *Aulacoseira islandica* (Frenette et al., 2008) and of *Aulacoseira baicalensis* (Bondarenko et al., 2006) have also been found. They predominantly colonize the lower surface of ice and its inner channels being confronted with low temperature and low light conditions.

As to Arctic snow and glacier algae (Leya et al., 2009), it could be shown that there exists an autochthonous flora that shows a fairly high species number. For Antarctica, Elster et al. (1999) report for Sverdrup Pass on 136 algal taxa, 84 of them belonging to eukaryotic algae with most species growing at depths of 3–4 cm. Broady (1976) reports for Signy Island on 162 taxa, 70 % of which belonging to chlorophyceyan, ulotrichophyceyan, xanthophyceyan, and bacillariophyceyan taxa. For further information, see also the list of found species by Mataloni et al. (2000). Probably, there exist some species that are endemic to their special habitat. Possmayer et al. (2011) could show that a *Chlamydomonas raudensis*, isolated from snow, does not grow at 24 °C. Hoham et al. (2006) report on new *Chloromonas* spp. on snow, and Ling (2001, 2002) describes new *Desmotetra* and *Chlorosarcina* species. Those taxa have probably evolved separately from algae of other biotopes (de Wever et al., 2009). The same observation of an endemic development of green algae probably holds for desert algae (Lewis and Lewis, 2005).

On snow, algal blooms are reported (Remias, 2012) as being formed by *Raphidonema nivalis* (green snow), by *Chlamydomonas nivalis* (red and orange snow), and by *Mesotaenium berggrenii* (gray snow). In another report, the biomass of *Chlamydomonas nivalis* on snow in California has been estimated to

33.10 kg biomass km⁻² (Painter et al., 2001). Green snow can also be formed by *Chloromonas*, *Chlorella*, and *Xanthonema* species and can turn to red snow by accumulation of pigments in cells. Yellow snow has been reported to consist of *Ochromonas* spp.; gray ice is formed by *Ancylonema nordenskiöldii* (Remias, 2012).

2.2. ADAPTION TO TEMPERATURE, IRRADIATION, AND SALINITY

2.2.1. Temperature

As to structural and physiological mechanisms that enable ice and snow algae to master extreme conditions and to settle their special niches successfully, research is but at the beginning, but some general insights can be given: diatoms living on and in marine ice have been shown to produce antifreeze proteins. Those ice-binding proteins (Mock and Junge, 2007) have been detected in psychrophilic strains of *Navicula glacei* but not in mesophilic counterparts (Janech et al., 2008). They show an interesting resemblance to antifreeze proteins of snow molds, bacteria, and fungi and avoid damage of membranes by formation of ice crystals. Ice-binding proteins have also been detected in *Fragilariopsis cylindrus* (Bayer-Giraldi et al., 2011; Krell et al., 2008) and in chlorophycean algae such as in *Chlamydomonas nivalis* (Komárek and Nedbalová, 2007).

Another countermeasure against freezing obviously is an increase of the amount of unsaturated fatty acids in cytoplasmic membranes: A temperate strain of *Stichococcus* was shown to produce less unsaturated fatty acids than an Antarctic strain (Chen et al., 2012). The same observation holds for *Chlamydomonas nivalis* (Komárek and Nedbalová, 2007; for references see also Liu et al., 2006). Pocock et al. (2011) report for *Chlamydomonas raudensis* in the Antarctic strain on a wider salinity range for growth compared to its mesophilic counterpart. Willem et al. (1999) have shown that there are differences in the α -tubulin amino acid sequences between two psychrophilic *Chloromonas* spp. and a mesophilic *Chlamydomonas reinhardtii* and interpret this as an adaption to cold conditions. Possmayer et al. (2011) report for a psychrophilic *Chlamydomonas raudensis* a 10 % cell death after change of ambient temperature from 10 to 24 °C for 12 h and a parallel increase in the production of heat-shock proteins.

In ice diatoms, there has been also found an increased level of unsaturated fatty acids in thylakoid membranes, but also amino acids and DMSP (dimethylsulfo-niopropionate) are reported for cryoprotectants (Mock and Junge, 2007; Margesin, 2012). For prokaryotic psychrophilic microorganisms, D'Amico et al. (2006) have reported that enzymes are up to ten times more active at low temperature than their homologues obtained from mesophilic organisms. This should be true also for eukaryotes.

2.2.2. Irradiation

As to algae living on snow and on other substrates, adaption to stress by light is achieved by increased production of mycosporine-like amino acids (MAA) (Sinha et al., 2001) and of shielding pigments. Those can be secondary carotenoids such

as β -carotene, astaxanthin, canthaxanthin, lutein, and echinenone (Leya et al., 2009; Vincent et al., 2004; Thomas and Duval, 1995), shielding against high light intensities and UV. Remias et al. (2011) report on *Mesotaenium berggrenii* that lives on bare surfaces of glaciers in alpine and polar regions to contain in cytoplasmic vacuoles a purpurogallin-derived secondary brownish vacuolar pigment that shields against UV and visible light. Lud et al. (2001) have studied the adaptation of the photosynthetic apparatus to increased levels of UVB in *Prasiola crispa*. It is interesting to note that in ice diatoms, pigments can fulfill also an opposite task: adaptation to low light conditions depends on an enhanced production of fucoxanthin (Mock and Junge, 2007).

Most snow algae form spores for overwintering. They can thrive only during a small period of the year when there forms a water film on snow. They then produce mucilage in order not to be washed away by melting water and to avoid desiccation (Remias, 2012).

2.2.3. Salinity

Adaptive mechanisms to increased salinity as, e.g., in the brine of marine ice, will be discussed elsewhere (Sect. 3.2.3).

3. Life at Elevated Temperatures

3.1. ALGAL TAXA

Thermophilic phototrophic eukaryotic algae can be encountered living on solid substrates as, e.g., in hot deserts and on salt plains but also in hot water, usually of an acid pH. As to hot deserts, most papers center on taxonomy of algae found, whereas physiological data concerning any possible adaptation of specimen to environmental stress are seldom found. In the central desert of Baja California, Mexico (summer mean temperature: 25.8 °C; maximum temperature higher than 50 °C), Flechtner et al. (1998) report on 49 species of eukaryotic algae, 37 belonging to chlorophycean, 10 to diatom, and 1 each to chrysophycean and to eustigmatophycean taxa. Among chlorophycean species there was a predominance of *Chlorella*, *Bracteacoccus*, *Myrmecia*, and *Chlorosarcinopsis* species, among diatoms of *Hantzschia amphioxys*, *Pinnularia borealis*, and *Luticola* species. Some indirect evidence to an adaptation of algae to the harsh environment is given by Lewis and Flechtner (2002, 2004) who have found a high autochthonous biodiversity of desert green algae. They conclude that those have evolved independently at least five times from freshwater ancestors.

In hot salt plains, chlorophycean genera as, e.g., *Dunaliella* spp., and diatoms such as *Navicula* spp., *Nitzschia* spp., and *Amphora* spp. are dominating (Kirkwood and Henley, 2006).

In aquatic habitats, acid conditions are often combined with an increased concentration of heavy metals and with elevated temperatures. In consequence, acidophily is often combined with thermophily and tolerance to heavy metals.

Main algal group living in those hot and acid environments is the Cyanidiophyceae, a sister group to the Rhodophyceae. They consist of at least six species (*Cyanidium caldarium*, *Cyanidioschyzon merolae*, *Galdieria partita*, *Galdieria daedala*, *Galdieria maxima*, *Galdieria sulphuraria*) that occur worldwide in hot acidic waters (pH 0.05–3.00; 42–56 °C) and volcanic calderas such as the Campi Flegrei caldera near Naples, in Yellowstone National Park, on Iceland, and the Azores (Seckbach, 1994). There are also human-made acidic environments such as acidic mine drainage as, e.g., the Rio Tinto system in Spain that is characterized by a red color due to iron and requires adaption to elevated temperatures, to an acid pH (0.9–3.0; mean: 2.2), and to elevated levels of heavy metals, such as Fe, Cd, Zn, Ni, and also As (Aguilera et al., 2007). Among Cyanidiophyceae, *Galdieria sulphuraria* holds a special position in being resistant not only to acid pH and heat (56 °C) but also to a high salt concentration (e.g., 1.5 M NaCl) and toxic metals (Barbier et al., 2005; Ciniglia et al., 2004; Weber et al., 2007). One *Cyanidium* strain has been reported to live in a nonacidic habitat (Dariencko and Hoffmann, 2010).

In Rio Tinto, besides Cyanidiophyceae, also other algal taxa have been found as being adapted to special conditions, belonging to bacillariophycean, euglenophycean, and chlorophycean taxa, such as *Dunaliella acidophila* (Seckbach and Oren, 2007), *Chlamydomonas acidophila* (Spikermann et al., 2007; Costas et al., 2007), *Klebsormidium* spp., *Stichococcus* spp., *Mesotaenium* spp. (Komárek and Nedbalová, 2007), *Euglena* spp. (Novis and Harding, 2007), and *Asteromonas gracilis*, as well as various diatom species and some rhodophytes. Interesting to note is that some of them show a rather high tolerance to Cd as, e.g., *Dunaliella viridis* up to 60 g L⁻¹ and diatom species up to 210 g L⁻¹.

3.2. ADAPTION TO TEMPERATURE, DRYNESS, SALINITY, AND ACIDITY

3.2.1. Temperature

There are various studies to analyze mechanisms of adaption to elevated temperatures. A *Chlamydomonas acidophila* living in a hot and acid milieu was shown to synthesize more heat-shock proteins in an acid environment than a mesophilic *Chlamydomonas reinhardtii* (Gerloff-Elias et al., 2006). According to Roberts (1998), hyperthermophilic organisms contain a reverse gyrase that induces a positive supercoiling of DNA and thus enhances its thermal stability. Samsonoff and MacColl (2001) show that there are differences in stability of high temperature and mesophilic red algae.

3.2.2. Dryness

Adaption of algae to dry conditions is achieved by different strategies such as production of special sheaths and excretion of extracellular carbohydrates and glycoproteins for retaining of water (Henley et al., 2001). Also, formation of cell

aggregates has been observed that probably diminishes the evaporation surface per biomass and is reported for *Dunaliella* spp., *Navicula* spp., *Nitzschia* spp., and *Amphora* spp. Another strategy is to withdraw cells from the surface of soil to depths of 0–1 cm, where some shielding is given against UVB, but yet enough light is still available for photosynthesis (Davey and Clarke, 1991). However, any aridophilic instead of aridotolerant features have not been proven beyond any doubt.

3.2.3. Salinity

For algae living on arid salt plains, the situation is somewhat different. They are halophilic by definition and can grow and complete their life cycle only after rain-falls when the salt concentration in pore water is lowered from about 30 % to below 15 % (Kirkwood and Henley, 2006). Those findings are supported by Clavero et al. (2001) who could show that *Amphora* sp. and *Climaconeis scopuloroides* isolated from saltern ponds (about 5 % salinity) showed in tests sexual reproduction only up to 3 % salinity.

Adaption to increased salinities in general can be achieved by different mechanisms (Oren, 2007). One strategy is to avoid loss of water in cells by increasing the cytoplasmic and vacuolar osmotic potential by the enhanced import of ions. However, this strategy risks to inhibit enzyme function so that another protective measure is the production of compatible solutes such as polyols (e.g., glycerol, mannitol, sorbitol), glycosides (e.g., trehalose, floridoside), amino acids (e.g., proline, glutamic acid), and other small molecules such as glycine-betaine that has been reported to stabilize the oxygen evolving part of photosynthesis (Weber et al., 2007; Oren, 2007). Those osmoprotectants are able to shield enzyme proteins from inorganic ions. A common compatible solute in marine diatoms and in other marine algae probably is DMSP, a precursor of dimethyl sulfide (Mock and Junge, 2007; Oren, 2007). Osmoprotectants found in *Dunaliella* spp. are mannitol, proline, and glycerol (Weber et al., 2007; see also Galinski and Trüper, 1994). In *Chlamydomonas* spp., accumulation of glycerol was observed when cells were stressed by NaCl (see also Henley et al., 2001); in *Galdieria sulphuraria*, proline, trehalose, and floridoside were detected (Weber et al., 2007). An additional measure was observed in *Dunaliella* spp. where the permeability of cytoplasmic membranes for glycerol is decreased by an increase of membrane sterols (Oren, 2007). Pick et al. (2006) report for *Dunaliella salina* under salt stress the production of two major plasma membrane proteins that they identify as homologous to eukaryotic carbonic anhydrase mediating the uptake of CO₂ and as homologous to human transferrin that is involved in the uptake of Fe³⁺ ions. Weber et al. (2007) observe in *Dunaliella salina* under similar conditions the upregulation of photosynthesis, mobilization of starch, and an increased synthesis of Na⁺-redox transporters. Probably the alga is able to maintain a low internal Na⁺ concentration by eliminating excess Na⁺. A similar mechanism is described by Tartari and Forlani (2008) for a psychrophilic *Xanthonema* sp.

3.2.4. Acidity

Adaption to acid conditions (Weber et al., 2007; Canganella and Wiegel, 2011, and references cited there) means to deal with a bunch of problems often occurring in combination. Thus, at an acid pH the solubility of toxic ions of heavy metals is increased. In the Rio Tinto ecosystem, element concentrations found for Fe are up to 20 g L^{-1} (Zettler et al., 2002; see also Aguilera et al., 2007). Probably, tolerance to heavy metals is achieved by an active sequestering of ions. For a *Cyanidioschyzon* sp. isolated from Yellowstone Park, As has been observed to be metabolized (Quin et al., 2009).

Another challenge encountered in an acidic milieu is the low availability of CO_2 . Interestingly, in *Galdieria sulphuraria* as well as in *Chlamydomonas acidophila*, a neutral pH in the cytosol was found (Weber et al., 2007). Thus, it is conceivable that in a neutral cytosol CO_2 is converted to HCO_3^- . Other adaptive strategies could be an increased affinity of RuBisCo to CO_2 or switching to the use of organic instead of inorganic carbon sources as has been shown for *Galdieria sulphuraria* (Oesterhelt et al., 2007).

4. Concluding Remarks

Extremophiles are a fascinating group of organisms that can tell us more about life, its strategies, and boundaries than others.

Accordingly, apart from scientific curiosity, the current interest in extremophilic organisms mainly centers on aspects derived from the fields of biotechnology, evolution of life, and exobiology. Thus, mechanisms of special adaption of membranes and enzymes (extremozymes) in extremophilic organisms to, e.g., coldness, heat, or acidic conditions, are of interest for biotechnological research.

As to scenarios concerning the evolution of life and cells, some extremophilic organisms may represent a sort of living fossils and thus give a hint to conditions under which early cells developed and were able to survive. Thus, deep-sea hydrothermal vents represent probably one of the oldest ecosystems on Terra.

Last but not least, the study of extremophilic organisms may contribute most interesting aspects to adaption mechanisms of life in general and therefore also to the field of exobiology in reminding us that the amplitude of conditions under which life can exist is far greater than we are inclined to deduce from our own set of favorable parameters.

Most studies on extremophiles have been done with prokaryotes, mainly with archaean species. Extremophily in eukaryotes is less well studied as measured by number of relevant papers. However, the question whether extremophilic traits in prokaryotes are more common and play a greater role than in eukaryotes is still open for discussion. At any rate, prokaryotes are far more versatile concerning different metabolism types and potential ecological niches than eukaryotes. But in terms of ecology, the role of extremophilic eukaryotes should not be underestimated.

Assumptions that prokaryotes in principle are able to settle more extreme environments than eukaryotes certainly hold for elevated temperatures (eukaryotes up to 57–60 °C: *Cyanidium caldarium*; Archaea: 121 °C) but probably not for other scenarios as, e.g., salinity. *Dunaliella viridis* shows optimum growth at 60 g NaCl L⁻¹. In the Dead Sea, total salt concentration is about 340 g L⁻¹, i.e., about ten times the marine mean, and *Dunaliella* species such as *D. parva* (Weber et al., 2007) are the only primary producers there. However, growth of *Dunaliella* species is probably possible only after rainfalls when high salt concentrations are diluted.

As to primary production in cold habitats, eukaryotic algae also play a considerable part: About 50 % of polar primary production is due to sea ice diatoms.

In the hot Rio Tinto ecosystem, eukaryotes make up about 60 % of the biomass with algal taxa such as *Chlamydomonas* spp., *Chlorella* spp., *Dunaliella* spp., *Euglena* spp., *Klebsormidium* spp., *Stichococcus* spp., *Mesotaenium* spp., and pennate diatoms.

In general, it is interesting to note that among extremophilic eukaryotes there are only few multicellular species (as, e.g., the tardigrades) and unicells are dominating. Among unicellular algal species, there are genera with extremophilic as well as mesophilic species, as, e.g., in *Chlamydomonas* sp. Other genera are made up of extremely specialized species: In *Dunaliella* sp. (Pick et al., 2006), *D. antarctica* thrives at subzero temperatures and is resistant to freezing, *D. acidophila* grows best at pH 0–1, *D. bardawil* tolerates exceptionally high irradiance, and *D. salina* is halotolerant.

5. References

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EXTREMOPHILIC MAGNETOTACTIC BACTERIA

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1. Introduction

Magnetotactic bacteria represent a diverse group of motile prokaryotes that biomineralize intracellular, membrane-bounded, and tens-of-nanometer-sized crystals of a magnetic mineral, either magnetite (Fe_3O_4) or greigite (Fe_3S_4) (Bazylinski and Frankel, 2004). These structures are called magnetosomes and cause cells to align along the Earth's geomagnetic field lines as they swim, a trait called magnetotaxis (Frankel et al., 1997). Magnetotactic bacteria are known to mainly inhabit the oxic-anoxic interface (OAI) of aquatic habitats (Bazylinski and Frankel, 2004), and it is currently thought that the magnetosomes function as a means of making chemotaxis more efficient in locating and maintaining an optimal position for growth and survival at the OAI (Frankel et al., 1997). Known cultured and uncultured magnetotactic bacteria are phylogenetically associated with the *Alpha*-, *Gamma*-, and *Deltaproteobacteria* classes of the *Proteobacteria*, the *Nitrospirae* phylum, and the candidate division OP3 of the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) superphylum (Amann et al., 2007; Kolinko et al., 2012; Lefèvre et al., 2012). Magnetotactic bacteria are generally thought to be ubiquitous in aquatic environments (Bazylinski and Frankel, 2004) as they are cosmopolitan in distribution and have been found in every continent (Bazylinski and Schübbe, 2007).

For many years, magnetotactic bacteria were thought to be restricted to habitats with pH values near neutrality and at ambient temperature, that is, no species was ever found to be present in extreme environments. Very recently, however, Lefèvre et al. (2010b) described an uncultured, moderately thermophilic magnetotactic bacterium in hot springs in northern Nevada with a probable upper growth limit of about 63 °C. In addition, this same group isolated several strains of obligately alkaliphilic magnetotactic bacteria from different aquatic habitats in California including the hypersaline, extremely alkaline Mono Lake (Lefèvre et al., 2011a). These strains had an optimal growth pH of >9.0. The

purpose of this chapter is to present what is known about the different types of extremophilic magnetotactic bacteria, how they might tolerate different stresses in their environment, and to discuss the potential existence of magnetotactic bacteria in other extreme environments.

2. Alkaliphilic Magnetotactic Bacteria

While magnetotactic bacteria have never been associated with strongly alkaline habitats, three strains of obligately alkaliphilic, anaerobic, and sulfate-reducing, magnetotactic bacteria belonging to the *Deltaproteobacteria* class, each with an optimal growth pH of 9.0–9.5, were recently isolated and grown in axenic culture (Lefèvre et al., 2011a). These new magnetotactic strains, designated ML-1, ZZ-1, and AV-1, were isolated from three different highly alkaline environments in California, USA. Strain ML-1 was isolated from the hypersaline, hyperalkaline Mono Lake. Mono Lake is a well-characterized lake located on the arid eastern side of the Sierra Nevada Mountains in California (Fig. 1a). The waters of this endorheic, monomictic basin exhibit high alkalinity (pH 9.2–10) and salinity (75–90 g/L) (Oremland et al., 2000). The high sodium and carbonate concentrations present at this site are derived from weathering of the surrounding volcanic rocks and hydrothermal inflow (Oremland et al., 2000; Kulp et al., 2007). Sulfate reduction accounts for 41 % of the mineralization of annual primary production in Mono Lake (Hoeft et al., 2004). At the site of the collection of water and sediment samples from which ML-1 was obtained, the pH was 9.8 and the salinity 68–70 ppt. Strain ZZ-1 was isolated from Soda Spring which is a small alkaline spring situated at the Desert Studies Center, a field station of the California State University system, located at the end of Zzyzx Road south of Interstate 15 in



Figure 1. Picture showing the sampling of sediment and water (a) at Mono Lake, a hypersaline, hyperalkaline endorheic lake situated in California. Shore of the lake and small islands is white due to the precipitation of carbonate minerals. (b) Sampling at the Great Boiling Springs (GBS) geothermal field in Gerlach, Nevada. On the first plan, we can see the sediment of the pool made of microbial mat. On the second plan of the picture, we can see the smoke coming from the evaporation of the hot water of the pool.

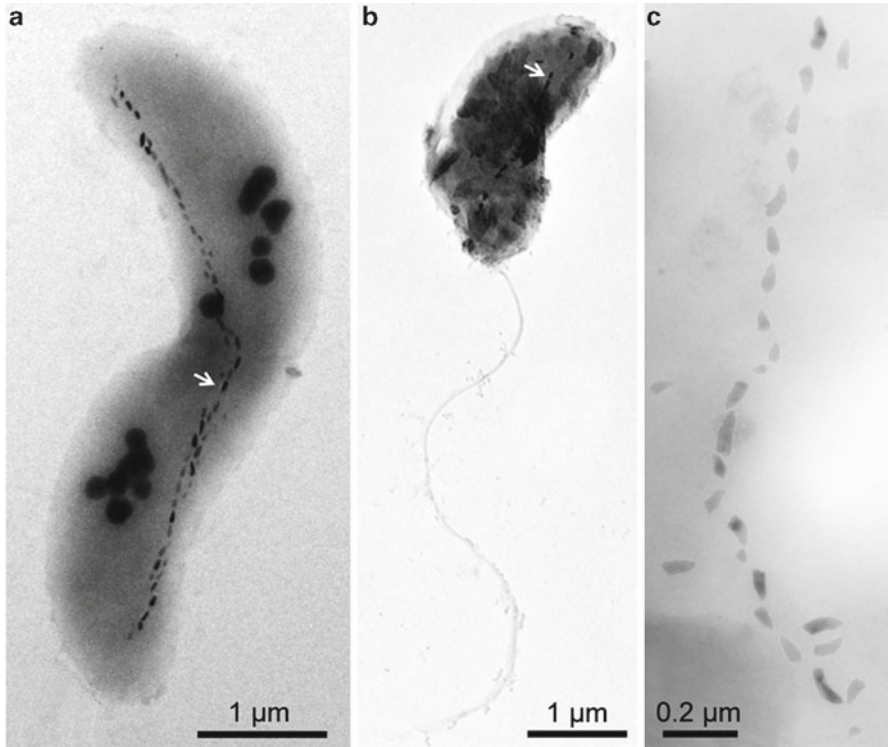


Figure 2. Transmission electron microscope (TEM) images of alkaliphilic magnetotactic bacteria. (a) An unstained magnetically purified cell from a brackish pool at Death Valley Junction (arrow indicates the magnetosome chain). (b) A stained cell grown in axenic culture from the hypersaline Mono Lake, California (arrow indicates the magnetosome chain). (c) Bullet-shaped magnetosome chain of strain ZZ-1 (Reprinted, with permission, from Lefèvre et al., 2011a).

California. Salinity at this location was ~27 ppt and the pH 9.5. The third site, where strain AV-1 was isolated from, is an unnamed small pond in Amargosa Valley situated at Death Valley Junction near the border of Nevada and California. This alkaline pond likely results from underground water flowing through the alkaline desert uplands of Ash Meadows National Wildlife Refuge (Wiemeyer, 2005; Al-Qudah et al., 2011) which is in close proximity. This pond is brackish with a salinity of 3 ppt and had a pH of 9.5 at the time of sampling.

After sampling, the mud and water collected from these three highly alkaline sites contained a significant population ($>10^3$ cells/mL) of magnetotactic bacteria of a single, morphological type based on light microscopic observations. Cells were helical, possessed a single polar flagellum, and contained one or two parallel chains of bullet-shaped magnetite-containing magnetosomes (Fig. 2). The 16S rRNA genes of magnetically purified, uncultured cells from Mono Lake and the brackish pool at Death Valley Junction were amplified and showed that these organisms were closely related (16S rRNA gene sequence identities $\geq 98.9\%$) to

the non-magnetotactic, alkaliphilic, and sulfate-reducing Deltaproteobacterium, *Desulfonatronum thiodismutans*, originally isolated from Mono Lake (Pikuta et al., 2003). Magnetically purified cells were used as inocula in a growth medium for the enrichment of anaerobic, alkaliphilic, and sulfate-reducing bacteria modified from Pikuta et al. (2003) to reflect the salinities of the sampling sites. Cells with the same morphology as those found in the mud and water samples from all sites grew in this growth media. All strains reduced sulfate and used formate and hydrogen as electron donors and were capable of chemolithoautotrophic growth with hydrogen as the electron donor with bicarbonate as the sole carbon source.

The presence of magnetotactic bacteria was previously reported in Lonar Lake in Maharashtra, India (Chavadar and Bajekal, 2009, 2010). This crater lake was formed as a result of a meteoritic impact and is a closed basin lake characterized by high alkalinity and salinity. However, no evidence of the presence of magnetosomes in these bacteria and no phylogenetic data were presented.

Alkaliphilic bacteria have a number of physiological problems to deal with living in such a harsh environment including maintaining their intracellular pH at about neutral while living in an external environment with a pH of >9.0. These organisms have developed a number of interesting ways to overcome these problems (Krulwich, 2006). In the specific case of alkaliphilic magnetotactic bacteria, how these microbes synthesize large number of magnetosomes in natural environments is particularly interesting in that at high pH, iron becomes extremely insoluble (Jimenez-Lopez et al., 2010), and thus, magnetotactic bacteria must possess highly efficient mechanisms of iron uptake under these conditions. The fact that strains ML-1, ZZ-1, and AV-1 exist in the reducing anoxic zone in these environments where iron is likely to be in the more soluble ferrous form, rather than the oxic zone of their habitat, probably obviates part of this problem.

3. Moderately Thermophilic Magnetotactic Bacteria

Most known cultured magnetotactic bacteria are mesophilic with regard to growth temperature and do not grow much above 30 °C (e.g., *Magnetospirillum* species, *Desulfovibrio magneticus* strain RS-1, *Magnetococcus marinus* strain MC-1, *Magnetospira thiophila* strain MMS-1, and *Candidatus Magnetovibrio blakemorei* strain MV-1; Schleifer et al., 1991; Sakaguchi et al., 2002; Bazylinski et al., 2012; Williams et al., 2012). In addition, virtually all studies on uncultured magnetotactic bacteria involved sampling sites that were at 30 °C and below. However, a recent environmental study demonstrated the existence of a moderately thermophilic magnetotactic bacterium, designated strain HSMV-1, found in mud and water samples collected from the Great Boiling Springs (GBS) geothermal field in Gerlach, Nevada (Lefèvre et al., 2010b; Fig. 1b). GBS is a series of hot springs that range from ambient temperature to ~96 °C (Anderson, 1978; Costa et al., 2009).

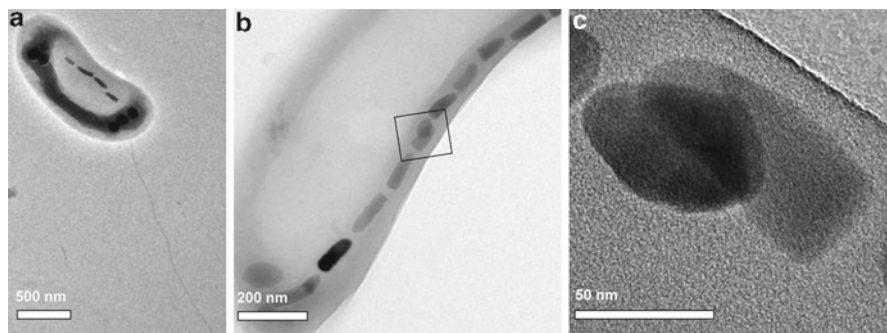


Figure 3. Transmission electron microscope (TEM) images of cells and magnetosomes of the thermophilic magnetotactic bacterium strain HSMV-1. **(a)** TEM image of unstained cell of HSMV-1 showing a single polar flagellum and a single chain of bullet-shaped magnetosomes. **(b)** Higher-magnification TEM image of the magnetosome chain. **(c)** High-magnification TEM image of magnetosomes (Reprinted, with permission, from Lefèvre et al., 2010b).

The geology, chemistry, and microbial ecology of the springs have been described in some detail (Anderson, 1978; Costa et al., 2009).

Microscopic examination of the samples collected at GBS showed the presence of a single morphotype of magnetotactic bacteria in samples taken from nine springs whose temperatures ranged from 32 to 63 °C. Cells were small (1.8 ± 0.4 by 0.4 ± 0.1 μm), Gram-negative, vibrioid-to-helicoid in morphology, and possessed a single polar flagellum (Fig. 3). Magnetotactic bacteria were not observed in springs that were 67 °C and higher, suggesting that the maximum survival and perhaps growth temperature for strain HSMV-1 is about 63 °C. When the water temperature in these pools was ambient, cells of strain HSMV-1 were not observed although other types of magnetotactic bacteria were present including greigite-producing, rod-shaped bacteria (Lefèvre et al., 2011b). Cells of HSMV-1 biomineralize a single chain of bullet-shaped magnetite magnetosomes that traverse the cells along their long axis (Fig. 3).

The 16S rRNA gene sequence of strain HSMV-1 places the organism phylogenetically in the phylum *Nitrospirae* with its closest relative in culture being *Thermodesulfovibrio hydrogeniphilus* (Haouari et al., 2008), a non-magnetotactic, thermophilic sulfate-reducing bacterium isolated from a terrestrial Tunisian hot spring with an optimal growth temperature of 65 °C.

Nash (2008) also reported the presence of thermophilic magnetotactic bacteria in microbial mats at about 45–55 °C adjacent to the main flow in Little Hot Creek and in other springs up to 58 °C all on the east side of the Sierras in California. Cells biomineralized bullet-shaped crystals of magnetite and were phylogenetically affiliated with the phylum *Nitrospirae*. Few additional details were provided (Nash, 2008). This indicates that thermophilic magnetotactic bacteria are not confined to the GBS and are likely present in hot springs around

the world. Moreover, these studies clearly show that some magnetotactic bacteria can be considered at least moderately thermophilic and extend the upper temperature limit for environments where magnetotactic bacteria exist and grow and where magnetosome magnetite is deposited.

4. Potential for Magnetotactic Bacteria in Other Extreme Environments

Magnetotactic bacteria are generally considered as gradient-loving, microaerophilic or anaerobic microorganisms that are primarily located at or just below the OAI in the aquatic environments. In theory, all aquatic environments having sediments or a water column stratified with the appropriate physical-chemical conditions, such as a suitable redox potential and the presence of enough soluble iron, could contain magnetotactic bacteria. Thus, there is no reason why acidophilic, piezophilic, halophilic, or psychrophilic bacteria could not have acquired the ability to biomineralize magnetosomes during their evolution. In fact, it is possible that these organisms have even been isolated in the past without the realization that the species were magnetotactic. For example, in an earlier section, we described three strains of alkaliphilic, sulfate-reducing magnetotactic bacteria as strains of *Desulfanotrum thiodismuans* based on 16S rRNA gene sequences and phenotypic characteristics. This raised the question whether the type species of *D. thiodismuans* was magnetotactic when first isolated from the environment but lost the magnetotactic trait (Lefèvre et al., 2011a). This is possible as many cultivated magnetotactic bacteria are known to lose this trait relatively easily in culture (Schübbe et al., 2003; Dubbels et al., 2004; Schüler, 2008), sometimes from the loss of a magnetosome genomic island in which the genes for magnetite biomineralization are located (Schübbe et al., 2003; Schüler, 2008). In turn, a larger, perhaps more interesting and important question is whether and how many magnetotactic prokaryotic organisms have been characterized from the environment or isolated and deposited in culture collections but have never been recognized as magnetotactic for various reasons. This may be most applicable to the sulfate-reducing bacteria, as another magnetotactic sulfate-reducing bacterium, *Desulfovibrio magneticus* strain RS-1, displays only a weak magnetotactic response and ability in biomineralizing magnetite magnetosomes particularly when grown on sulfate as a terminal electron acceptor (Pósfai et al., 2006).

4.1. POTENTIAL FOR THE EXISTENCE OF HALOPHILIC MAGNETOTACTIC BACTERIA

There are no specific reports of halophilic magnetotactic bacteria; however, there are several studies that report the existence of magnetotactic bacteria in hypersaline environments (having salinity levels greater than that of seawater ~35 ppt). Multicellular magnetotactic prokaryotes (MMP) such as *Candidatus*

Magnetoglobus multicellularis thrive in the hypersaline Araruama lagoon which has a maximum salinity of 60 ppt (Martins et al., 2009). MMPs and other types of magnetotactic bacteria are also present in the hypersaline Salton Sea (salinity ~50 ppt (Lefèvre et al., 2010a, 2012; Lefèvre and Bazylinski, unpublished). The site from where the sample containing magnetotactic bacteria at the Salton Sea were collected was a reddish pool containing large numbers of halophilic prokaryotes as determined by microscopic observation. One species from this environment was isolated in culture, strain SS-5, but this strain does not seem to require high concentration of salt to grow. The discovery of the alkaliphilic magnetotactic strain ML-1 in the Mono Lake increased the upper limit of salinity where magnetotactic bacteria can be found (Lefèvre et al., 2011a). Indeed, samples were collected in a site where the salinity was 68 ppt. However, like strain SS-5, high salinity is not crucial for the growth of ML-1. Thus, some magnetotactic bacteria can be considered moderately halophilic, and it is possible that halophilic magnetotactic bacteria exist as some halophilic strains are known to have similar metabolism than known magnetotactic bacteria (e.g., Ollivier et al., 1994) and to be phylogenetically related to magnetotactic bacteria (e.g., strain SS-5 related to *Thiohalocapsa marina*; Lefèvre et al., 2012).

4.2. POTENTIAL FOR THE EXISTENCE OF PIEZOPHILIC AND PSYCHROPHILIC MAGNETOTACTIC BACTERIA

The deep-sea piezosphere includes the volume of the deep sea at a depth of 1,000 m and greater, with hydrostatic pressures of over 100 atmospheres or 10 megapascal (1 atm = 1.013 bar = 0.1 MPa), and accounts for about 75 % of the total ocean volume (Fang et al., 2010). A study showed the presence of living magnetotactic bacteria in shallow hemipelagic sediments, collected at the depth of 598 m, from the Santa Barbara Basin in the eastern Pacific with a temperature of 8 °C (Stolz et al., 1986). There is no phylogenetic data available from this study, only the morphology of the cells and the shape of their magnetosomes were reported. Cells were vibrioid and rod-shaped but the most common observed morphology was coccoid. Magnetosome crystals in these bacteria were composed of magnetite and were cuboidal, rectangular, or irregular in shape. It was also reported the presence in the surface sediments of the Santa Barbara Basin of biogenic ultra-fine-grained, single-domain magnetite. These particles were also composed of magnetite with cuboidal, prismatic, and teardrop-shaped (commonly named bullet-shaped) morphologies. This study was the first to describe magnetotactic bacteria in deep sediments, and it was suggested that these microorganisms are the source of the primary remanence carrier in marine sediment.

Later, Petermann and Bleil (1993) indicated the systematic findings of magnetotactic bacteria in pelagic and hemipelagic sediments of the eastern South Atlantic. In this study, they described different morphologies (cocci, spirilla, vibrioid, and rod-shaped) of magnetotactic bacteria at water depths to about

3,000 m in the African continental margin (off Namibia, between the equator and 30°S) and on the Walvis Ridge in a pelagic environment (about 1,400 km off the coast) situated on a seamount at a water depth of 1,007 m. They showed that the number of magnetotactic bacteria in cores that were stored at an ambient temperature of around 25 °C during 12 h declined and those that survived showed less swimming bacteria. Magnetotactic bacteria could still be detected after several months in samples stored in a refrigerator at approximately 2 °C. It was concluded that at least some magnetotactic bacteria might be at least facultatively psychrophilic. Moreover, they sampled magnetotactic bacteria from intertidal sediments of the North Sea and brought them to 3,100 m water depth; after 24 h, no decrease in numbers or swimming activity of individuals was detected after recovery, indicating that these magnetotactic bacteria are piezotolerant. In sum, these studies suggest that the possibility of the existence of even obligate piezophilic and psychrophilic bacteria cannot be dismissed.

4.3. POTENTIAL FOR THE EXISTENCE OF ACIDOPHILIC MAGNETOTACTIC BACTERIA

Acidophiles are defined as those bacteria having an optimal growth pH of 3 (Baker-Austin and Dopson, 2007). To our knowledge, there are no reports involving investigating the presence of magnetotactic bacteria in highly acidic environments such as acid mine drainage or bogs. The formation of magnetite under these conditions might pose a problem for prokaryotes as magnetite produced through chemical means or through biologically induced biomineralization (Frankel and Bazylinski, 2003) by non-magnetotactic bacteria (e.g., dissimilatory iron-reducing bacteria) does not appear to be thermodynamically favored at very low pH (e.g., Bell et al., 1987). However, despite living at very low pH, acidophilic bacteria must maintain an intracellular pH of near neutrality (Baker-Austin and Dopson, 2007), and thus, it seems like it may be possible for certain acidophilic species to be capable of biomineralizing intracellular magnetosomes. The fact that iron is more soluble and thus more bioavailable under acidic conditions, it seems likely that a magnetotactic bacterium in such environment should not have a problem taking up enough iron for magnetosome formation.

4.4. POTENTIAL FOR THE EXISTENCE OF MAGNETOTACTIC BACTERIA ON MARS

Magnetotactic bacteria have had a major impact on the field of astrobiology. Magnetite crystals morphologically similar to those present in some magnetosomes of magnetotactic bacteria living in the present have been found in the Martian meteorite ALH84001 (Thomas-Keprta et al., 2000, 2001, 2002; Clemett et al., 2002). These crystals, referred to as “magnetofossils,” have been used as evidence

for the past presence of magnetotactic bacteria in the meteorite ALH84001 as well as on Earth about two billion years ago (Chang and Kirschvink, 1989). The presence and interpretation of these crystals in Martian meteorite ALH84001 have evoked great controversy and debate. If the magnetite crystals were indeed biogenic, the implication was that bacterial life had existed on ancient Mars (McKay et al., 1996; Thomas-Keprta et al., 2000, 2001, 2002; Buseck et al., 2001; Clemett et al., 2002; Weiss et al., 2004). In turn, this debate has led to a number of criteria to be used to distinguish biogenic magnetite from inorganically produced magnetite (Thomas-Keprta et al., 2000; Arató et al., 2005; Kopp and Kirschvink, 2008; Jimenez-Lopez et al., 2010; Gehring et al., 2011; Kind et al., 2011).

The discovery and isolation of obligately alkaliphilic magnetotactic bacteria is discussed in a previous section and clearly demonstrates that some magnetotactic species can be considered extremophilic. Because magnetotactic bacteria had never been considered to inhabit extreme environments, highly alkaline habitats have apparently not been searched for magnetofossils. Chemical analyses of soil samples of Mars indicate a period of highly alkaline conditions on the planet in the past (Kempe and Degens, 1985; Hecht et al., 2009; Kounaves et al., 2010). Moreover, equilibrium modeling based on measured Ca^{2+} and Mg^{2+} concentrations was consistent with carbonate equilibrium for a saturated solution (Hecht et al., 2009; Kounaves et al., 2010), and thus, carbonate buffering appears to be significant in some Martian soils as it is in Mono Lake. Mono Lake has been used by researchers at a number of institutions, including the National Aeronautics and Space Administration (NASA), as a model for extreme environments that might be comparable to those on planet Mars (Kempe and Kazmierczak, 1997). It would be interesting to determine whether bullet-shaped magnetite crystals like those in strains ML-1 are incorporated and preserved as magnetofossils in carbonate minerals, such as the unusual carbonate structures known as tufas, abundant in Mono Lake, as they appear to do in sedimentary carbonates in marine environments (McNeill et al., 1988; Sakai and Jige, 2006) and in carbonates in the Martian meteorite ALH84001 (Thomas-Keprta et al., 2002). Between 2018 and 2023, a mission launch by the NASA, the Mars sample return mission (MSR), will aim to collect rock and dust samples from Mars and to return them to Earth for analysis. This mission might reveal the existence of such magnetofossils.

5. Conclusions and Future Directions

Almost 50 years after the initial discovery of magnetotactic bacteria by Salvatore Bellini in 1963 (2009a, b) and more than 30 years after the rediscovery and formal publication of these organisms by Richard P. Blakemore (1975), it is only recently that extremophilic magnetotactic bacteria were observed and described. This clearly raises the possibility that magnetotactic microorganisms might exist in other extreme environments that have never been sampled and examined for their presence. Do magnetotactic bacteria exist in environments characterized by very high

pressure, by extreme cold, highly acidic, or highly saline? To address this question, more sampling missions in extreme environments need to be done by researchers using specific culture- and non-culture-based techniques. However, based on the recent results described in this chapter, the potential of finding magnetotactic bacteria in other extreme environments is high. Moreover, considering the relatively small number of groups that study these intriguing organisms, it seems that the known diversity of magnetotactic bacteria is seriously underestimated.

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TARDIGRADES: AN EXAMPLE OF MULTICELLULAR EXTREMOPHILES

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1. Introduction

Tardigrades (tardi, “slow” and grade, “walker”), also known as “water bears” or “moss piglets,” were first discovered in 1773 by the German zoologist Johann August Ephraim Goeze (Romano, 2003). Nowadays, there are hundreds of species known (Gridetti and Bertolani, 2011). Tardigrades are the classical polyextremophilic survivors of multi-extreme environments and are thus commonly considered as an appropriate model for astrobiological studies based on their high survival ability to desiccation, ionizing radiation, and under a wide range of temperature and pressure conditions (Horikawa, 2012). The segmented body of tardigrades has a bilateral symmetry plan with eight legs with hooked claws or sucking disks on their legs. They are hydrophilic microscopic invertebrates; meaning that all tardigrade species are aquatic and require a film of water to be active. Reproduction occurs sexual or by parthenogenesis. Females are larger in size than the male and the life span of tardigrades ranges from 30 months to 12 years. Tardigrades inhabit a variety of extreme habitats as diverse as damp pools, algal mats, marine and deep-sea abysses, fresh- and saltwater, and terrestrial ecosystems. They usually live semi- or epibenthic (living on corals and algal species, on mud, and in moist moss cushions, lichens, bark, life litter, and in soil). Their food, which enters via the buccal apparatus, consists of algae, diatoms, and bacteria. Some tardigrades are carnivorous species and consume invertebrates such as rotifers, protozoa, and nematodes. Among their predators are fungi and other parasites such as mites, but also larvae, spiders, and nematodes.

2. Tolerance of Tardigrades to Extreme Environmental Conditions

In an active state, tardigrade species inhabiting a terrestrial environment require at least a film of water around them. However, when deprived of that water, they can enter an ametabolic state called cryptobiosis, which can further be subdivided into five categories based on the factors that induce it, such as cryobiosis (induced by freezing), thermobiosis (low and high temperatures), osmobiosis

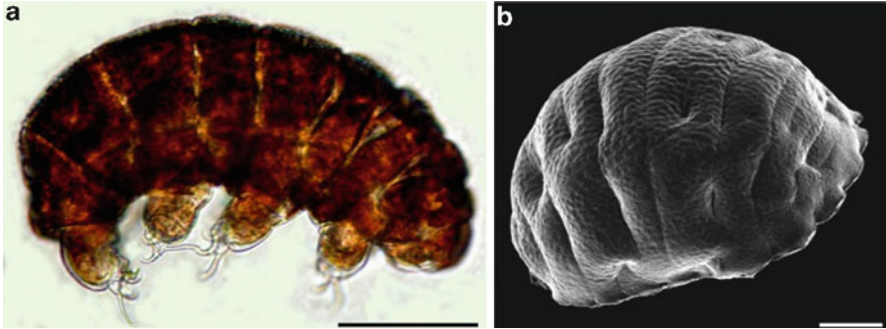


Figure 1. A light micrograph of a hydrated adult (a) and a scanning electron micrograph of an anhydrobiotic adult (b) of the tardigrade *Ra. varieornatus*. Scale bars = 100 μm (a) and 20 μm (b) (Reproduced from Horikawa (2012) with permission from Springer).

(extremely high osmolarity), anoxybiosis (lack of oxygen), and anhydrobiosis (lack of water) (Wright et al., 1992; Takamatsu et al., 1997; Jönsson and Bertolani, 2001). At the organismic level, the distinction between anhydrobiosis, cryobiosis, and osmobiosis is often unclear because more than one process may be involved in the entry of cryptobiosis (Islam and Schulze-Makuch, 2007). For example, lower temperatures that induce extracellular freezing in an organism will also have a desiccating effect on the cells (Jönsson, 2003). Tardigrades can survive many years in a desiccated state (Jönsson and Bertolani, 2001). In the natural environment of tardigrades, the lack of water is usually thought to be the cause that initiates the change into a dormant state (anhydrobiotic state). The dormant state is referred to as a tun (Fig. 1b). This ability provides the opportunity for tardigrades to colonize extremely challenging environments such as deserts, high mountains, abyssal plains, and cold polar regions. Dispersal of tardigrades, especially of the dormant stages such as cysts, tuns, and eggs, usually occurs by wind or by floodwater, melting snow, and rain in a terrestrial environment. As is the case with nematodes and bdelloid rotifers, tardigrades can enter cryptobiosis at any stage of their life cycle (Bertolani et al., 2004). These water bears resemble closely *Onychophora*, *Euarthropoda*, and *Annelida* (Islam and Schulze-Makuch, 2007). However, tardigrades have small body sizes (generally 0.5–1 mm), and it is generally thought that the ancestor of this taxon was relatively large *Articulata* (Schmidt-Rhaesa 2001). If that assumption is correct, the small size and the absence of certain organs such as a dorsal heart, segmental coelomic cavities, and metanephridia can be understood as specialized adaptations of tardigrades to their environment.

It is without doubt that these animals are masters in adaptation, especially to drastically changing environmental conditions: they have been reported to survive extreme temperatures such as down to $-272\text{ }^{\circ}\text{C}$ and up to $151\text{ }^{\circ}\text{C}$ and pressures of up to 6,000 bar (Jönsson et al., 2008). Their efficient DNA repair

system helps them to survive these very severe environmental conditions. It was also reported (Jönsson et al., 2008; Rebecchi et al., 2009) that tardigrades being sent to space survived exposure to low temperature, cosmic and solar radiation, microgravity, and vacuum. Based on their high survival ability under these environmental stresses, they are ideal analogues for candidates for extraterrestrial life forms (Seckbach, 2013; Chela-Flores, 2011; Horikawa, 2012).

3. The Tardigrade Tool Set for Extreme Environmental Conditions

Adaptation in a biological sense is a genetically controlled characteristic that increases an organism's ability to survive and reproduce in its environment. Anhydrobiosis is the most common form of cryptobiosis, and anhydrobiotic organisms are able to survive almost complete dehydration; 95–99 % of the water content is lost in many cases (Drinkwater and Crowe, 1991; Danks, 2000). Body water content of terrestrial tardigrades decreases to between 1 and 3 wt%, which is associated with body contraction (Horikawa et al., 2006, 2008). The contracted animal (tun) does not show any visible signs of life, but can resume activity when exposed in a drop of water (Horikawa, 2012). Extreme desiccation tolerance is observed in many taxa ranging from unicellular organisms to higher invertebrates such as vegetative tissues of higher plants (resurrection plants), yeasts, bacteria, fungal spores, protozoans, eggs of turbellaria, nematodes, rotifers, tardigrades, springtails, cysts of primitive crustaceans including the brine shrimp (*Artemia salina*), and larvae of a chironomid midge (Watanabe, 2006; Takamatsu et al., 1997; Ricci, 1998; Clegg, 2001; Wharton, 2002). The anhydrobiotic state of animals is usually smaller than 1 mm and only occurs in invertebrates (Takamatsu et al., 1997). Alpert (2000) suggested that desiccation-tolerant animals may be small in size because of physical stresses associated with drying. The organism's cells shrink as a result of drying and the whole animal must reduce in size. All animals that tolerate anhydrobiosis or desiccation as adults adopt distinctive or curled shapes as they dry such as being observed for the tun in tardigrades and the coiling of the body in soil nematodes (e.g., *Scottinema lindsayae*, *Eudorylaimus antarcticus*, and *Plectus antarcticus*). The curling would be prevented by rigid external or internal skeletons.

Many species entering the anhydrobiotic state can remain viable for significant time periods. For example, Steiner and Albin (1946) reported a 39-year survival of the nematode *Tylenchus polyhypnus* after laboratory preservation. Reports of recovery from the anhydrobiotic state for insects include 17 years, for crustaceans 16 years, and for tardigrades and rotifers 9 years (Baumann, 1922; Clegg, 2001; Bertolani et al., 2004). This may not be the limit though. There are reports that tardigrades and rotifers were recovered from a dry moss sample taken from an Italian museum after 120 years of preservation, although the individual organisms underwent “quivers in several zones of their body” (Franceschi, 1948; Jönsson, 2003).

Since extreme water loss causes critical damage to cells, tardigrades and other anhydrobiotic animals must protect their cells during the anhydrobiotic state. Cells of organisms, which are adapted to survive anhydrobiosis, usually contain large amounts of low-molecular-mass compatible solutes (Yancey et al., 1982; Somero and Yancey, 1997). Among these solutes, trehalose, the disaccharide of glucose, plays a considerable role (Drinkwater and Crowe, 1991; Eleutherio et al., 1993; Clegg, 2001). Trehalose has a high solubility, a low reactivity, and a low tendency to crystallize. It is a nonreducing sugar and thus is less harmful to cells and tissues than reducing sugars such as glucose, even at extremely high concentrations (Drinkwater and Crowe, 1991; Pannowitz et al., 2003). It substitutes for bound and free water and thus maintains the structures of cell membranes and proteins, but also leads to metabolic shutdown. The disaccharide trehalose has been found in concentrations of up to 10–20 wt% in several anhydrobiotic animals, including nematodes (Madin and Crowe, 1975), insect larvae (Watanabe et al., 2003), and embryos of *Artemia salina* (Clegg, 1962). However, only relatively small amounts of trehalose have been found in the anhydrobiotic state of tardigrades (Westh and Ramlov, 1991; Horikawa et al., 2006).

Thus, other biochemical processes may be occurring to protect tardigrades in the anhydrobiotic state. Heat shock proteins (Hsps) are conceivably good candidates, because they can serve as molecular chaperones. The Hsp70 family and the Hsp60 chaperone complexes, understood as “heat shock proteins,” are upregulated by heat stress, although they also play a vital role in folding nascent proteins under nonstress conditions (Ellis and Hartl, 1999). Ellis and Hartl (1999) and MacRae (2000) reported that these molecules participate in unfolding and relocalization of proteins damaged by stresses, assist in the folding of newly synthesized proteins, and protect them from denaturation and aggregation and aid in their renaturation, plus influence the final intracellular location of mature proteins (Ellis and Hartl, 1999; MacRae, 2000). Hsps are also implicated in observed tolerance against various other stresses such as low and high temperatures, oxidation, anoxia, and heavy metals.

Other candidates are late embryogenesis abundant (LEA) proteins, which may work as molecular chaperons helping other proteins being protected from denaturation (Wise and Tunnacliffe, 2004; Horikawa, 2012). LEA proteins are expressed in tardigrades (e.g., Schokraie et al., 2010) and some other anhydrobiotic animals.

4. Tardigrades: Candidates for Panspermia?

Tardigrades are astonishingly recalcitrant to environmental stresses and can survive in the dormant state even in space conditions for a limited time. Thus, they appear to be able to survive more harsh conditions than most of the bacteria can (Table 1). Microbial organisms have often been invoked in various panspermia scenarios, the transport of organisms from one planetary body to the other. Any

Table 1. Known environmental limits for extremophiles for growth or tolerance.

Environmental parameter	Bacteria and Archaea	Eukaryotes	Example environments
Temperature	113 °C, <i>Pyrolobus fumarii</i> , 121 °C, Strain 121 Bacterial growth at about -15 °C	~60 °C algae (e.g., <i>Cyanidium caldarium</i> and <i>Galdieria sulphuraria</i>) some fungi Himalayan midge at -18 °C	Submarine hydrothermal systems, geothermal hot springs Brine pockets in sea ice at about -30 °C
pH	~pH 0, acidophilic archaea such as <i>Ferroplasma</i> sp. pH 13, <i>Plectonema</i> , pH 10.5 <i>Natronobacterium</i>	pH 0, fungi such as <i>Cephalosporium</i> , <i>Cyanidium</i> , and <i>Galdieria</i> pH 0-4 pH 10, many species of protists and rotifers	Acid mine drainage, geothermal sulfurous sites (e.g., Yellowstone, hot springs) Soda lakes, peridotite-hosted hydrothermal systems (e.g., Lost City vent)
Desiccation	Growth in 35 % NaCl, archaea and bacteria, such as halobacteria	Molds and yeasts such as <i>Zygosaccharomyces rouxii</i> (growth in high sugar content)	Deep-sea brines, soda lakes, evaporate ponds, dry soils, and rocks, food with high solute content
Pressure	High diversity of bacteria and archaea in deep ocean trenches including piezophiles	High diversity of invertebrates and fishes in ocean trenches, tardigrades survive 10-day exposure to space vacuum	11,100-m deep Marianas Trench
Radiation	10,000-11,000 grays (gamma radiation), <i>Deinococcus radiodurans</i>	German cockroach (<i>Blattella germanica</i>) and tardigrades can survive exposure to radiation above 1,000 grays	No natural source of radiation on Earth at levels tolerated by <i>Deinococcus radiodurans</i>

Modified from Schulze-Makuch and Irwin (2008).

kind of organism that would undertake this type of journey would have to survive a series of hazards, including (1) survival of the meteorite impact that ejects the organism into space from the planet of its origin; (2) maintenance of viability for long durations of time inside the meteoritic material; (3) intense UV and cosmic radiation, cold, and vacuum; and (4) the shock and heat of impact on the planetary body to which the organism is transferred (Schulze-Makuch et al., 2008).

A huge hypervelocity impact by a large space object would be required to lift up a rock fragment from the surface of a terrestrial planet like Earth, which will include intense heating of ejected material and a large accelerative force. Nevertheless, a significant fraction of bacterial spores survived this kind of stress (Nicholson et al., 2006). Dormant stages of tardigrades have not been tested this way, but could be expected to survive as well. The next challenge would be the space environment with its high doses of UV irradiation and particle radiation, extremely low temperatures, and space vacuum. Both dormant stages of bacteria and tardigrades have been shown to survive space conditions for a significant time (Horneck et al., 1994; Jönsson et al., 2008; Rebecchi et al., 2009), particularly if embedded in salt or a thin dust layer (Mancinelli et al., 1998). Finally, any surviving organism will face the landing on a new planet. The thicker the atmosphere, the more likely the impactor, on which the organism did hitch a ride, would break apart with the result that it would slow down the speed of the resulting fragments in the lower atmospheric layers (Schulze-Makuch et al., 2008). This would raise the chances of survival for the organism. Davies (1996) analyzed this scenario for the Mars-Earth case and concluded that the survival of microorganisms would be plausible.

Likelihoods of microbial survival of the different steps involved in a panspermia scenario were provided by Mileikowsky et al. (2000) and Clark (2001). A critical parameter is travel time in space, but under a scenario of exchange between terrestrial planets of the same solar system such as Venus, Earth, and Mars, it can be assumed that a significant portion of microbes would survive. Would tardigrades be able to survive as well? We do not know for sure and appropriate tests would have to be conducted. But even if they do, they would likely not survive for the long-term. While microbes would be able to live in a viable state and feed on trace organic compounds or even inorganic sources at the target location, given a minimum requirement of suitable environmental conditions, it is very difficult to imagine that tardigrades, after leaving the dormant stage – even if they survive panspermia – would find a hospitable location where other organisms are present upon which they can feed. Also, as multicellular organisms, they do have higher demands on habitable environmental conditions than bacteria, when they are in an active state.

Thus, while the dormant stage of tardigrades is amazingly recalcitrant to a wide diversity of stresses and a prime example how hardy even multicellular life forms can be, we doubt that we would find them away from their home planet on Earth. Even if ejected by a meteorite impact and the tardigrades would survive the ejection, there would be no food sources and no habitable conditions for

them, and any surviving tardigrades would eventually die in the dormant form. Microbes, on the other hand, might find habitable conditions elsewhere, like in the near surface of Mars (Houtkooper and Schulze-Makuch, 2007). Nevertheless, tardigrades may be an intriguing example to study how far life, especially more complex life, is capable to adapt to harsh conditions, some of which might be found in extraterrestrial environments.

5. Summary

Life on Earth exhibits an amazing diversity and adaptability. Tardigrades are one pronounced example of organisms that exhibit resistance to multiple environmental stresses such as low temperature, desiccation, high radiation doses, and extreme pressure. The tool set that tardigrades employ to overcome these multiple stresses is even more amazing and still not fully explored. Based on the hardiness of their dormant stage, it can be imagined that viable organisms could be transported from Earth to some other planet (such as Mars) via an asteroid impact. However, today it seems that habitable conditions for tardigrades are not likely to be found outside of Earth; thus, we would not expect to find viable organisms except on Earth. Nevertheless, tardigrades inform us how adaptable life and how rich its toolset can be, which in turn can inform us what forms of life may exist elsewhere in the universe.

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**PART VIII:
FINAL COMMENTS**

Chela Flores

Biodata of **Julian Chela-Flores**, author of “*Polyextremophiles: Summary and Conclusions.*”

Professor Julian Chela-Flores was born in Caracas, Venezuela, and studied in the University of London, England, where he obtained his Ph.D. in quantum mechanics (1969). He was a Researcher at the Venezuelan Institute for Scientific Research (IVIC) and Professor at Simon Bolivar University (USB), Caracas, until his retirement in 1990. During his USB tenure, he was Dean of Research for 6 years. He is a Fellow of the Latin American Academy of Sciences, the Academy of Sciences of the Developing World, and the Academy of Creative Endeavors (Moscow), and a Corresponding Member of the Venezuelan “Academia de Fisica, Matematicas y Ciencias Naturales.” His current positions are Staff Associate of the Abdus Salam International Center for Theoretical Physics (ICTP), Trieste; Research Associate, Dublin Institute for Advanced Studies (DIAS); and Professor Titular, Institute of Advanced Studies (IDEA), Caracas. His particular area of expertise is astrobiology, in which he is the author of numerous papers. He organized a series of Conferences on Chemical Evolution and the Origin of Life from 1992 to 2003. In 2001, he published the book: *The New Science of Astrobiology from Genesis of the Living Cell to Evolution of Intelligent Behavior in the Universe*, reprinted as a paperback in 2004. Its second edition was published with Springer in 2011 as *The Science of Astrobiology: A Personal Point of View on Learning to Read the Book of Life.*” He is also the author of “*A Second Genesis: Stepping-Stones Towards the Intelligibility of Nature*” (2009) with World Scientific Publishers.

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POLYEXTREMOPHILES: SUMMARY AND CONCLUSIONS

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The search for extraterrestrial life is encouraged by a comparison between organisms living in severe environmental conditions on Earth and the physical and chemical conditions that exist on some Solar System bodies. Astrobiology raises the possibility of life elsewhere in the Solar System (cf., the chapter by Joseph Seckbach – “[Life on the Edge and Astrobiology: Who Is Who in the Polyextremophiles World?](#)”).

The extremophiles that could tolerate more than one factor of harsh conditions are called polyextremophiles. There are unicellular and even multicellular organisms that are classified as hyperthermophiles (heat lovers), psychrophiles (cold lovers), halophiles (salt lovers), barophiles (living under high pressures), and acidophiles (living in media of the lower scale of pH). At the other end of the pH scale, they are called alkaliphiles (namely, microbes that live at the higher range of the pH scale). Thermoacidophilic microbes thrive in elevated thermo-environments with acidic levels that exist ubiquitously in hot acidic springs.

Most of the organisms that we know thrive in normal environments that we consider to be ambient habitats. Extremophiles are among the microorganisms living on the edge of life under severe conditions. In recent years, microorganisms have been discovered living in extreme environments, such as very high temperature (up to 115 °C) (cf., the chapter by William D. Orsi And Virginia P. Edgcomb, “Microbial Eukaryotes in Marine Oxygen Minimum Zones”). Survival is also particularly relevant for very low temperatures (~ -20 °C, as discussed from various points of view in the chapters by V. Edgcomb et al., “Protist Community Structure and Dynamics in a Seasonally Anoxic Fjord: Saanich Inlet British Columbia”; Hanhua Hu, “[Adaptation of Antarctic Freshwater Green Algae to Extreme Environments](#)”; Jacqueline Goordial, Guillaume Lamarche-Gagnon, Chih-Ying Lay And Lyle Whyte, “Left Out in the Cold: Life in Cryoenvironments”; Thomas Leya, “[Snow Algae: Adaptation Strategies to Survive on Snow and Ice](#)”).

In addition, polyextremophiles can also withstand a variety of stresses; among them we mention both ends of the pH range, very strong acidity vs. high alkalinity, saturated salt solutions, and high hydrostatic pressure (Yutaka Kawarabayasi, “Acido- and Thermophilic Microorganisms, Their Features and Identification of Novel Enzymes or Pathways” and Hiroaki Minegishi, “Halophilic, Acidophilic, and Haloacidophilic Prokaryotes”). We know that life exists on Earth in almost every ecological niche. One of the prerequisites for life is the

availability of liquid water, sources of energy, and a reasonable supply of organic molecules. From our experience with the Earth biota, wherever there is water, there is a good opportunity of finding living organisms.

In hypersaline areas (such as the Dead Sea, Israel), we find halophilic bacteria and algae that can balance the osmotic pressure of hypertonic external solutions (cf., the three well-documented chapters by Aharon Oren – “[Two Centuries of Microbiological Research in the Wadi Natrun, Egypt: A Model System for the Study of the Ecology, Physiology, and Taxonomy of Haloalkaliphilic Microorganisms](#)”; “[Life in Magnesium- and Calcium-Rich Hypersaline Environments: Salt Stress by Chaotropic Ions](#)”; and finally, “[Life at High Salt and Low Oxygen: How Do the *Halobacteriaceae* Cope with Low Oxygen Concentrations in Their Environment?](#)”). In addition, this book covers this topic in Helga Stan-Lotter’s chapter “[Survival Strategies of Halophilic Oligotrophic and Desiccation-Resistant Prokaryotes.](#)” Halophilic bacteria were further discussed in the chapters by Virginia P. Edgcomb and William D. Orsi, “[Microbial Eukaryotes in Hypersaline Anoxic Deep Sea Basins](#)”, as well as the chapter by Horia Banciu and Dimitry Y. Sorokin, “[Adaptation Mechanisms in Haloalkaliphilic and Natronophilic Bacteria.](#)”

Recently, the segmented microscopic animals, tardigrades (0.1–1.5 mm), have been under investigation, as demonstrated by the well-documented chapter “[Tardigrades: An Example of Multicellular Extremophiles](#)” written by Dirk Schulze-Makuch and Joseph Seckbach. These “water bears” are polyextremophilic and are able to tolerate a temperature range from about 0 °C to +151 °C (much more than any other known microbial prokaryotic extremophile). But even low Earth orbit microorganisms would be exposed to extremely low temperatures: tardigrades can survive being heated for a few minutes to 151 °C or being chilled for days at –200 °C, or for a few minutes at –272 °C, 1° warmer than absolute 0. These extraordinary temperatures were discovered by an ESA project of research into the fundamental physiology of the tardigrade, named TARDIS. Tardigrades are also known to resist high radiation. The chapter by Kim M. Webb and Jocelyne DiRuggiero further discusses the tolerance of high radiation – “[Radiation Resistance in Extremophiles: Fending Off Multiple Attacks](#)”. Part 7 of this book provides multiple examples of the extraordinary adaptations that are known in microbiology, including fungi, algae, and magnetotactic bacteria.

To conclude, many other valuable chapters make up this timely and comprehensive volume that demonstrate the current relevance of the subject of polyextremophiles in a wide range of subjects that the readers will discover by themselves, supplementing those that we have listed above, as well as other topics from the space sciences, biochemistry, genetics, ecological situations, and in human activities. But from our personal point of view, the most striking and appealing aspect of this work is the fact that life on Earth is evidently ubiquitous, and it suggests how life may emerge and adapt itself in other worlds (Chela-Flores, 2011).

This argument is particularly compelling, especially now that we have a highly developed NASA robot on Mars, Curiosity, capable of searching for extinct microorganisms that according to our viewpoint would be some kind of

polyextremophiles (Mars Science Laboratory, 2012). The argument in this book is in addition significant, since with two major probes, the current Kepler Mission (Borucki et al., 2011) and FINESSE-type of spectroscopic analysis (Swain, 2010), we will be able to begin probing if some of the Kepler worlds in our galactic neighborhood would be in habitable zones of their stars and if they have produced unusually high fractions of biogenic gases, as that the cyanobacteria were able to pump out since the Archean. After the new abundant gas from photosynthesis, oxygen, was able to saturate surficial ferrous compounds (banded-iron formations), the Earth was transformed from an oxygen-poor atmosphere to an oxygen-rich one: “the great oxidation event” took place around 2.4 Ga before the present.

An alternative name to the oxygenation of the atmosphere was given by the late Lynn Margulis and Doron Sagan (Margulis and Sagan, 1987), who called this atmospheric phenomenon the “great oxygen holocaust” from the point of view of those ancient polyextremophiles that were anaerobic and could not survive on a world that was rapidly transforming itself into a multicellular-friendly environment from the surviving aerobes. This book is a treasure trove for microbiologists, but especially for astrobiologists that need to anticipate what sort of organisms may have evolved elsewhere.

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ERRATUM

ADAPTATION IN HALOALKALIPHILES AND NATRONOPHILIC BACTERIA

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The title for this chapter should read “Adaptation Mechanisms in Haloalkaliphilic and Natronophilic Bacteria” instead of “Adaptation in Haloalkaliphiles and Natronophilic Bacteria”.

ERRATUM

DEEP HYPERSALINE ANOXIC BASINS AS MODEL SYSTEMS FOR ENVIRONMENTAL SELECTION OF MICROBIAL PLANKTON

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No permission has been received for the use of the Bathymetric maps of two Mediterranean DHABs (Figure 1) and the image of the seafloor, as taken during a mapping dive of the ROV Jason (Figure 3) and therefore Chapter 22 Fig. 1 and Fig. 3 have been greyed out.

Figure 1. Bathymetric maps of two Mediterranean DHABs. Brines occur in a depth of around 3,550 and 3,500 m for (a) Discovery Basin and (b) Urania Basin, respectively.

Figure 3. Image of the seafloor (*left*) showing the transition (interphase) from seawater into the brine layer (*right, dark area*) (The picture was taken during a mapping dive of the ROV Jason (WHOI)).

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